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RESEARCH TOPICS

NEUROLOGICAL FUNCTIONS OF THE MASTERSWITCH PROTEIN KINASE – GSK-3

Hosted by
Oksana Kaidanovich-Beilin, Jean-Martin Beaulieu, Richard S. Jope and Jim R. Woodgett

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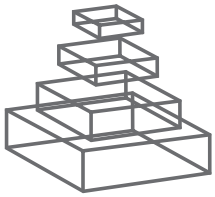

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NEUROLOGICAL FUNCTIONS OF THE MASTERSWITCH PROTEIN KINASE – GSK-3

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The functions of the brain that allow us to think, feel, move, and perceive the world are the result of an exchange of information within a network composed of millions of specialized cells called neurons and glia. Neurons use neurotransmitters and other extracellular messengers to communicate with each other, and to constantly update and re-organize their network of connections in a process known as neural plasticity. In order to respond to these extracellular signals, neurons are equipped with specialized receptors that can recognize a single neurotransmitter a bit like a lock would recognize a key. They do this by activating or inhibiting a class of specialized signaling proteins and second messengers. Typically, signaling proteins are themselves organized in networks or pathways in which they activate or inhibit each other in order to integrate the mass of information received by a single cell and to regulate the biological functions of this cell. As we can see, rather than simply being a network of neurons, the brain can be seen as a sort of "Russian doll" in which each neuron is at the same time a part of networks with other neurons and the receptacle of many networks composed of signaling proteins.

Two individual genes encode two paralogous signaling proteins: Glycogen Synthase Kinase -3 alpha and beta (GSK-3a, GSK-3b), named for its ability to phosphorylate a key metabolic enzyme of glycogen synthesis, glycogen synthase. This unique "glamour and gloom" protein kinase, has been intriguing many researches for over 30 years by its unusual features, still unknown mechanisms of its activation, its regulation by multiple "key" intracellular pathways, and its capacity to influence the functions of many substrates. Since GSK-3 was discovered, there has been significant progress in elucidating its regulatory roles in the neuron and the structure and functions of the brain.

Lithium has been used as a gold standard in the treatment of bipolar disorder for 60 years; and "GSK-3's renaissance" in psychiatry began with the discovery of GSK-3 as lithium's intracellular target. Since then, GSK3 has been implicated in the pathogenesis of mood disorders, schizophrenia, Alzheimer's disease, ADHD, multiple sclerosis, Fragile X syndrome and Huntington disease. Connections to these and other diseases has led over the last 10 years to the generation of multiple types of GSK-3 inhibitors as promising therapeutic treatments for the aforementioned pathological conditions.

During last couple years new genetic models have been generated, including conventional and conditional mouse models, allowing the discovery of new roles of GSK-3 in the mechanism of neurotransmitter action, neurodevelopment, learning and memory formation, GSK-3's gene - effect on mouse behavior, and other functions. Thus, GSK-3 has been well-established as an intracellular second messenger for several neurotransmitter systems, and as an important therapeutic target of mood stabilizers, antipsychotics and psychomimetic drugs.

The proposed Specific Topic for Frontiers in Neuroscience will be focused on the latest advances from leading laboratories in this area, subdivided into 5 topics: (1) GSK-3 history, mechanism of regulation, substrate specificity and comparison between the brain function of two GSK-3 genes through new animal models and cell biology approaches; (2) role of GSK-3 in neurodevelopment and neuronal structure; (3) involvement of GSK-3 in synaptic functions, learning and memory, and in serotonin and dopamine pathways; (4) role of GSK-3 in neuroinflammation, and application to the pathogenesis of multiple sclerosis, AD, schizophrenia, Fragile X, brain tumors, stroke and bipolar disorder; (5) development of GSK-3 inhibitors and their application in psychiatry, including special discussion about the mechanism of lithium action...

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Neurological functions of the masterswitch protein kinase – GSK-3

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I know that I know nothing (Socrates, 469 BC–399 BC)

This Research Topic is dedicated to a rather unique cellular regulator termed Glycogen Synthase Kinase-3 (GSK-3). In the late 1970s, this protein serine kinase was first discovered through its ability to activate the ATP-Mg-dependent form of type-1 protein phosphatase (Factor A activity) and to phosphorylate the key rate-limiting metabolic enzyme that catalyzes the last step of glycogen synthesis, glycogen synthase. From five chromatographically separable glycogen synthase kinases, the third (GSK-3) was subsequently demonstrated to mediate the effect of insulin on activation of glycogen synthesis: insulin leads to inactivation of GSK-3 which relieves its inhibitory phosphorylation of glycogen synthase. Molecular cloning in 1990 revealed the existence of two distinct genes for GSK-3, which encode two paralogous signaling proteins termed GSK-3α and GSK-3β (reviewed in Kaidanovich-Beilin and Woodgett, 2011). GSK-3 has intrigued many researchers for over 30 years by its unusual features, mechanisms of regulation, its participation within at least four major signaling systems, and its capacity to influence the functions of more than 100 substrates (reviewed in Kaidanovich-Beilin and Woodgett, 2011).

While GSK-3 was initially identified as a regulator of metabolism, a significant role in neuroscience has since emerged. The first indication of an important brain function was made in 1992 by Hanger et al. (1992) who showed that GSK-3 phosphorylates the microtubule-associated protein tau at sites relevant to the pathology of Alzheimer's disease, further supported by studies by Takahashi et al. (1994) who demonstrated that Tau was indeed a physiological substrate of GSK-3, which at that time was also called tau protein kinase I (TPKI) (reviewed in Kremer et al., 2011; Medina et al., 2011; Takashima, 2012). These findings were important milestones for initiating interest in GSK-3 in the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease (see Gomez-Sintes et al., 2011; Kremer et al., 2011; Medina et al., 2011; Takashima, 2012).

In parallel to the aforementioned discoveries, GSK-3 was shown to be a major regulator of the Wnt signaling pathway. In 1995, studies in *Xenopus laevis* showed that the expression of a catalytically inactive mutant of GSK-3 induces duplication of the dorsal axis, a phenotype similar to earlier studies of lithium ion treatment of *Xenopus* embryos. A molecular explanation for this relationship was discovered in 1996 when GSK-3 was shown to be inhibited by lithium (see chapter by Valvezan and Klein, 2012). Lithium was a gold standard in the treatment of bipolar disorder (reviewed by Freland and Beaulieu, 2012). This finding poured gas onto the GSK-3 fire in neuroscience research. Subsequent multiple studies have shown associations between dysregulation of GSK-3-mediated signaling pathways and the pathogenesis of bipolar disorder (reviewed in Jope, 2011), Fragile X syndrome (reviewed in Mines and Jope, 2011), schizophrenia (reviewed in Emamian, 2012), brain tumors (reviewed in Mills et al., 2011), and stroke (reviewed in Chuang et al., 2011). Several independent studies have also shown the involvement of GSK-3 in the action of the monoamine neurotransmitters serotonin and dopamine that are central targets for antidepressant and antipsychotic drugs (reviewed in Beaulieu et al., 2011; Polter and Li, 2011) therefore suggesting that GSK-3 may constitute an important "missing link" between disease mechanisms and treatments for mental illnesses.

In 2000, the first mouse knockout model of GSK-3 (beta) was reported (reviewed in Kaidanovich-Beilin and Woodgett, 2011), and provided the first of many tools for physiological analysis of GSK-3's roles. Subsequent transgenic models of GSK-3 have allowed its role to be elucidated in the mechanism of monoamine neurotransmitter action (reviewed in Beaulieu et al., 2011; Polter and Li, 2011) and synaptic plasticity (reviewed in Bradley et al., 2012), neurodevelopment (reviewed in Kim and Snider, 2011; Cole, 2012), neuronal morphogenesis (reviewed in Kim et al., 2011), apoptosis, and neuronal survival (reviewed in Gomez-Sintes et al., 2011) as well as neuroinflammation (reviewed in Beurel, 2011).

Hence a single pair of signaling molecules can be found at the center of multiple neuronal functions. Indeed, these roles positioned GSK-3 as a potential therapeutic target for both psychiatric and neurodegenerative diseases and a series of pharmacological inhibitors with alternative modes of actions have been generated over last two decades (reviewed in Eldar-Finkelman and Martinez, 2011). We certainly hope that these molecules will prove useful and safe in the clinic as there are many unmet needs in both psychiatry and dementia-related medicine. That said, study of GSK-3 in the brain has uncovered many important facets of brain biology and there is clearly much more to be revealed.

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GSK-3: functional insights from cell biology and animal models

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Glycogen synthase kinase-3 (GSK-3) is a widely expressed and highly conserved serine/threonine protein kinase encoded in mammals by two genes that generate two related proteins: GSK-3α and GSK-3β. GSK-3 is active in cells under resting conditions and is primarily regulated through inhibition or diversion of its activity. While GSK-3 is one of the few protein kinases that can be inactivated by phosphorylation, the mechanisms of GSK-3 regulation are more varied and not fully understood. Precise control appears to be achieved by a combination of phosphorylation, localization, and sequestration by a number of GSK-3-binding proteins. GSK-3 lies downstream of several major signaling pathways including the phosphatidylinositol 3′ kinase pathway, the Wnt pathway, Hedgehog signaling and Notch. Specific pools of GSK-3, which differ in intracellular localization, binding partner affinity, and relative amount are differentially sensitized to several distinct signaling pathways and these sequestration mechanisms contribute to pathway insulation and signal specificity. Dysregulation of signaling pathways involving GSK-3 is associated with the pathogenesis of numerous neurological and psychiatric disorders and there are data suggesting GSK-3 isoform-selective roles in several of these. Here, we review the current knowledge of GSK-3 regulation and targets and discuss the various animal models that have been employed to dissect the functions of GSK-3 in brain development and function through the use of conventional or conditional knockout mice as well as transgenic mice. These studies have revealed fundamental roles for these protein kinases in memory, behavior, and neuronal fate determination and provide insights into possible therapeutic interventions.

Keywords: GSK-3, signal transduction, animal models, behavior

CHARACTERIZATION OF GSK-3

Glycogen synthase kinase-3 (ATP:protein phosphotransferase, E.C. 2.7.1.37) is a serine/threonine protein kinase, belonging to the CMCG family of proline-directed kinases (Cyclin-dependent kinases (CDKs), Mitogen-activated protein kinases (MAPKs), Glycogen synthase kinases (GSKs), and CDK-like kinases (CLKs). GSK-3 is a monomeric, second messenger-independent protein kinase that was first discovered through its ability to activate the ATP–Mg-dependent form of type-1 protein phosphatase ("Factor A") and to phosphorylate the key rate-limiting metabolic enzyme that catalyzes the last step of glycogen synthesis, glycogen synthase (GS; Embi et al., 1980; Rylatt et al., 1980; Vandenheede et al., 1980).

GSK-3 is a highly conserved protein kinase and has orthologs in plants, fungi, worms, flies, sea squirts, and vertebrates: isoenzymes from species as distant as flies and humans display more than 90% sequence similarity within the protein kinase domain (reviewed in Ali et al., 2001). GSK-3/F_A has been purified by several groups (Hemmings et al., 1982; Woodgett and Cohen, 1984; Tung and Reed, 1989), and was molecularly cloned based on partial peptide sequencing (Woodgett, 1990). Two rat brain cDNAs encoding GSK-3 were isolated that corresponded to isoenzymes designated GSK-3 alpha(α) and GSK-3 beta(β), with apparent M_r of 51,000 Da (483 aa in humans) and 47,000 Da (433 aa), respectively (Woodgett, 1990). Genes encoding GSK-3α and β are located on mouse chromosome 7/human chromosome 19 and on mouse chromosome 16/human chromosome 3, respectively (see **Figure 1**; based on http://genome.ucsc.edu). Mammalian GSK-3 is therefore represented by two paralogous proteins. Overall, GSK-3α and β share 85% overall sequence homology, including 98% amino acid sequence identity within their kinase domains (Woodgett, 1990). This latter fact makes the likelihood of development of small molecule inhibitors that can discriminate the two forms very unlikely. Despite their catalytic domain similarity, GSK-3 isoenzymes diverge in their N- and C-termini. For example, the two gene products share only 36% identity in the last 76 C-terminal residues (Woodgett, 1990). GSK-3α has an extended glycine-rich N-terminal region that has been proposed to function as a pseudosubstrate (Dajani et al., 2001). Interestingly, while many species such as fish, amphibians, and lizards harbor both isoforms, birds have only GSK-3β and appear to have selectively lost GSK-3α (Alon et al., 2011).

GSK-3 is expressed ubiquitously and both gene products are found in virtually all mammalian tissues. The kinase is highly expressed in the brain (Woodgett, 1990; Yao et al., 2002; Perez-Costas et al., 2010), both in neurons and glia (Ferrer et al., 2002). In the developing brain, the presence of GSK-3 is high at E18 and

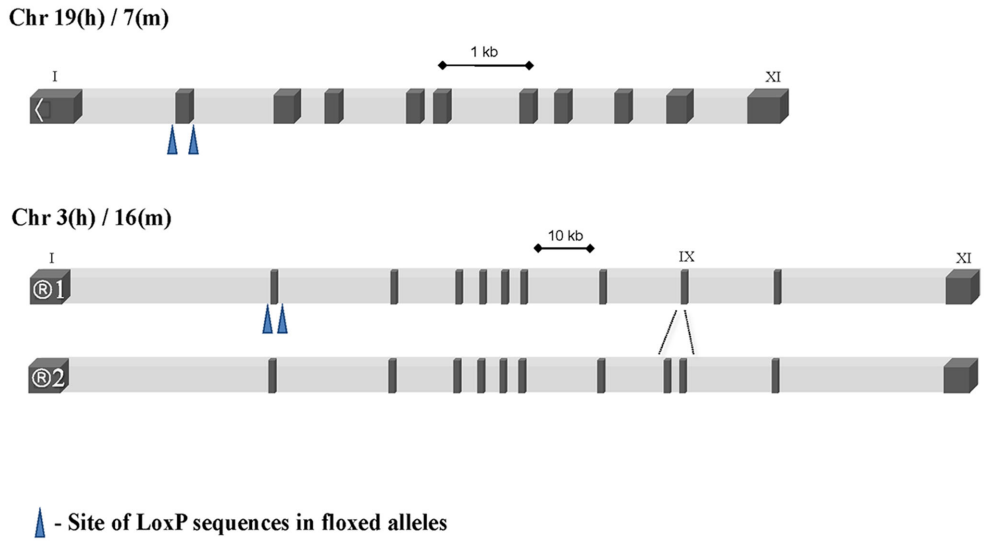

FIGURE 1 | Intron/exon structure of the two mammalian GSK-3 genes indicating the differential splice of GSK-3β and location of LoxP recombination sites in conditional alleles.

peaks at P8, decreasing somewhat after that period (Takahashi et al., 1994). In adult brain, GSK-3α is especially abundant in the hippocampus, cerebral cortex, striatum, and cerebellum (based on Allen Brain Atlas). GSK-3β is expressed in nearly all brain regions, although there are marked regional differences of GSK-3β mRNA levels in the human brain (Pandey et al., 2009). As a caution, the glycine-rich (and hence purine-rich) region of GSK-3α may distort comparative analysis of RNA expression between it and GSK-3β.

In certain cell types of the brain, alternative splicing between exon 8 and 9 of GSK-3β leads to the generation of an additional "long" form containing a 13 amino acid insert within the catalytic domain (GSK-3β2; see **Figure 1**). This insert is located between residues 303 and 304 of GSK-3β, and is flanked by two proximal α-helices of kinase subdomains X and XI (Hanks and Hunter, 1995; Mukai et al., 2002). This alternatively spliced isoform of GSK-3β in rodents (Mukai et al., 2002; Yao et al., 2002) and in human (Lau et al., 1999; Schaffer et al., 2003; Kwok et al., 2005) has been implicated in neuronal-specific functions. The short form of GSK-3β is ubiquitously expressed in the body, including the developing and adult nervous system (Takahashi et al., 1994; Leroy and Brion, 1999). By contrast, GSK-3β2 is predominantly expressed in the neural tissues, with highest levels in the developing brain and persistence into adulthood (Mukai et al., 2002; Wood-Kaczmar et al., 2009).

GSK-3 REGULATION

An unusual feature of GSK-3 is that the kinase displays high activity in cells under resting/unstimulated conditions (Sutherland et al., 1993; Stambolic and Woodgett, 1994; Woodgett, 1994) and is one of few protein kinases that is inhibited by extracellular signals that induce a rapid and reversible increase in serine phosphorylation of GSK-3 causing a decrease in enzymatic activity. For example, growth factor, insulin, or serum treatment decreases GSK-3 activity by 30–70% within 10 min (Sutherland et al., 1993; Welsh and Proud, 1993; Saito et al., 1994; Stambolic and Woodgett, 1994; Sutherland and Cohen, 1994; Cross et al., 1995; Eldar-Finkelman et al., 1995). The mechanisms of GSK-3 regulation are varied and not yet fully understood; precise control appears to be achieved by a combination of phosphorylation, localization, and sequestration by a number of GSK-3-binding proteins (reviewed in Frame and Cohen, 2001; Doble and Woodgett, 2003; Jope and Johnson, 2004; Kockeritz et al., 2006).

REGULATION THROUGH PHOSPHORYLATION

GSK-3 is dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. The activity of GSK-3 is positively regulated by phosphorylation on a "T loop" tyrosine residue within subdomain VIII (Tyr279 for GSK-3α and Tyr216 for GSK-3β; Hughes et al., 1993; Lochhead et al., 2006). The kinase is negatively regulated by N-terminal phosphorylation of serine residues of the enzyme (Ser21 for GSK-3α and Ser9 for GSK-3β; Sutherland et al., 1993; Sutherland and Cohen, 1994). p38 MAPK can also inactivate GSK-3β via phosphorylation within its C-terminal region at Ser389 and Thr390 (Thornton et al., 2008). p38 MAPK-mediated phosphorylation of GSK-3β occurs primarily in the brain and thymocytes (Thornton et al., 2008). In addition, Thr43 of GSK-3β may be phosphorylated by Erk, resulting in GSK-3 inhibition (Ding et al., 2005).

From the crystal structure, it has been proposed that unphosphorylated Tyr276/Tyr216 act to block the access of primed substrates (as discussed below). Indeed, the structure of phosphorylated GSK-3β (Bax et al., 2001) shows that phosphorylated Tyr216 undergoes a conformational change that allows substrates to bind the enzyme. Previous studies, however, led to conflicting conclusions as to whether tyrosine phosphorylation of GSK-3 is catalyzed by GSK-3 itself (autophosphorylation) or by a distinct tyrosine kinase (Hughes et al., 1993; Kim et al., 1999; Lesort et al., 1999;

Wang et al., 2003; Cole et al., 2004a). In support of the autophosphorylation model, at least in mammals, Lochhead et al. (2006) showed that newly synthesized GSK-3β autophosphorylated itself on tyrosine and that this event could be prevented by exposure to GSK-3 inhibitors.

In contrast to tyrosine phosphorylation, regulation of N-terminal serine phosphorylation is only conserved in GSK-3 homologs from mammals, *Xenopus,* and *Drosophila*, but not in yeast, higher plants, *Dictyostelium,* or *Caenorhabditis elegans*. The phosphorylation state of serine residues of both isoenzymes is dynamic, involving phosphorylation by several protein kinases and dephosphorylation by protein phosphatase-1 (PP-1; Sutherland et al., 1993; Saito et al., 1994; Stambolic and Woodgett, 1994; Cross et al., 1995; Eldar-Finkelman et al., 1995; Welsh et al., 1998; Zhang et al., 2003). N-terminal domain serine phosphorylation of GSK-3α and GSK-3β leads to inhibition of its activity (Sutherland et al., 1993; Saito et al., 1994; Stambolic and Woodgett, 1994; Cross et al., 1995; Eldar-Finkelman et al., 1995; Welsh et al., 1998; Grimes and Jope, 2001; Zhang et al., 2003). Phosphorylation of GSK-3 within its N-terminal region creates a "pseudosubstrate" which intramolecularly binds to a "phosphoprotein binding pocket" within the active site of the kinase, suppressing activity by occluding primed substrate access to the binding pocket (Frame et al., 2001).

This inhibitory mechanism is induced by agonists such as neurotrophins and growth factors that activate protein kinases that act on the N-terminal domain of GSK-3 such as PKB/Akt, p90rsk, cyclic-AMP-dependent protein kinase, p70 S6 kinase, as well as regulators of phosphatase-1 (Sutherland et al., 1993; Stambolic and Woodgett, 1994; Alessi et al., 1996; Li et al., 2000; Svenningsson et al., 2003; see **Figure 2**). For example, insulin leads to inhibition of GSK-3 via insulin receptor substrate-1-dependent induction of phosphatidylinositol 3′ kinase (PI3K), which then stimulates PKB/Akt (Cross et al., 1995). GSK-3 has previously been shown to catalyze serine phosphorylation of IRS-1 and IRS-2, interfering with receptor-mediated tyrosine phosphorylation by the insulin receptor, effectively attenuating insulin receptor signaling via a negative feedback loop (Eldar-Finkelman and Krebs, 1997; Sharfi and Eldar-Finkelman, 2008; **Figure 2**). Moreover, recent studies have revealed novel bi-directionality in the interaction of PKB/Akt and GSK-3 whereby genetic ablation of GSK-3 significantly suppresses PKB/Akt phosphorylation (Lu et al., 2011), indicating a possible novel feedback loop in PKB/Akt/MAPK network (**Figure 2**).

Growth factors, such as EGF and PDGF can also inhibit GSK-3 activity through the phosphatidylinositol 3′ kinase (PI3K) pathway (Stambolic and Woodgett, 1994; Shaw and Cohen, 1999), as well as through induction of the MAPK cascade (Saito et al., 1994; Brady et al., 1998). Ser9/21 phosphorylation of GSK-3 can be modified by amino acid deprivation through mammalian target of rapamycin (mTOR; Armstrong et al., 2001; Krause et al., 2002; Terruzzi et al., 2002) or in response to agonists that elevate the intracellular levels of cAMP through cyclic-AMP-dependent protein kinase (PKA; Fang et al., 2000; Li et al., 2000; **Figure 2**). The PKA-anchoring

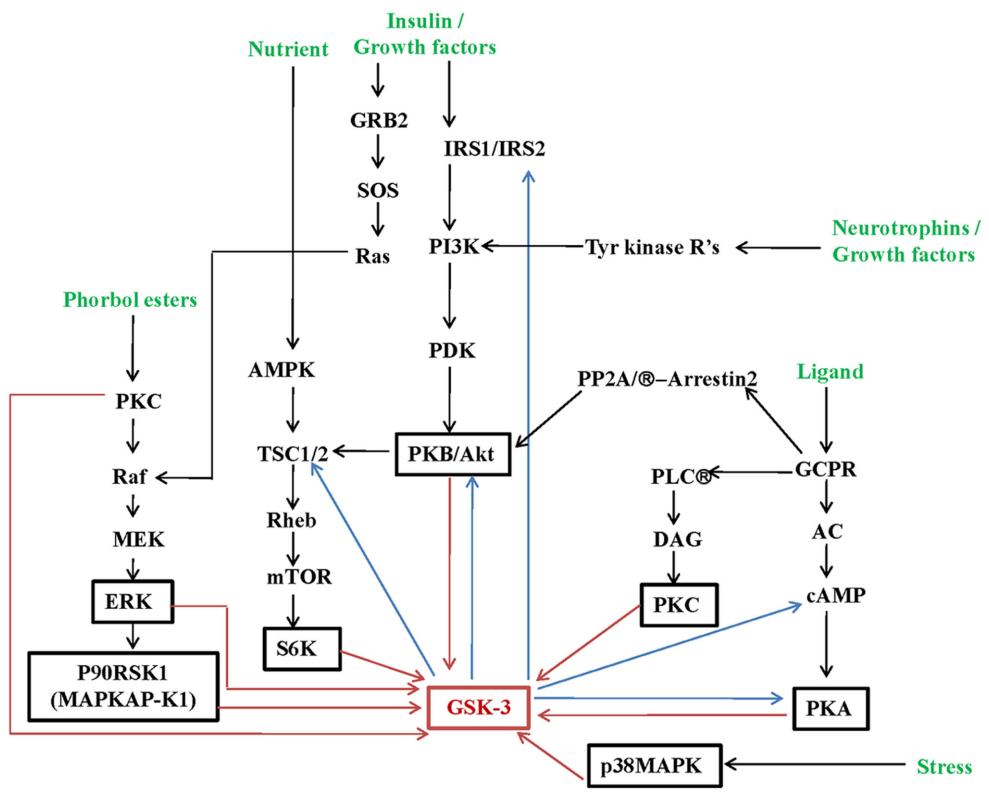

FIGURE 2 | Summary of regulatory signaling inputs into GSK-3.

protein 220 binds both GSK-3 and PKA and hence facilitates GSK-3 phosphorylation by this protein kinase (Tanji et al., 2002). PKC agonists can also regulate GSK-3 (Ballou et al., 2001; Fang et al., 2002), however certain PKCs may preferentially regulate GSK-3β but not GSK-3α (Goode et al., 1992).

REGULATION THROUGH COMPLEX FORMATION

Wnts are secreted glycolipoproteins that activate canonical and non-canonical (β-catenin independent) Wnt signaling cascades, which are essential for early embryonic patterning, cell fate, cellular polarity, cell movement, cell proliferation as well as adult homeostasis in both vertebrates and invertebrates (Logan and Nusse, 2004; Moon et al., 2004; Salinas, 2005; MacDonald et al., 2009). The canonical Wnt signaling pathway employs a distinct mechanism for regulating GSK-3 that is independent of N-terminal domain serine phosphorylation or tyrosine phosphorylation and, instead, relies on protein:protein interactions and intracellular sequestration. Thus, the canonical Wnt pathway comprises phylogenetically conserved proteins: the Wnt receptor, Frizzled; co-receptor, low-density lipoprotein receptor-related protein (LRP) 5/6; scaffolding proteins, Disheveled (Dvl), Axin, Adenomatous polyposis coli (APC) and GSK-3, β-catenin, and casein kinase-1 (CK1; see **Figure 3**).

In cells a small fraction (<5–10%) of cellular GSK-3 is associated with a scaffolding protein termed Axin (Lee et al., 2003; Benchabane et al., 2008). These molecules are joined by others to create a "destruction complex" comprising Axin, APC, CK1, GSK-3, and β-catenin (Zeng et al., 1997; Hart et al., 1998; Ikeda et al., 1998). Within this machine, CK1 phosphorylates Ser45 of β-catenin, which generates a priming site for subsequent GSK-3 phosphorylation on Thr41 (Amit et al., 2002; Hagen and Vidal-Puig, 2002; Hagen et al., 2002; Liu et al., 2002; Sakanaka, 2002; Yanagawa et al., 2002) and subsequently Ser37 and Ser33; resulting in β-catenin recognition by β-TrCP (an E3 ubiquitin ligase subunit), and subsequent ubiquitin-mediated proteasomal degradation of β-catenin (Aberle et al., 1997; Amit et al., 2002; Liu et al., 2002; He et al., 2004). This results in the maintenance of very low levels of β-catenin within the cytoplasm and nucleus of cells (significant amounts of β-catenin are associated, in epithelial cells, with the cadherin adhesion molecules but this fraction is effectively sequestered and does not play a role in Wnt signaling). In addition to β-catenin, both Axin and APC are phosphorylated by GSK-3. Phosphorylation of Axin by GSK-3 increases its stability and binding to β-catenin (Ikeda et al., 1998; Jho et al., 1999; Yamamoto et al., 1999). Phosphorylation of APC increases its affinity to β-catenin (Rubinfeld et al., 1996). Both events promote β-catenin phosphorylation and degradation complex stability.

Wnt ligand induces binding of the seven-pass transmembrane receptor Frizzled and the LRP5/6 co-receptor which leads to the recruitment of Dvl and induction of LRP 5/6 phosphorylation by GSK-3 and CK1. This creates a high affinity binding site for Axin (He et al., 2004; Zeng et al., 2005; Mi et al., 2006; Niehrs and Shen, 2010). Recruitment of Axin to the receptor proteins results in functional dissolution of the destruction complex allowing the stabilization and accumulation of β-catenin. The now stable β-catenin translocates to the nucleus where it binds with members of the TCF/LEF family of DNA-binding proteins, resulting in transcriptional activation of certain targets genes.

β-catenin is dephosphorylated primarily by protein phosphatase (PP) 2A (Su et al., 2008). APC may also act to interfere with PP2A dephosphorylation of β-catenin (Su et al., 2008). APC also facilitates Axin degradation (Lee et al., 2003; Takacs et al., 2008). PP-1 activity leads to dephosphorylation of Axin, antagonizing CK1 phosphorylation as well as negatively regulating GSK-3-Axin binding, promoting complex disassembly (Luo et al., 2007).

Several molecular mechanisms have been proposed to explain how canonical Wnt signaling may interfere with GSK-3-dependent phosphorylation of β-catenin (reviewed in Kimelman and Xu, 2006). For example, the intracellular domain of LRP6 may act as a direct inhibitor of GSK-3 (Mi et al., 2006; Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). A recent study suggested an important role of multi-vesicular endosomes in the canonical Wnt pathway (Taelman et al., 2010). In cells harboring a constitutively activated mutant of LRP6, sequestration of GSK-3 into these membrane-bound organelles was observed, leading to insulation of GSK-3 from other components of the pathway (Taelman et al., 2010). This trafficking machinery required β-catenin which formed a feed-forward loop by facilitating GSK-3 sequestration (Taelman et al., 2010). Whether this mechanism plays a role in physiological Wnt signaling has yet to be determined.

Both mammalian isoforms of GSK-3 function equivalently in Wnt signaling and are entirely redundant (Doble et al., 2007). Indeed, retention of just one of the four GSK-3 alleles is sufficient to maintain low levels of β-catenin in the absence of Wnt, reiterating the fact that only a small fraction of GSK-3 is tightly associated with Axin and therefore relevant to Wnt signaling.

GSK-3 can also associate with other proteins, e.g., GSK-3-binding protein (GBP or FRAT; Li et al., 1999; Fraser et al., 2002) and GSKIP (Chou et al., 2006); however, the roles of FRAT and GSKIP in GSK-3 biology have yet to be defined. GSK-3 is also a part of interacting complex of proteins involved in Hedgehog (Hh) pathway, regulating a key transcription factor of Hh signaling – cubitus interruptus (Ci; Price and Kalderon, 2002).

REGULATION THOUGH INTRACELLULAR LOCALIZATION

In addition to binding proteins in the cytoplasm, there are differences in patterns of subcellular localization of the GSK-3 isozymes (Hoshi et al., 1995; Franca-Koh et al., 2002; Bijur and Jope, 2003). GSK-3 is largely considered as a cytoplasmic protein, but the kinase can also be detected in the nucleus and mitochondria where it is more active compared with the larger cytoplasmic fraction (Bijur and Jope, 2003). Nuclear localization of GSK-3 is dynamic and dependent on the cell cycle (being highest during S-phase; Diehl et al., 1998). Activity is also rapidly increased during apoptosis (Bijur and Jope, 2001). The mechanisms governing intracellular localization of GSK-3 are not fully elucidated. Activated PKB/Akt has been reported to decrease nuclear levels of GSK-3 (Bijur and Jope, 2001). Binding of FRAT 1 to GSK-3 facilitates nuclear export (Franca-Koh et al., 2002). The viral tumor-associated latent nuclear antigen binds GSK-3 and acts to enrich it in the nuclear fraction (Fujimuro et al., 2003). As mentioned previously, GSK-3α has an N-terminal extension compared to GSK-3β. One role of this extra domain may be to provide a level of regulation to

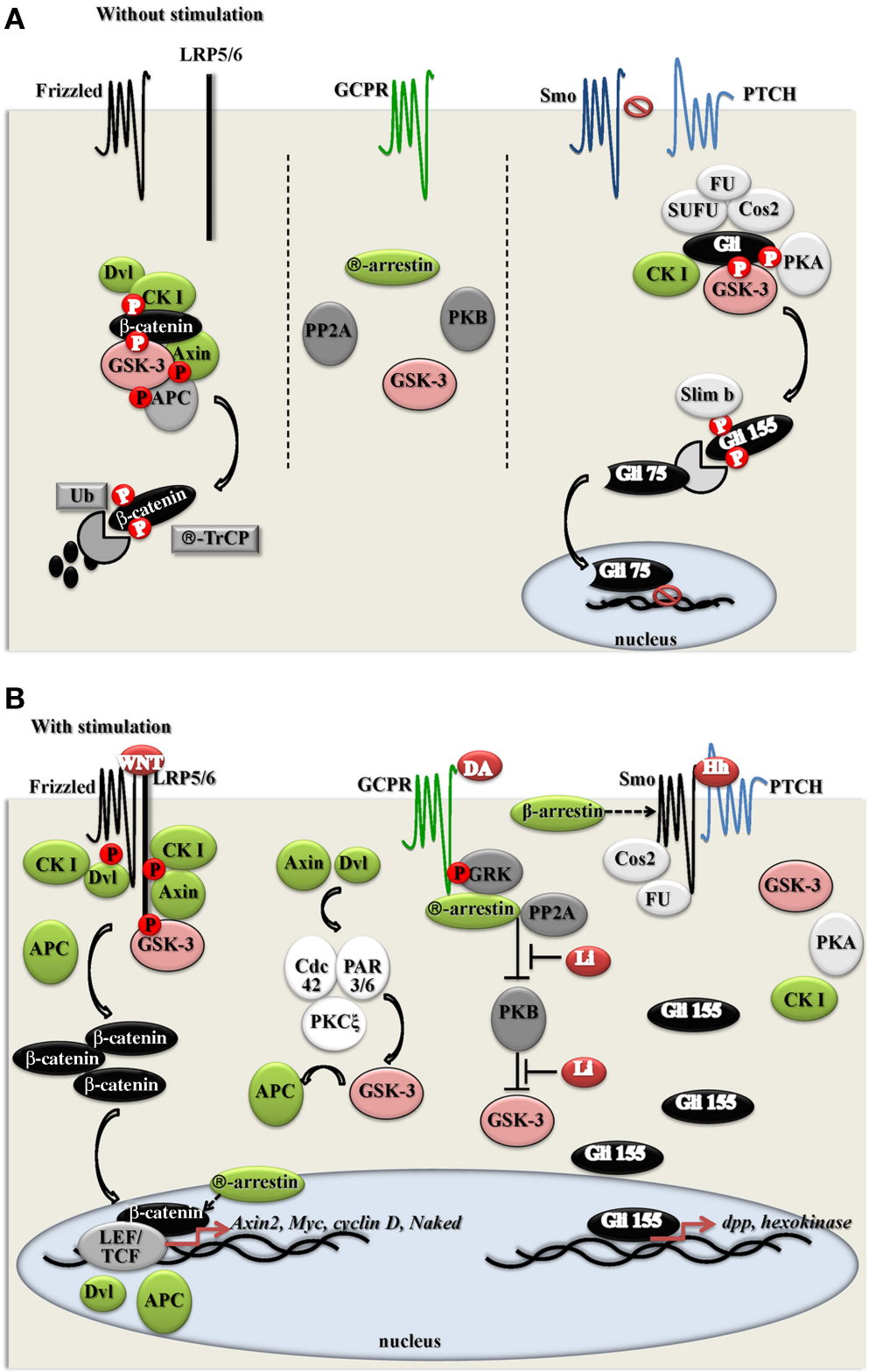

FIGURE 3 | Interaction between different intracellular pools of GSK-3 and protein complexes, involved into Wnt, Hedgehog (Hh), GCPR, and PAR3/6–Cdc42–PKC pathways. (A); resting conditions. (B); activated conditions.

nuclear transport of this isoform (Azoulay-Alfaguter et al., 2011). Notably, deletion of N-terminus of GSK-3 beta reduces its nucleus accumulation (Meares and Jope, 2007). These studies indicate that at least a fraction of GSK-3 may be regulated by intracellular compartmental shuttling.

The finding that GSK-3 acts downstream of multiple signaling pathways that have distinct effects on cells and tissues presents a conundrum. How might signal selectivity be achieved if a protein common to multiple pathways was a required intermediary? The elegant cellular solution to this is to fractionate GSK-3 between scaffolding proteins or other structures such that each system has its own population of GSK-3 molecules "assigned" to it. This effectively insulates the signals and requires that the GSK-3 subpopulations do not intermingle or exchange. It is still an open question why so many important pathways evolved with a common component, a subject of speculative commentary (McNeill and Woodgett, 2010).

GSK-3 SUBSTRATES

The determination of the crystal structure of GSK-3β provided further insight into the molecular nature of the regulation of GSK-3 and its predilection for primed, pre-phosphorylated, substrates (Dajani et al., 2001; ter Haar et al., 2001). GSK-3 shares common features with other protein kinases and has a small N-terminal lobe mostly consisting of β-sheets and a large C-terminal lobe essentially formed of α-helices (Noble et al., 2005). The ATP-binding pocket is located between the two lobes and is so highly conserved between the two isoforms that discrimination between the two protein kinases by an ATP analog-based inhibitor is highly unlikely (Bain et al., 2007).

GSK-3 is one of only a handful of the over 500 known protein kinases that has a strong (500- to 1000-fold) preference for substrates that are already primed by phosphorylation at a proximal serine/threonine to the GSK-3 target residue (Thomas et al., 1999). The phosphorylated residue within the presumptive substrate slots into to a "phosphate-binding" pocket that comprises three crucial basic residues – Lys205, Arg96, and Arg180 (Bax et al., 2001; Dajani et al., 2001; ter Haar et al., 2001). These three residues are conserved in all GSK-3 homologs identified to date, suggesting conservation of the priming phosphate-binding site and the substrate specificity of GSK-3 in all organisms. Binding of the priming phosphate of the substrate to this pocket on GSK-3 induces a conformational change, aligning the substrate for subsequent phosphorylation.

The majority of GSK-3 substrates exhibit an absolute requirement for prior phosphorylation by another kinase at a "priming" residue located C-terminal to the site of subsequent phosphorylation by GSK-3 (Fiol et al., 1987). GSK-3-catalyzed phosphorylation of these substrates occurs at the fourth (Fiol et al., 1990) or fifth (Cole et al., 2006) serine or threonine residue N-terminal to the primed site ($pS/T_1XXXpS/T_2$), where the first pS/T_1 (Ser or Thr) is the target residue, X is any amino acid (but often Pro), and the last pS/T_2 is the site for priming phosphorylation. Thus, the primed Ser/Thr is recognized by the positively charged "binding pocket" on GSK-3 which facilitates the correct orientation of the substrate within the active site of the kinase. Several protein kinases have been shown to act as priming enzymes for GSK-3 phosphorylation, including CDK-5 (Sengupta et al., 1997; Noble et al., 2003; Li et al., 2006), PAR-1 (Nishimura et al., 2004), casein kinase-1 (Amit et al., 2002), casein kinase-2 (Picton et al., 1982; DePaoli-Roach et al., 1983), PKA (Singh et al., 1995), and PKC (Liu et al., 2003). In the case of several substrates, the residue phosphorylated by GSK-3 acts to prime an additional Ser/Thr residue N-terminal to it. This can lead to a zippering effect where multiple residues become phosphorylated by GSK-3. Certain substrates apparently dodge the requirement for prior phosphorylation including c-Jun (Boyle et al., 1991), c-Myc (Saksela et al., 1992), histone H1.5 (Happel et al., 2009), and MARK2/PAR-1 (Kosuga et al., 2005; Timm et al., 2008). In these cases, acidic residues or peptide conformations may substitute for the effect of the priming phosphate.

To prove that an *in vitro* identified protein is an *in vivo* physiological substrate of GSK-3 the target has to meet several criteria (Frame and Cohen, 2001). These include phosphorylation of the protein at the appropriate residues by the protein kinase *in vitro* and under conditions known to modulate that kinase *in vivo* and selective reduction in those phosphorylation sites upon treatment with a specific inhibitor of the protein kinase (or via gene knockout/RNAi). To date, over 100 cytoplasmic and nuclear proteins have been identified as substrates of GSK-3 although not all of these meet the Frame and Cohen criteria as *bona fide* targets (reviewed in Doble and Woodgett, 2003; Jope and Johnson, 2004; Kockeritz et al., 2006; Sutherland, 2011; see **Table 1**).

With respect to biological processes, GSK-3 substrates may be classified into several groups of proteins/transcriptional factors/regulatory enzymes that have roles in processes such as metabolism, cellular architecture, gene expression, neurobiological processes, synaptogenesis, neurodevelopment, axonal growth and polarity, immune response, circadian rhythms, and neuronal/cellular survival (reviewed in Frame and Cohen, 2001; Doble and Woodgett, 2003; Jope and Johnson, 2004; Kockeritz et al., 2006; Sutherland, 2011; see **Table 1**).

GSK-3 SUBSTRATES RELATED TO CIRCADIAN RHYTHMS

Circadian (from the Latin *circa diem* meaning "about a day") rhythms occur with a periodicity of about 24 h and enable organisms to adapt and anticipate environmental changes. Circadian control provides an evolutionary advantage to organisms in adapting their behavior and physiology to the appropriate time of day (reviewed in Wijnen and Young, 2006; Sahar and Sassone-Corsi, 2009). Feeding behavior, sleep-wake cycles, hormonal levels, and body temperature are just a few examples of physiological circadian rhythms. Dysregulation of the cycle is associated with the onset and development of numerous human diseases, including sleep disorders, depression, and dementia.

From a molecular standpoint, circadian rhythms are regulated by transcriptional and post-translational feedback loops generated by a set of interplaying "clock" proteins. The positive limb of the mammalian clock machinery is comprised of CLOCK and BMAL1, which are transcription factors that heterodimerize through their PAS domains and induce the expression of clock-controlled genes by binding to their promoters at E-boxes. Cryptochromes (Cry 1, Cry2) and Period genes (Per1, Per2, Per3) are clock-controlled genes that encode proteins that form the

Table 1 | GSK-3 substrates.

Substrate name	GSK-3's phosphorylation site (in bold) S/TxxxS/T	Biological process/relevance to disease	Reference
METABOLIC ENZYMES AND SIGNALING PROTEINS			
ATP-citrate lyase	**T446**xxx**S450**xxxS454	Fatty acid biosynthesis	Hughes et al. (1992), Benjamin et al. (1994)
Glycogen synthase	**S640**xxx**S644** xxx**S648**xxx **S652**xxxS656	Glycogen metabolism, diabetes	Rylatt et al. (1980), Parker et al. (1983), Dent et al. (1989)
Pyruvate dehydrogenase	**. . .?...**	Metabolism	Hoshi et al. (1996)
Phosphocholine cytidylyltransferase (CTP)	C-terminus . . .?...	CDP-choline pathway	Cornell et al. (1995)
κ-casein	. . .?...	Milk protein	Donella-Deana et al. (1983)
Protein phosphatase-1 G-subunit	**S38**xxx**S42**xxxS46	Signaling/protein dephosphorylation	Dent et al. (1989)
Protein phosphatase inhibitor-2	T72xxxS86	Signaling/protein dephosphorylation	Aitken et al. (1984), DePaoli-Roach (1984)
Axin	**S322**xxx**S326**xxxS330	Wnt pathway, development, cancer	Ikeda et al. (1998), Jho et al. (1999), Yamamoto et al. (1999)
Axil	. . .**?...**	Member of axin family	Yamamoto et al. (1998)
Adenomatous polyposis coli (APC)	**S1501**xxxS1505 **S1503**xxxS1507	Wnt pathway, development, cancer	Rubinfeld et al. (1996), Ikeda et al. (2000), Ferrarese et al. (2007)
Cubitus interruptus/Gli	**S852**xxxS856 **S884**xxx**S888**xxxS892	Hedgehog pathway, development, cancer	Jia et al. (2002), Price and Kalderon (2002)
eIF2B (Eukaryotic initiation factor 2B)	**S535**xxxS539	Growth, cancer	Welsh et al. (1998), Wang et al. (2001), Woods et al. (2001)
Amyloid precursor protein (APP)	**T743**xxx? **T668**xxx?	Neuronal function, Alzheimer's disease	Aplin et al. (1996)
Presenilin-1	**S397**xxx**S401xxx?** **S353**xxx**S357xxx?**	Alzheimer's disease	Kirschenbaum et al. (2001), Twomey and McCarthy (2006)
Heterogeneous nuclear ribonucleoprotein D	**S83**xxxS87	Transcriptional regulator	Tolnay et al. (2002)
Phosphatase interactor targeting protein K (PITK)	**S1013**xxxS1017	PP-1 targeting subunit, modulates phosphorylation of hnRNP K	Kwiek et al. (2007)
p21 CIP1	**T57**xxx?	Cell cycle, apoptosis	Rössig et al. (2002)
Insulin receptor substrate 1	**S332**xxxS336	Diabetes, growth, cancer	Eldar-Finkelman and Krebs (1997), Liberman and Eldar-Finkelman (2005)
Insulin receptor substrate 2	**S484**xxxS488	Diabetes	Sharfi and Eldar-Finkelman (2008)
P75 NGF receptor	. . .?...	Neurotrophin signaling	Taniuchi et al. (1986)
Mcl-1	**S140**xxxT144	Growth, apoptosis, cancer	Maurer et al. (2006), Ding et al. (2007)
Cyclic-AMP-dependent protein kinase – RII subunit	**S44**xx**S47**xxx?	cAMP pathway, hormonal responses	Hemmings et al. (1982)
Cyclin D1	**T286**xxx?	Cell cycle, cancer	Diehl et al. (1998)
Cyclin E	**T380**xxx?	Cell cycle	Welcker et al. (2003)
Myelin basic protein	**T97**xxx?	Myelination of nerves in CNS	Yu and Yang (1994)
Cry2	**S553**xxxS557	Circadian rhythm	Harada et al. (2005), Kurabayashi et al. (2010)
Per2	. . .?...	Circadian rhythm	Kaladchibachi et al. (2007)
Nucleoporin p62	C-terminus . . .?...	Cell division	Miller et al. (1999)
PTEN (phosphatase and tensin homolog)	**S362**xxx**S366**xxxS370	Cell proliferation, migration and growth, cell survival	Al-Khouri et al. (2005)

(Continued)

Table 1 | Continued

Substrate name	GSK-3's phosphorylation site (in bold) S/TxxxS/T	Biological process/relevance to disease	Reference
Lipoprotein receptor-related protein 6	**S1490**xxx? **S1590**xxx? **T1572**xxx?	Wnt pathway, development., cancer	Zeng et al. (2005); Mi et al. (2006); Niehrs and Shen (2010)
TSC2 (tuberous sclerosis 2)/Tuberin	**S1337**xxx **S1341**xxxS1345	Tumor suppressor	Inoki et al. (2006)
CDC25 (cell division cycle)	**S76**xxxT80	Cell cycle, cancer	Kang et al. (2008)
RBL2/p130	**S948**xxxS952 **S962**xxxS966 **S982**xxxT986	Growth, cell cycle, cancer	Litovchick et al. (2004)
Voltage dependent anion channel	**T51**xxxT55	Apoptosis, cancer	Pastorino et al. (2005)
CTPS (cytidine triphosphate synthetase	**S571**xxxS575	Cell growth, cancer	Higgins et al. (2007)
Mdm2 (murine double minute 2)	**S240**xxxS244 **S254**xxxS258	Growth	Kulikov et al. (2005)
Calcipressin/RCN1 (regulators of calcineurin)	**S113**xxxS117	Neuronal regulator of calcineurin, growth, Alzheimer's disease, down syndrome	Hilioti et al. (2004)
Gephyrin	**S270**xxx?	GABA transmission, neuronal functions	Tyagarajan et al. (2011)
Mixed lineage kinase-3	**S789**xxx **S793**xxx?	c-Jun and p38 MAPK pathways, apoptosis, neurodegenerative disease	Mishra et al. (2007)
OMA-1	**T339**xxxT239	Oocyte maturation	Nishi and Lin (2005)
p27Kip1	**...?...**	Cell cycle regulator	Surjit and Lal (2007)
Polypyrimidine track-binding protein-associated-splicing factor (PSF)	**T687**	Alternative splicing, T cell function/activation	Heyd and Lynch (2010)
Zinc finger CCHC domain-containing protein 8 (Zcchc8)	**T492**xxx**?**	RNA metabolism	Gustafson et al. (2005)
SC35	**...?...**	Alternative splicing	Hernandez et al. (2004)
STRUCTURAL PROTEINS			
DF3/MUC1 (high molecular weight mucin-like glycoprotein)	**S40**xxxS44	Wnt pathway, β-catenin, and E-cadherin complex	Li et al. (1998)
Dynamin I	**T774**xxxT778	Endocytosis, neuronal function	Hong et al. (1998), Clayton et al. (2010)
Kinesin light chains (KLCs)	**S611**xxx**S615**xxx?	Axonal transport, mitosis, meiosis	Morfini et al. (2002)
Microtubule-associated protein 1B	**S1260**xxx? **T1265**xxx? **S1388**xxxS1392	Neuronal functions	Lucas et al. (1998), Trivedi et al. (2005), Scales et al. (2009)
Microtubule-associated protein 2C	**T1620**xxx? **T1623**xxx?	Neuronal functions	Sánchez et al. (2000)
Tau	**S208**xxxT212 **T231**xxx**T235**xxx? **S396**xxx? **S404**xxx?	Microtubule stabilization, neuronal functions, Alzheimer's disease	Hanger et al. (1992), Yang et al. (1993), Woods et al. (2001), Cho and Johnson (2004)
Paxillin	**S126**xxxS130	Cell adhesion and migration	Cai et al. (2006)
Collapsin response mediator protein 2	**T509**xxx**T514**xxx **S518**xxxS522	Neuronal functions, axonal growth, neuronal polarity, Alzheimer's disease	Cole et al. (2004b), Yoshimura et al. (2005)
Collapsin response mediator protein 4	**T509**xxx**T514**xxx **S518**xxxS522	Neuronal function, axonal growth	Cole et al. (2004b), Alabed et al. (2010)

(Continued)

Table 1 | Continued

Substrate name	GSK-3's phosphorylation site (in bold) S/TxxxS/T	Biological process/relevance to disease	Reference
Neural cell adhesion protein (NCAM)	. . .?...	Cell–cell adhesion, neurite outgrowth, synaptic plasticity, learning, and memory	Mackie et al. (1989)
Neurofilament L	**S502**xxx**S506**xxx? **S603**xxx?	Cell cytoskeleton, axonal growth, axonal diameter	Guan et al. (1991); Yang et al. (1995), Sasaki et al. (2002)
Neurofilament M	**S666**xxx?		
Neurofilament H	**S493**xxx?		
Ninein	. . .?...	Centrosomal functions, brain development, tumorigenesis	Hong et al. (2000), Howng et al. (2004)
Telokin kinase-related protein	**S15**xxx?	Stabilization of smooth muscle myosin filaments	Krymsky et al. (2001)
CLIP-associated protein 1 (CLASP 1)	. . .**594–614**. . ..	Neuronal functions	Wittmann and Waterman-Storer (2005), Kumar et al. (2009)
CLASP 2 (CLIP-associated protein 2)	**S533**xxx**S537**xxxS541	Cell migration, neuronal function	Watanabe et al. (2009)
Focal adhesion kinase	**S722**xxxS726	Cell cycle, survival, migration, cancer	Bianchi et al. (2005)
Microtubule affinity-regulating kinase-2/PAR-1	**S212**xxx?	Neuronal function, axonal growth	Kosuga et al. (2005), Timm et al. (2008)
Polycystin 2	**S76**xxxS80	Growth, survival, polycystic kidney disease	Streets et al. (2006)
Dystrophin	. . .**?...**	Cytoskeleton of muscle fibers	Michalak et al. (1996)
Stathmin/oncoprotein 18 (STMN1)	**S31**xxx?	Microtubule polymerization and dynamics	Moreno and Avila (1998)
von Hippel–Lindau (VHL)	**S68**xxxS72	Oxygen sensor, tumor of CNS, kidney, eyes	Hergovich et al. (2006)
TRANSCRIPTION FACTORS			
β-catenin	**S33**xxx**S37**xxx **T41**xxx**S45**	Wnt pathway, development, cancer	Yost et al. (1996), Ikeda et al. (1998)
δ-catenin	**T1078**	Cell adhesion	Oh et al. (2009)
CCAAT/enhancer-binding protein αC/EBPα)	**T222**xxx**T226**xxx	Cell proliferation, growth, differentiation	Ross et al. (1999), Liu et al. (2006)
C/EBPβ (CCAAT/enhancer-binding protein β)	**T179**xxx**S184**xxxT188 **S177**xxx**S181**xxx **S185**xxx**T189**xxx	Cell proliferation, growth, differentiation	Tang et al. (2005), Zhao et al. (2005)
Cyclic AMP response element-binding protein (CREB)	**S129**xxxS133	Metabolism, neuronal function, memory formation, diabetes	Fiol et al. (1994), Bullock and Habener (1998)
GATA4	. . .2–116. . . . . .2–205. . .	Embryogenesis, myocardial differentiation and function	Morisco et al. (2001)
Hypoxia-inducible factor-1	**S551**xxx**T555**xxx? **S589**xxx?	Growth, cancer	Mottet et al. (2003), Flugel et al. (2007)
Heat shock factor-1 (HSF1)	**S303**xxxS307	Stress (heat) response	Chu et al. (1996)
c-Myc	**S62**xxx?	Growth, cancer, oncogenes	Saksela et al. (1992), Sears et al. (2000)
L-myc	**T58**xxxS62		
N-myc downstream regulated gene 1	**T342**xxxT346 **T352**xxxT356 **T362**xxxT366	Stress and hormone response, cell growth and differentiation, cancer	Murray et al. (2004)
c-Jun, Jun B, Jun D	**T239**xxxT243	Growth and cancer	Boyle et al. (1991), Nikolakaki et al. (1993), Woodgett et al. (1993), Morton et al. (2003)
c-Myb	**T572**xxx?	Hematopoiesis, tumorigenesis.	Kitagawa et al. (2009)
Nuclear factor of activated T cells c (NFATc)	SP2 domain SP3 domain	Immune system response	Beals et al. (1997), Neal and Clipstone (2001)

(Continued)

Table 1 | Continued

Substrate name	GSK-3's phosphorylation site (in bold) S/TxxxS/T	Biological process/relevance to disease	Reference
Nuclear factor κB (NF-κB) p65subunit precursorp105 of p50 subunit	**S468**xxx? **S903**xxx**S907**xxx?	Stress response, immune response, synaptic plasticity and memory, cancer, inflammation, autoimmune diseases	Demarchi et al. (2003), Buss et al. (2004)
Notch 1C	…?…	Development, cell–cell communication, cancer.	Foltz et al. (2002), Espinosa et al. (2003)
p53	**S33**xxxS37 **S315**xxx? **S376**xxx?	Cell cycle regulator, cancer.	Turenne and Price (2001), Qu et al. (2004)
Snail	**S97**xxx **S101** **S108**xxx **S112**xxx **S116**xxx**S120**xxx?	Epithelial to mesenchymal transition regulator	Zhou et al. (2004)
Activator protein 1 (AP-1)	…?…	Differentiation, proliferation, apoptosis	de Groot et al. (1993)
Glucocorticoid receptor	**T171**xxx?	Stress and immune response, development, metabolism	Rogatsky et al. (1998)
Microphthalmia-associated transcription factor	**S298xxx**	Melanocyte and osteoclast development	Takeda et al. (2000)
NeuroD	…?…	Central nervous system development	Moore et al. (2002)
BCL-3	**S394**xxxS398	Growth and cancer	Viatour et al. (2004)
Bmal1	**S17**xxx**T21**xxx?	Circadian rhythm	Sahar et al. (2010)
Rev-erb α	**S55**xxx**S59**xxx?	Circadian rhythm	Yin et al. (2006)
Timeless	…?…	Circadian rhythm	Martinek et al. (2001)
Clock	**S427**xxxS431	Circadian rhythm	Spengler et al. (2009)
SMAD1	…?…	Embryonic pattern formation, TGFβ signaling	Fuentealba et al. (2007)
SMAD3	**T66**xxx?	TGFβ signaling, development, cancer	Guo et al. (2008)
Neurogenin 2 (Ngn2)	**S231**xxx? **S234**xxx?	Neuronal function, motor neurons, development	Ma et al. (2008)
BCLAF1 (Bcl-2 interacting transcriptional repressor)	**S531**xxx?	Apoptosis, cancer	Linding et al. (2007)
Myocardin	**S455**xxx**S459**xxx **S463**xxx**S467**xxx? **S624**xxx**S628**xxx **S632**xxx**S636**xxx?	Development, cardiac hypertrophy	Badorff et al. (2005)
Histone H1.5	**T10**	Chromosome condensation	Happel et al. (2009)
Nascent polypeptide associated complex	**T159**	Transcriptional coactivator, bone development.	Quelo et al. (2004)
Nuclear factor E2-related factor 2	**…?…**	Antioxidant response, cell survival	Salazar et al. (2006), Rada et al. (2011)
SKN-1	**S393**xxxS397	Oxidative stress, detoxification	An et al. (2005)
Sterol regulatory element-binding protein	**T426**xxx**S430**xxx?	Lipid and cholesterol metabolism	Sundqvist et al. (2005)
MafA/RIPE3β1	**…?…**	Regulates insulin gene expression in β cells of pancreas, pancreatic development	Han et al. (2007)

negative limb of the circadian machinery. PER and CRY proteins are classically thought to translocate into the nucleus to inhibit CLOCK:BMAL1 mediated transcription, thereby closing the negative feedback loop (reviewed in Sahar and Sassone-Corsi, 2009).

GSK-3 is expressed in the primary center of circadian rhythm regulation – the suprachiasmatic nucleus (SCN) of hypothalamus (Iitaka et al., 2005). GSK-3αmRNA is found at higher levels in the mouse SCN than GSK-3β (Iwahana et al., 2004). The expression of both GSK-3αprotein and the phosphorylated form of GSK-3 have a daily rhythm on the SCN, with peak expression of GSK-3α at ZT5 (Iwahana et al., 2004). Lithium treatment reduces the expression of GSK-3α in the SCN at CT5 and CT 11 (Iwahana et al., 2004).

Lithium has been shown to lengthen the period of circadian rhythms in a wide range of experimental systems, including unicellular organisms, insects, mice, and humans (Abe et al., 2000; Iwahana et al., 2004; reviewed in Engelmann, 1988). The GSK-3 ortholog in *Drosophila*, Shaggy (Sgg), plays a central role in determining circadian period length in flies (Martinek et al., 2001). For example, mutation of GSK-3 in *Drosophila* causes period lengthening (Martinek et al., 2001). Sgg (GSK-3) phosphorylates Timeless and regulates nuclear translocation of the Period/Timeless heterodimer (Martinek et al., 2001).

GSK-3 has also been demonstrated to phosphorylate and regulate the stability of "core" circadian rhythm genes in mammals. GSK-3 together with another serine kinase, DYRK1A, phosphorylates CRY2 at Ser 557 and 553 (respectively) resulting in degradation of CRY2 (Harada et al., 2005; Kurabayashi et al., 2010). GSK-3 phosphorylates BMAL1 (Ser17/Thr21) and these events control the stability of the proteins and the amplitude of circadian oscillation (Sahar et al., 2010). Moreover, GSK-3 has been found to phosphorylate Rev-erbα (Yin et al., 2006), as well as Clock (Spengler et al., 2009). GSK-3 interacts with Per2 *in vitro* and *in vivo*, phosphorylates Per2 *in vitro* and promotes nuclear translocation of Per2 (Iitaka et al., 2005; Kaladchibachi et al., 2007). Overexpression of GSK-3 caused a ∼2 h advance in the phase of mPER2 (Iitaka et al., 2005). Genetic depletion of two alleles of GSK-3β in combination with deletion of one allele of GSK-3α in synchronized oscillating mouse embryonic fibroblasts (3/4 GSK-3α/β KO MEFs) resulted in a significant delay in the period of endogenous clock mechanism, particularly in the cycling period of Per 2 (Kaladchibachi et al., 2007). In contrast, one study revealed that siRNA knockdown of GSK-3β or treatment with GSK-3 inhibitors (CHIR 99021 and 1-azakenpaullone) shortened the circadian rhythm (Hirota et al., 2008); however, the same study observed prominent period lengthening by lithium in another experimental system (Hirota et al., 2008). Nevertheless, pharmacological inactivation of GSK-3 by a related molecule (kenpaullone) induced a phase delay in Per2 transcription (Kaladchibachi et al., 2007).

ANIMAL MODELS OF GSK-3

Several genetic approaches have been used to generate mutant mice for GSK-3: conventional knockouts and knock-ins (all tissues), conditional knockouts (tissue-specific), and transgenic mice (**Table 2**). Use of mice harboring genetic inactivation or overexpression of one or both of the GSK-3 genes has proven a powerful means to study GSK-3 function in brain development, morphology, neurogenesis, memory and learning, sensorimotor function, sociability, emotionality as well as depressive-like animal behaviors. The listings below are not exhaustive as they focus on publications describing findings relevant to brain functions. There are many more investigating the role of GSK-3 in other tissues (including mammary gland, liver, heart, etc.).

Table 2 | Animal models of GSK-3.

Type of approach	Mouse design	Mouse name	Characterized by (reference)
CONVENTIONAL			
Knockout	Deletion of exon 2 (ATP-binding loop) of GSK-3α	GSK-3α KO	MacAulay et al. (2007), Kaidanovich-Beilin et al. (2009), Lee et al. (2011), Lipina et al. (2011)
Knockout	Deletion of exon 2 (ATP-binding loop) of GSK-3β	GSK-3β KO	Hoeflich et al. (2000)
Knockout	Deletion of exon 2 (ATP-binding loop) of GSK-3β	GSK-3β HET	Hoeflich et al. (2000), Beaulieu et al. (2004), O'Brien et al. (2004), Beaulieu et al. (2008), Bersudsky et al. (2008), Kimura et al. (2008)
TRANSGENIC			
Knock-in	Mutations GSK-3 α^{S21A}, β^{S9A}	GSK-3α, β [S21A,S9A] KI	McManus et al. (2005), Eom and Jope (2009), Ackermann et al. (2010), Mines et al. (2010), Polter et al. (2010)
CONDITIONAL			
Double shRNA knockdown	GSK-3α/β shRNA (shGSK-3-dh$^{+/flox}$) × Nestin-Cre	Nestin-Cre/shGSK-3-dh$^{+/\Delta}$	Steuber-Buchberger et al. (2008)
Conditional knockout	GSK-3α/GSK-3β $^{flox/flox}$ × Nestin-Cre	Nestin-GSK-3α + β KO	Kim et al. (2009)
Double shRNA knockdown	GSK-3β shRNA	DG–GSK-3β knockdown (shRNA)	Omata et al. (2011)
TRANSGENIC			
Dominant-negative (DN) GSK-3β	K85RGSK-3β × CamkII-tTA-Cre	DN-GSK-3β	Gomez-Sintes et al. (2007)
Overexpression of GSK-3β	TetO GSK-3β × CamkII-tTA-Cre	Tet/GSK-3β	Lucas et al. (2001), Hernandez et al. (2002), Engel et al. (2006), Hooper et al. (2007)
Overexpression of constitutively active GSK-3β [S9A]	S9AGSK-3β in Thy-1 gene vector	GSK-3β [S9A]	Spittaels et al. (2000), Spittaels et al. (2002), Prickaerts et al. (2006)
Overexpression of GSK-3β	*Xenopus* GSK-3β × mouse prion promoter MoPrP.Xho	PrpGSK-3β^{L56} and PrpGSK-3β^{L64}	O'Brien et al. (2011)

CONVENTIONAL KO MICE

The first GSK-3 gene to be knocked out was GSK-3β (Hoeflich et al., 2000). These animals die late in development either due to hepatic apoptosis (Hoeflich et al., 2000) or a cardiac patterning defect (double outlet, right ventricle; Kerkela et al., 2008).

GSK-3β heterozygous (HET) mice are viable, morphologically normal and have been tested extensively. These animals exhibit a lithium-mimetic, anti-depressant-like state (Beaulieu et al., 2004; O'Brien et al., 2004). Notably, the anti-depressant-like behavior in GSK-3β HET mice effectively normalizes the depressive behavior caused by serotonin deficiency (Beaulieu et al., 2008). Exploratory activity in these animals is reduced although general locomotion remains normal (O'Brien et al., 2004). GSK-3β HET animals show reduced responsiveness to amphetamine treatment (Beaulieu et al., 2004; O'Brien et al., 2004), but have increased morphine-induced locomotion (Urs et al., 2011). Sensorimotor function as well as coordination and balance are normal in GSK-3β HET mice (O'Brien et al., 2004; Bersudsky et al., 2008). GSK-3β HET mice demonstrate increased anxiety (Bersudsky et al., 2008) and reduced aggressive behavior (Beaulieu et al., 2008). Recent studies by Kimura et al. (2008) have revealed the importance of GSK-3β in memory reconsolidation in adult brain. Mice heterozygous for GSK-3β exhibit retrograde amnesia (Kimura et al., 2008). These animals have reduced memory reconsolidation but normal memory acquisition, suggesting that they might be impaired in their ability to form long-term memories.

In contrast to GSK-3β null mice, animals lacking GSK-3α are viable and exhibit improved insulin sensitivity and hepatic glycogen accumulation on the ICR background (MacAulay et al., 2007). However, these anti-diabetic properties are not significant on the C57BL6 background (Patel et al., 2011). Similar to GSK-3βHET mice (Beaulieu et al., 2004, 2008; O'Brien et al., 2004), GSK-3α mutants have decreased exploratory activity, decreased immobility time, and anti-aggression behavior (Kaidanovich-Beilin et al., 2009). GSK-3α KO animals also have abnormal behavioral features that are unique to mice lacking the GSK-3α gene, such as decreased locomotion, increased sensitivity to environmental cues, decreased social motivation, and novelty; impaired sensorimotor gating, associative memory, and coordination (Kaidanovich-Beilin et al., 2009). GSK-3α KO mice also exhibit decreased numbers of Purkinje cells in the cerebellum (Kaidanovich-Beilin et al., 2009), as well as decreased dendrite length and surface, but show no changes in spine density in the frontal cortex (Lee et al., 2011).

CONDITIONAL KNOCKDOWN MODELS

Two studies have employed shRNA knockdown to suppress expression of the two GSK-3 genes in mouse brain. Nestin-Cre was employed to drive shRNA expression in the brain progenitor compartment by excising LoxP flanked transcriptional stop sites. This approach resulted in partial reduction of GSK-3α and β protein levels (60 and 50%, respectively) in whole brain lysate (Steuber-Buchberger et al., 2008). These mice have partial embryonic or neonatal lethality (50% of expected offspring). The surviving double-shGSK-3α and β knockdown animals exhibited ∼50% of the body weight of littermate controls (Steuber-Buchberger et al., 2008).

In an alternative approach, lentivirus-expressing short-hairpin RNAs targeting GSK-3β were injected bilaterally into the hippocampus to inactivate GSK-3β in the dentate gyrus (Omata et al., 2011). These DG–GSK-3β knockdown mice showed decreased immobility time in both forced swim and tail suspension tests (TST), while the locomotor activity of these animals was unchanged (Omata et al., 2011). This technique achieved 30% suppression of GSK-3β in the hippocampus, sufficient to yield an anti-depressant-like behavior in the mice (Omata et al., 2011).

DOMINANT-NEGATIVE MUTANTS

Dominant-negatively acting mutants interfere with the endogenous proteins by soaking up downstream targets or upstream regulators. This approach has been used to generate conditional transgenic expression of a dominant-negative (DN) form of GSK-3β in the brain (Gomez-Sintes et al., 2007). Mutation of a critical residue involved in ATP-binding, Lys85, to Arg inactivates the protein kinase activity of GSK-3β (Dominguez et al., 1995). Double transgenic mice were generated that expressed dominant-negative GSK-3β in a tetracycline-repressible manner under control of a promoter that is active in the postnatal forebrain (CamKIIα-tTA × K85RGSK-3β). These DN–GSK-3β mice grew normally and showed no evidence of tumor formation (Gomez-Sintes et al., 2007). However, these animals exhibited increased levels of apoptosis in the brain regions involved in motor control as well as showing behavioral deficits in motor coordination (Gomez-Sintes et al., 2007). Suppression of the DN–GSK-3 transgene by doxycycline administration restored normal GSK-3 activity and resulted full reversal of the motor and of the neuronal apoptosis phenotypes (Gomez-Sintes et al., 2007).

OVEREXPRESSION OF GSK-3β

Overexpression of GSK-3β has been postulated to be embryonic lethal as viable transgenic animals show only modest levels of the exogenously engineered gene (Brownlees et al., 1997). Mice overexpressing GSK-3β in the forebrain have been generated by placing the transgene under the control of a tetracycline response element that is induced by administration of doxycycline (Lucas et al., 2001). These Tet/GSK-3β mice have decreased levels of nuclear β-catenin, increased phosphorylation of tau in Alzheimer's disease-relevant epitopes (correlated with somatodendritic accumulation of microtubule-unbound tau in hippocampal neurons), increased neuronal cell death, and reactive astrocytosis and microgliosis (Lucas et al., 2001). Behavioral characterization of Tet/GSK-3β mice revealed that these animals have impaired acquisition of reference memory in a novel object recognition task (Engel et al., 2006) and impaired spatial learning (Hernandez et al., 2002). Moreover, Tet/GSK-3β mice have reduced LTP induction, a deficit that was rescued by chronic treatment with lithium (Hooper et al., 2007). Thus, mice with conditional overexpression of GSK-3 in forebrain neurons (Tet/GSK-3β) recapitulate aspects of Alzheimer's disease neuropathology such as tau hyperphosphorylation, apoptotic neuronal death, and reactive astrocytosis, as well as spatial learning deficits. Moreover, these sequelae can be completely reverted by restoration of GSK-3 activity by silencing of transgene expression indicating that these biological defects, at

least, may be responsive to therapeutic intervention (Engel et al., 2006).

Transgenic mice have also been generated that overexpress a mutant form of GSK-3β in which the inhibitory N-terminal phosphorylation site is mutated [S9A] (Spittaels et al., 2000, 2002). This form of the kinase cannot be inhibited by agonists of pathways that promote phosphorylation of this site (such as PI3K, cAMP, etc.). These mice are characterized by microcephaly and higher neuronal density due to reduction of the volume of the somatodendritic compartment (dendrites and cell bodies) of pyramidal neurons in the cortex (Spittaels et al., 2002). The levels of MAP2 were also significantly decreased in the brain and spinal cord of GSK-3β [S9A] mice (Spittaels et al., 2002). However, postnatal overexpression of this non-inhibitable form of GSK-3β in neurons did not alter behaviors of the mice in terms of general cognition and aging and they showed only a minor decline in psychomotor capability (Spittaels et al., 2002). Subsequent characterization revealed that mice with constitutive overexpression of GSK-3β [S9A] showed hypophagia, reduced water consumption, increased general locomotor activity, and increased ASR (acoustic startle response; Prickaerts et al., 2006). GSK-3β [S9A] mice showed reduced immobility times in the forced swim test (FST) but this is likely related to the hyperactivity of these animals (Prickaerts et al., 2006). There were no differences in baseline and stress-induced increases of plasma adrenocorticotrophic hormone and corticosterone levels in GSK-3β [S9A] mice (Prickaerts et al., 2006). Biochemical examination in these animals revealed upregulation of Akt1 together with downregulation of PPP2R3A (regulatory subunit of PP2A) and GSK-3α in the striatum, as well as increased brain-derived neurotrophic factor (BDNF) in the hippocampus (Prickaerts et al., 2006). In summary, mice overexpressing active GSK-3β [S9A] represent a model for studying hyperactivity, hyperreactivity, and disturbed eating patterns; aspects which recapitulate some of the symptoms observed in the manic phase of bipolar disorder patients, ADHD, and schizophrenia.

GSK-3α, β MUTANT KNOCK-IN MICE

As mentioned above, mutation of the N-terminal phosphorylation sites of GSK-3 renders the protein kinase insensitive to inhibition by that mode of regulation (although the kinase remains sensitive to Wnt regulation, for example). Mice have been generated in which the phosphorylation sites of the endogenous alleles have been replaced by non-phosphorylatable alanine (GSK-αS21A, βS9A). Since serine phosphorylation of GSK-3 is increased by lithium, anti-psychotic drugs, anti-depressants, etc., this model is attractive to use for studying the mechanism of action of aforementioned drugs and related pathological conditions. GSK-αS21A, βS9A knock-in mice have normal development and growth, with no signs of metabolic abnormalities/insulin resistance (McManus et al., 2005). However, these animals have a drastic (40%) impairment in neurogenesis, which is not increased/rescued by co-administration of fluoxetine and lithium (Eom and Jope, 2009). Expression of vascular endothelial growth factor (VEGF), but not BDNF, was reduced in GSK-αS21A, βS9A knock-in mice, suggesting that a deficiency in external support for neural precursor cells might contribute to impaired neurogenesis (Eom and Jope, 2009).

GSK-αS21A, βS9A knock-in mice exhibited increased susceptibility to hyperactivity and a heightened response to a novel environment (Polter et al., 2010). Moreover, these animals revealed increased susceptibility to amphetamine-induced hyperactivity, which was partially reversed by chronic lithium administration (Polter et al., 2010). Besides being sensitive to hyperactivity, these knock-in mutant mice displayed mild anxiety, had increased immobility time in FST and TST and were highly susceptible to stress-induced depressive-like behavior (Polter et al., 2010). In contrast, studies by another group revealed decreased immobility time in FST, indicating a phenotype less prone to depression (Ackermann et al., 2010). LTD in the mutants was found to be abnormal and emotion-associated memory was impaired (Polter et al., 2010). The knock-in animals also demonstrated impaired social preference (Mines et al., 2010). Additional studies revealed increased curiosity in these animals, associated with less sensitivity to application of chronic mild stress as well as decreased HPA axis activity (Ackermann et al., 2010).

CONDITIONAL KNOCKOUT MOUSE MODELS

Alleles of GSK-3α and β have been generated in which exon 2 (containing essential residues for ATP-binding) are flanked by LoxP (flox) sites (**Figure 1**). Tissue-specific expression of Cre recombinase allows selective excision of the critical exon and inactivation of the allele(s). Combination of a floxed GSK-3β gene on a GSK-3α null background has been employed to generate total GSK-3 nullizygous cells in the developing nervous system via nestin-Cre mediated excision (Nestin-GSK-3α, β KO; Kim et al., 2009). Selective deletion of both GSK-3α and β in neural precursor cells results in dramatic hyperproliferation of neuronal progenitors along the entire rostrocaudal extent of the neuraxis. The progenitor expansion was at the expense of neurogenesis, intermediate neural progenitors, and post-mitotic neurons indicating effective inhibition of the differentiation process (Kim et al., 2009). The morphological abnormalities were accompanied by dysregulation of β-catenin, Hedgehog and Notch signaling as well as loss of polarity of cell division (Kim et al., 2009).

LESSONS LEARNED FROM COMPARING BETWEEN GSK-3 ANIMAL MODELS

Ten different GSK-3 animal models have been described to date (**Table 2**). All of them display some kind of neuroanatomical and behavioral abnormalities (**Tables 3–6**). However some of them have not been fully characterized yet and require further examinations. Overall, analysis of different animal models supports *in vivo* role of GSK-3 in the regulation of fundamental brain functions (emotionality, sociability, learning and memory, and neurogenesis, etc.).

The differences in behavioral results between different GSK-3 animal models may be accounted for by the alternative design of the models, and/or by strain and gender differences, varying methodology, and animal house-keeping environment.

Well described and presented is effect of genetic GSK-3 manipulations on depressive-like phenotype in mice (**Table 3**). In all three models, inactivation of GSK-3α or β genes (**Table 3**) revealed anti-depressive phenotypes, supporting studies with GSK-3 inhibitors. However, a similar effect was found in mice

Table 3 | Emotionality behaviors of GSK-3 mutant mice.

Behavioral test	Type of test	GSK-3α KO	GSK-3α HET	GSK-3β HET	GSK-3αβ knock-in	GSK-3β [S9A] overexpression	DG–GSK-3β knockdown (shRNA)
Anxiety	EPM	▲ In females only (1)		▲ (2, 3)	Mild ▲ (6)		
	O-maze				▼ (11)		
	Light–dark box	≠ In males (1)					
Emotionality	Open field 5 min	▲ (Both genders) (1)					
Locomotor activity	Open field 30 min	▼ (1)	≠ (10)	≠ (4, 5, 2)	▲ (6, 11)	▲ (8)	≠ (12)
Exploratory activity/curiosity	Open field 30 min	▼ (1)		▼ (2, 5)	≠ (6)	▲ (8)	
	Light–dark box				▲(11)		
Depression-like behavior	Learned helplessness				Stress-induced ▲ (6)		
	FST	▼ (1)		▼ (2, 4, 5)	▲ (6), ▼ (11)	▼ (8)	▼ (12)
	TST	▼ (1)		▼(2, 4, 5)	▲ (6)		▼ (12)
Stress reactivity		Stress response▲ (1), ▼corticosteroids (1)			General ▼ (11) ≠ (6), stress response ▲(6), ▲body weight, food, and fluid intake (11), ▼ (11) ≠ (6), corticosteroids, ▼ACTH (11)	≠ Corticosteroids (8)	

▲, *Increased;* ▼, *decreased;* ≠, *same/no changes.*

References for the comparison tables are: 1. Kaidanovich-Beilin et al. (2009), 2. Bersudsky et al. (2008), 3. Beaulieu et al. (2008), 4. Beaulieu et al. (2004), 5. O'Brien et al. (2004), 6. Polter et al. (2010), 8. Prickaerts et al. (2006), 10. Lipina et al. (2011), 11. Ackermann et al. (2010), 12. Omata et al. (2011).

Table 4 | Sociability behaviors of GSK-3 mutant mice.

Type of behavior	Type of the test	GSK-3α KO	GSK-3β HET	GSK-3αβ KI
Sociability and social novelty	Social interaction test	▼ (1)		Impaired social preference to Str2 (7)
Aggression	Resident intruder	▼ (1)	▼ (Beaulieu et al., 2008)	

▼, *Decreased.*

References for the comparison tables are: 1. Kaidanovich-Beilin et al. (2009), 7. Mines et al. (2010).

overexpressing GSK-3β (GSK-3β S9A mice; **Table 3**). The contradictory result in GSK-3β S9A mice was explained by increased locomotor activity in these animals, which may affect performance in the FST and its interpretation.

It is important to mention that different GSK-3 animal models have employed different "Cre" promoters. Activation of specific "Cre" recombinases may happen at different stages of embryogenesis (or after birth), thus may affect specific neuronal populations (post-mitotic or precursors), which may affect structure and function of adult brain. For example, dominant-negative GSK-3β and Tet/GSK-3β mice have been generated by using CamkIIα-Cre, compare to GSK-3β S9A mice which have been created by using Thy-1 gene promoter (**Table 2**).

Moreover, there are different approaches have been used to generate mice with overexpression of GSK-3β gene. In all three models with overexpression of GSK-3β, different constructs for GSK-3β gene itself were used (**Table 2**): intact GSK-3β in Tet/GSK-3β mice versus point mutated form of GSK-3β (S9A) in GSK-3β[S9A] animals. GSK-3 has complex mechanisms of regulation, thus, is it likely that overexpression of wild type protein has different effects on specific brain functions than Serine 9 mutated forms of the protein, depending on the relative importance of "phosphorylation" as the regulatory mechanism in specific brain process/stimulation and structure.

Comparison between GSK-3α KO and GSK-3α + β serine phosphorylation site KI mice revealed similar impaired sociability in both models, despite different genetic approaches being used. These data indicate that both the protein level of GSK-3α as well as serine phosphorylation of GSK-3 are important aspects for neuronal circuits responsible for social interaction.

Moreover, studying and analyzing genetic animal models may be used to make predictions about long-term usage of GSK-3

Table 5 | Memory, informational process, pharmacology, coordination behaviors of GSK-3 mutant mice.

Type of behavior	Type of the test	GSK-3α KO	GSK-3α HET	GSK-3β HET	GSK-3αβ KI	GSK-3β [S9A] overexpression	Tet/GSK-3β	DN–GSK-3β
Amphetamine response	OF + amp	▲		▼ (4)	▲ (6)			▼ (16)
Response to morphine	OF + morph			▲ (23)				
Information processing	PPI/ASR	▲ PPI (1)	≠ (10)	≠ (2, 5)		▲ASR (8)		
	LI	▲ NPE in KO (1)	≠ (10)					
Long-term memory	FC	▼ (1)		Impaired memory reconsolidation (9)	▲ (6), Enhanced emotion-associated memory			
	Passive avoidance	≠ (1)						
	LTP/LTD				abnormal LTD in the ventral hippocampus (6)		▼ (20)	
Spatial memory							Impaired (22)	
Coordination, balance	Rotarod	▼ (1)		≠ (2, 5)				▼ (16)
Motor learning	Rotarod	≠ (1)		≠ (2, 5)				

▲, *Increased;* ▼, *decreased;* ≠, *same/no changes.*

References for the comparison tables are: 1. Kaidanovich-Beilin et al. (2009), 2. Bersudsky et al. (2008), 4. Beaulieu et al. (2004), 5. O'Brien et al. (2004), 6. Polter et al. (2010), 8. Prickaerts et al. (2006), 9. Kimura et al. (2008), 10. Lipina et al. (2011), 16. Gomez-Sintes et al. (2007), 20. Hooper et al. (2007), 22. Hernandez et al. (2002).

inhibitors (as therapeutic agents). For example, the well characterized GSK-3 inhibitor – lithium – has a diverse spectrum of effects after long-term treatment of patients, including tremor and death of Purkinje cells. Of note, similar changes in cerebellar structure and function were observed in GSK-3α KO and dominant-negative GSK-3β mice (**Tables 5** and **6**).

Thus, comparative analysis of different animal models may be very informative, however critical and combinatory approach needs to be used to make correct interpretation and right conclusions.

PATHOGENESIS OF NEUROLOGICAL DISORDERS THOUGH CROSSBREEDING OF GSK-3 MUTANT MICE AND OTHER NEUROLOGICAL MUTANTS

To study the role of GSK-3β in the pathogenesis of Alzheimer's disease, particularly with respect to the mechanism of tauopathy, double transgenic mice have been generated by inter-breeding mice overexpressing GSK-3β [S9A] with transgenic mice that overexpress the longest isoform of human protein tau (Spittaels et al., 1999, 2000). Biochemical examination of brain and spinal cord extracts from double transgenic mice revealed that the amount of protein tau associated with microtubules was reduced compared to mice harboring only hTau40 (Spittaels et al., 2000). Moreover, unbound tau protein was hyperphosphorylated suggesting that additional phosphorylation of tau in the double transgenic mice reduces its binding capacity to microtubules, but fails to cause tau filament formation (Spittaels et al., 2000). Importantly, virtually all of the pathological defects observed in the hTau40 transgenic mice (Spittaels et al., 1999) were rescued by the mild overexpression of GSK-3β, such as the reduction by about an order of magnitude of the number of axonal dilations in the brain and spinal cord, the reduction in axonal degeneration and muscular atrophy, as well as the alleviation of most motoric complication (Spittaels et al., 2000).

DISC1 (*Disrupted-in-Schizophrenia-1)* is one of the best characterized genetic risk factors for schizophrenia (reviewed in Harrison and Weinberger, 2005; Chubb et al., 2008; Brandon et al., 2009; Jaaro-Peled et al., 2009). One breakpoint of a chromosomal *t*(1;11)(q42.1;q14.3) translocation has been identified within DISC1 gene, which co-segregates in a Scottish family with major mental illness, including schizophrenia, bipolar disorder, and major depression (Millar et al., 2000; Blackwood et al., 2001). DISC1 has been demonstrated to play a role in essential brain functions from embryonic development through to adulthood (reviewed in Jaaro-Peled et al., 2009), such as neurogenesis, neuronal migration, neurite outgrowth, spine development, neurotransmitter signaling, cytoskeletal organization, cell cycle, signal transduction, intracellular transport/exocytosis, etc.(reviewed in Chubb et al., 2008; Brandon et al., 2009; Jaaro-Peled et al., 2009). DISC1 appears to act as a coordinating hub or scaffold protein and has multiple intracellular interacting proteins including GSK-3 (Camargo et al., 2007; Mao et al., 2009; Lipina et al., 2011).

GSK-3 acts as an important downstream component in the etiology of schizophrenia (reviewed in more detail elsewhere in this

Table 6 | Neurogenesis, anatomical changes, biochemical, histological, and molecular characterizations of GSK-3 mutant mice.

Type of analysis	Type of test	GSK-3α KO	Tet/GSK-3β	GSK-3αβ KI	GSK-3β [S9A] overexpression	Nestin-GSK-3αβ KO	DN–GSK-3β
Neuro genesis	BrdU [3H] thymidine incorporation			▼ Proliferation (14)	≠ Proliferation (8)	▲ Proliferation (15), ▼ differentiation (15)	
Apoptosis necrosis	TUNEL, caspase IHC		▲ Apoptosis (17)	≠ Apoptosis (14)	≠ Apoptosis (18), ≠ necrosis (18)		▲ Apoptosis (16)
Neuro anatomical changes	MRI	▲ Cerebellum (1)			▼ Cerebellum, cerebrum, hippocampus, cortex (18)		
	Histology	▼ Dendrite length and surface area in the frontal cortex (12), ≠ in spine density (12), ≠ dendritic arborization (12), ▼Purkinje cells (1)	▲ Microgliosis (17), ≠ tau fibrils (17, 22)		▲ Neuronal density in cortex (18), ≠ number of cortical neurons (18), ▼ caliber of the proximal and distal part of the apical dendrites (18), ▼ size of the cell body of pyramidal neurons (18)	▼ Tuj1, MAP2, SMI32, NeuN (15), ▲Nestin, Pax6 (15)	
	Brain weight	▲ Brain (1)			▼ Brain and spinal cord (18, 8)		
Biochemical molecular	Western blot RTPCR		▲ pTau (17), ▼ nuclear β-catenin (17)	▼ VEGF (14), ≠ BDNF (14)	▼ MAP2 (18), ▲ pTau in old mice (19), ▼GSK-3α, ▼PPP2R3A and ▲Akt1 in striatum (8), ▲BDNF in hippocampus (8)	▲ β-catenin, Axin, c-jun, Gli1, Gli2, Patched, Hes1, Hes5, NICD, c-myc, N-myc (15)	▼pTau (16), ≠ β-catenin (16)

▲, *Increased;* ▼, *decreased;* ≠, *same/no changes.*

References for the comparison tables are: 1. Kaidanovich-Beilin et al. (2009), 8. Prickaerts et al. (2006), 12. Omata et al. (2011), 14. Eom and Jope (2009), 15. Kim et al. (2009), 16. Gomez-Sintes et al. (2007), 17. Lucas et al. (2001), 18. Spittaels et al. (2002), 19. Spittaels et al. (2000), 22. Hernandez et al. (2002).

Special Topic series). There are several lines of evidence supporting the involvement of GSK-3 in the pathogenesis of schizophrenia. Polymorphisms in GSK-3 genes have been associated with schizophrenia (Souza et al., 2008; Benedetti et al., 2010). Dysregulation of the PKB/Akt/GSK-3 signaling pathway has been found in subjects with schizophrenia (reviewed in Koros and Dorner-Ciossek, 2007; Lovestone et al., 2007; Beaulieu et al., 2009; Freyberg et al., 2009). For example, phosphorylation of GSK-3β Ser9 is reduced in the peripheral lymphocytes and brains of schizophrenia patients (Emamian et al., 2004). Drugs that influence the DA and 5-HT systems indirectly affect the activity of GSK-3 (this topic is reviewed in greater detail in another chapter in this Special Topic series). In the dopaminergic system, anti-psychotic, and psychotomimetic drugs alter GSK-3 function (Mai et al., 2002; Svenningsson et al., 2003; Beaulieu et al., 2004; Emamian et al., 2004; Li et al., 2007). Lithium is used to augment anti-psychotic treatment in schizophrenia patients (Kang et al., 2004; Gould, 2006). Moreover, GSK-3 inhibitors can rescue schizophrenia-like behaviors in mice (Beaulieu et al., 2004; Lipina et al., 2011).

Several mouse models for Disc1 have been described (reviewed in Jaaro-Peled et al., 2009). For example, overexpression of the N-terminal portion of Disc1 in the dentate gyrus causes interaction with GSK-3, suppressing activity, and perturbing ability to down-regulate the Wnt/β-catenin pathway resulting in proliferation of neuronal progenitors (Mao et al., 2009). Treatment with a GSK-3 inhibitor, SB216763 rescued the behavioral effects of lentivirally induced DISC1 suppression in the adult dentate gyrus (Mao et al., 2009).

An ENU-induced mutant of Disc1, Disc1-L100P, exhibits schizophrenia-related behaviors in mice (Clapcote et al., 2007). Pharmacological as well as genetic inactivation of one allele of GSK-3α reverses pre-pulse inhibition (PPI) and latent inhibition (LI) deficits as well as normalizing the hyperactivity of Disc1-L100P mutants (Lipina et al., 2011). In parallel to these observations, interaction between DISC1 and GSK-3α and β is reduced in Disc1-L100P mutants (Lipina et al., 2011). At the histological level, genetic inactivation of GSK-3α partially corrected neurite outgrowth and spine development abnormalities in the frontal cortex induced by the Disc1-L100p mutation (Lee et al., 2011).

SUMMARY

The emergence of sophisticated animal models with tissue and developmentally selective expression of GSK-3 has allowed direct assessment of the roles of this protein kinase in a variety of neurological processes and conditions. Clearly, the complexity of brain development and disease pathogenesis requires the use of animal models to examine the biological role of candidate components and with the numbers of candidate genes for neurological illness increasing, allows relatively rapid assessment of genetic interactions through inter-breeding of variant alleles.

While GSK-3 was first implicated in a neurological disorder in 1992 through its capacity to phosphorylate residues on Tau that are associated with neurofibrillary tangles in AD, the potential importance of this kinase in brain function and disease took off with the identification by Klein and Melton of GSK-3 as a direct target of lithium (Klein and Melton, 1996; Stambolic et al., 1996). Since that time, there has been enormous expansion of understanding of this protein kinase with respect to regulation, roles in normal development, and in pathophysiology. However, despite this tsunami of knowledge, there are many remaining questions including the therapeutic reality of modulating GSK-3 in these disorders. Since the beneficial effects of lithium on stabilization of bipolar disorder is achieved at serum levels that reduce GSK-3 levels by only 25% (noting that there are likely several other targets of this drug) and the behavioral phenotypes of Disc1L110P mutations are alleviated by knocking out only one allele of GSK-3α (Lipina et al., 2011), subtle drug modulation of GSK-3 may be sufficient for therapeutic benefit in humans.

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Functions of GSK-3 signaling in development of the nervous system

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Glycogen synthase kinase-3 (GSK-3) is central to multiple intracellular pathways including those activated by Wnt/β-catenin, Sonic Hedgehog, Notch, growth factor/RTK, and G protein-coupled receptor signals. All of these signals importantly contribute to neural development. Early attention on GSK-3 signaling in neural development centered on the regulation of neuronal polarity using *in vitro* paradigms. However, recent creation of appropriate genetic models has demonstrated the importance of GSK-3 to multiple aspects of neural development including neural progenitor self-renewal, neurogenesis, neuronal migration, neural differentiation, and synaptic development.

Keywords: GSK-3, neural progenitor, neuronal migration

INTRODUCTION

Glycogen synthase kinase-3 (GSK-3), a serine–threonine kinase, was originally identified as an important component of the glycogen metabolism pathway. Later, however, it was found to be an important regulator in multiple intracellular contexts. Recent studies using pharmacological and genetic approaches have suggested important functions for GSK-3 in an array of neural developmental activities including neural progenitor homeostasis, neuronal migration, neurite growth/specification, and synapse development. Further, recent data indicate that the Disrupted in Schizophrenia protein, DISC1, acts in part via GSK-3 suggesting that dysregulated GSK-3 activity during development may contribute to the pathogenesis of schizophrenia. Multiple proteins that play important roles during neural development have been identified as either GSK-3 substrates or binding partners (**Table 1**).

ISOFORMS OF GSK-3 IN THE NERVOUS SYSTEM

In mammals, the GSK-3 family consists of two members, GSK-3α and GSK-3β that show 98% sequence identity within their kinase domains and overall share 85% amino acid sequence identity (reviewed in Doble and Woodgett, 2003). Both GSK-3 isoforms are highly expressed in the nervous system including brain and spinal cord. There are two splice variants of the GSK-3β and one variant GSK-3β2 is highly expressed in the developing nervous system (Mukai et al., 2002). It is interesting that GSK-3β2 is only expressed in differentiated neurons, not in glial cells while GSK-3β1 is expressed in glial cells (Wood-Kaczmar et al., 2009). Further, GSK-3β2 is upregulated in PC12 cells when induced by nerve growth factor to differentiate into a neuronal phenotype (Goold and Gordon-Weeks, 2001). These findings suggest that differential regulation of GSK-3β isoform levels may be important in the initiation of neuronal differentiation and the maintenance of neurons during nervous system development.

Most functional studies of GSK-3 functions in mammalian neural development have relied on pharmacological inhibitors in dissociated preparations. However, since pharmacological GSK-3 inhibitors target ATP-binding pockets of both isoforms, distinctive functional analysis of each isoform using pharmacological inhibitors is not possible. Recently, studies in gene targeted mice have begun to yield decisive information regarding the roles of GSK-3 isoforms in neural development. Gene targeting of GSK-3β leads to late embryonic lethality via liver apoptosis or heart defects (Hoeflich et al., 2000; Kerkela et al., 2008). Surprisingly, the developing nervous system of GSK-3β null mice including the brain and spinal cord exhibits grossly normal morphology (Kim et al., 2006). GSK-3β heterozygous (+/−) mice, however, show multiple neurobehavioral abnormalities including aggressive behaviors, increased anxiety, reduced movement, and poor memory processing (O'Brien et al., 2004; Beaulieu et al., 2008; Kimura et al., 2008). Further, overexpression of GSK-3β in the brain results in hyperactivity and mania (Prickaerts et al., 2006). Finally GSK-3β is important in signaling downstream of dopamine receptors (Beaulieu et al., 2004). These results demonstrate that the GSK-3β isoform has important functions in regulation of behavior, probably via regulation of signaling downstream of neurotransmitter receptors.

GSK-3α null mice are viable and fertile (MacAulay et al., 2007) and show no abnormalities of gross anatomy in the developing brain (Kim et al., 2009; Yokota et al., 2010). However, young adult GSK-3α null mice show decreases in the size and number of cerebellar Purkinje cells and changes in social interaction and locomotion (Kaidanovich-Beilin et al., 2009). GSK-3α deletion also partially rescues the dendritic abnormality in cortical neurons of DISC1-L100P mutant mice that show reduced spine density compared to wild type mice (Lee et al., 2011). Further, GSK-3α null mice are hypersensitive to insulin (MacAulay et al.,

Table 1 | Glycogen synthase kinase-3-interacting proteins associated with neural development and diseases.

Name	Neural development	Diseases
Amyloid precursor protein	Neurogenesis, neuron migration, synaptic development	Alzheimer
APC	Neurogenesis, neuron migration, neurite growth/polarization	Autism, cancer
Axin	Neurite growth/polarization	Cancer
β-catenin	Progenitor renewal, neurogenesis, neuron migration, neurite growth/polarization, synaptic development	Cancer
CDK5	Neurogenesis, neuron migration, synaptic development	Alzheimer, Parkinson, Huntington, ALS
c-Jun	Progenitor apoptosis, neuron migration, neurite growth/polarization	Cancer, Alzheimer, Parkinson
CLASP1, CLASP2	Neurite growth/polarization	
c-Myb	Progenitor renewal, neuronal apoptosis	Cancer, liver disease
c-Myc	Progenitor renewal, neurite growth/polarization	Cancer, Alzheimer
CREB	Progenitor renewal, neurogenesis, neuron migration, neurite growth/polarization, synaptic development	Alzheimer, Huntington
CRMP2	Neurite growth/polarization	Alzheimer
cyclin D1	Progenitor renewal, neurogenesis	Cancer
δ-catenin	Neurite growth/polarization, synaptic development	Cri-du-Chat Syndrome
DISC1	Progenitor renewal, neuron migration, neurite growth/polarization synaptic development	Schizophrenia, autism
Dvl	Neurite growth/polarization	Cancer
eIF2B	Neurite growth/polarization	Leukoencephalopathy
Kinesin light chain	Progenitor renewal, neuron migration, neurite growth/polarization	Alzheimer, ALS
LRP6	Dopaminergic neuron differentiation	Heart disease
MAP1B	Neurite growth/polarization	Fragile X syndrome, giant axonal neuropathy, Alzheimer, Parkinson
MAP2	Neurite growth/polarization	Alzheimer
Myelin basic protein	Myelination	MS, seizure
NCAM	Neuron migration, neurite growth/polarization	Depression, Alzheimer, Parkinson
NeuroD	Neurogenesis, neurite growth/polarization	
Neurogenin2	Neurogenesis, neurite growth/polarization	
NFAT	Progenitor renewal, neurogenesis, neurite growth/polarization	Cancer, Alzheimer, heart disease, kidney disease
Ninein	Progenitor renewal, neurite growth/polarization	
P53	Neurite growth/polarization, neuronal apoptosis	Cancer
Presenilin 1	Neuron migration, neurite growth/polarization, synaptic development	Alzheimer
PTEN	Progenitor renewal, neurogenesis, neuron migration, neurite growth/polarization, synaptic development	Autism, cancer, Cowden syndrome
SMAD1	Neurite growth/polarization	
Tau	Neurite growth/polarization	Alzheimer
TSC2	Neuron migration, neurite growth/polarization	Tuberous sclerosis, autism

2007), suggesting the potential roles of GSK-3α in hypothalamic regulation. Using brain lysates of GSK-3α null, β null, and double knockout mice, a recent study has demonstrated isoform-specific activities on substrate phosphorylation in the brain. Each isoform produces distinct phosphorylation patterns of CRMP2, CRMP4, and Tau (Soutar et al., 2010). Together, these results indicate that GSK-3α isoform has important specific functions in postnatal neuroanatomy and behavioral regulation.

The lack of gross brain developmental abnormalities in GSK-3α null and GSK-3β null mice suggests that either isoform might compensate for the other in the regulation of differentiation, survival, and morphogenesis of neurons and glia. Indeed, axon and dendritic morphogenesis is normal in cultured hippocampal neurons from GSK-3β null embryonic brains (Kim et al., 2006). However, genetic elimination of GSK-3 using an shRNA that targeted both GSK-3 isoforms lead to strong changes in axon and dendritic morphogenesis in cultured hippocampal neurons (Kim et al., 2006). Further, the possibility of compensation has now been confirmed *in vivo*. Conditional elimination of both GSK-3α and β at an early stage (∼E10) of mouse nervous system development results in dramatic effects on murine brain development (Kim et al., 2009).

REGULATION OF NEURAL PROGENITORS

There are several types of neural progenitors in rodent developing brains (**Figure 1**). Radial neural progenitors are a major population of neural progenitors and occupy the ventricular zone in the developing brain. They display long radial processes to form a scaffold covering from apical ventricular membrane to pial surface.

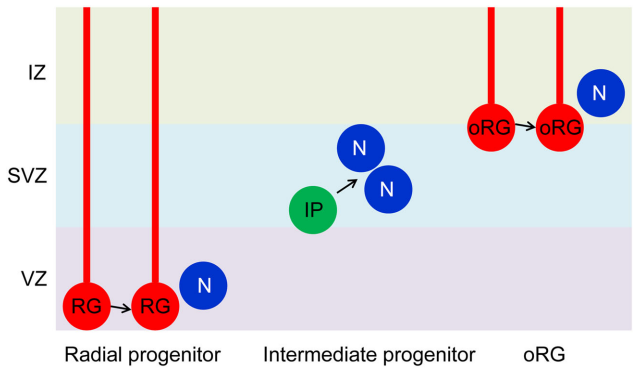

FIGURE 1 | Neural progenitors in the developing brain. Radial glial progenitors (RG) arise early in development from neuroepithelial cells. They are located in the ventricular zone (VZ) and have long radial processes that extend form VZ to the pial surface. A RG divides symmetrically to self-renew and asymmetrically to generate a daughter RG and a post-mitotic neuron or an intermediate progenitor (IP). IPs localize in the subventricular zone (SVZ) and divide symmetrically to produce neurons. There is another type of progenitors (oRG) in the superficial layer of SVZ or in the intermediate zone (IZ). Like RGs, oRGs have glial processes and can self-renew as well as generate neurons. For more information on oRG, see a recent review by Kriegstein and colleagues (Lui et al., 2011). The role of GSK-3 in regulating radial progenitors is now established. Functions related to IPs and oRGs remain to be investigated.

This scaffold supports neuronal migration. Intermediate progenitors in the subventricular zone execute only the final neurogenic division. In addition to radial and intermediate progenitors, there are other types of neural progenitors more prominent in higher species. Importantly, recent studies show existence of a greatly expanded oRG (outer subventricular zone progenitors) in human (Hansen et al., 2010) and mice (Wang et al., 2011). oRG progenitors are located in the outer region of the subventricular zone in rodent. Unlike intermediate progenitors, this type of progenitor can undergo self-renewal as well as symmetric neurogenesis.

EFFECTS OF GSK-3α AND β DELETION

Recent pharmacological evidence has strongly implicated GSK-3 signaling in the regulation of embryonic stem cell self-renewal (Sato et al., 2004; Umehara et al., 2007; Ying et al., 2008; Bone et al., 2009). Further, DISC1 implicated in some familial cases of schizophrenia and depression, regulates neural progenitor proliferation and may act in part via regulation of GSK-3 activity (Mao et al., 2009). These observations underscore the importance of determining GSK-3 functions in relation to neural progenitor self-renewal and neurogenesis during brain development *in vivo*.

The functions of GSK-3 in mammalian brain development remained unclear because GSK-3 single isoform mutants had not been associated with major brain developmental abnormalities (see above). The results, however, did not exclude possible compensatory effects of one GSK-3 isoform for the other in single null mutant animals (Doble et al., 2007). To explore GSK-3 functions definitively, a recent study pursued a conditional knockout strategy in which a GSK-3β floxed allele was bred onto GSK-3α null background to allow tissue specific elimination of GSK-3 signaling (Kim et al., 2009). This study used a mouse strain expressing Cre recombinase under the control of the neural specifying elements of the nestin promoter (Tronche et al., 1999) to induce recombination around E10 in neuroepithelial cells as they convert to radial progenitors and initiate neurogenesis (Yoon et al., 2008; Yokota et al., 2009). The study conclusively demonstrated that elimination of both GSK-3 isoforms in the developing brain lead to a massive increase in neural progenitor proliferation. This increase was indicated by infolding of the developing cortical wall, major increases in numbers of cells that expressed mitotic markers, and a dramatic expansion of the population of neural progenitors expressing Sox2. GSK-3 double knockout brains showed increased progenitor division, premature re-entry and shortening of the cell cycle.

Glycogen synthase kinase-3 deficient cells appeared to be locked into the radial progenitor phase as generation of both intermediate neural progenitors and post-mitotic neurons was markedly suppressed. In contrast to the expansion of Sox2-positive radial progenitors, the population of intermediate neural progenitors marked with Tbr2 was dramatically down-regulated in GSK-3 double knockout brains (Kim et al., 2009). Consistent with this finding, only around one-third of the normal number of neurons was generated in GSK-3 double knockout brains. Although deletion of GSK-3 prevented progression of most cells beyond the radial progenitor phase, a few progenitors were still capable of differentiation as manifested by expression of Tbr2 and markers of post-mitotic neurons. Whether some progenitors can truly progressed independently of GSK-3 signaling or whether the differentiated cells represented a population of radial progenitors that had some persistent GSK-3 proteins due to either late or incomplete deletion of GSK-3 remained unclear.

MECHANISMS ASSOCIATED WITH GSK-3 REGULATION OF NEURAL PROGENITORS

Multiple extracellular signals and intracellular signaling mechanisms that regulate neural progenitor proliferation and differentiation have been identified (reviewed in Corbin et al., 2008). The mechanisms that have received the most attention in the dorsal telencephalon are β-catenin/LEF1 and Notch signaling (**Figure 2A**). Whether these mechanisms operate independently or are coordinately regulated is an interesting question. GSK-3 has the potential to influence both mechanisms. It is important to emphasize that GSK-3s are also key components in pathways downstream of two extracellular signals, FGF2 and Shh, which also strongly regulate progenitor proliferation in the developing telencephalon (**Figure 2A**; Vaccarino et al., 1999; Raballo et al., 2000; Komada et al., 2008; Shimizu et al., 2008).

β-*Catenin signaling and GSK-3*

It is well known that regulation of β-catenin is critical to control of progenitor proliferation in multiple regions of the developing nervous system (Chenn and Walsh, 2002; Machon et al., 2003, 2007; Zechner et al., 2003; Woodhead et al., 2006; Gulacsi and Anderson, 2008). As expected, β-catenin was increased substantially in GSK-3 double knockout brains, consistent with the critical importance of GSK-3 in phosphorylating β-catenin in the destruction complex that mediates canonical Wnt signaling (Kim et al.,

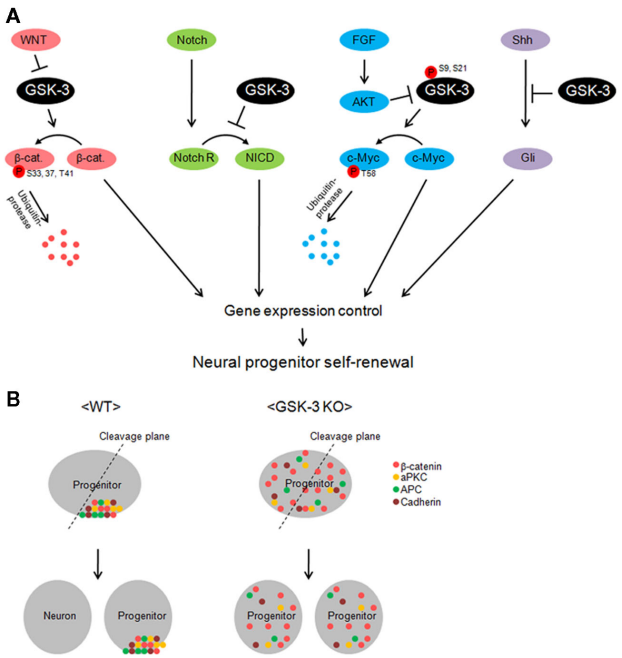

FIGURE 2 | Glycogen synthase kinase-3 is a key regulator of multiple signaling pathways in neural progenitors. (A) GSK-3 controls gene induction by regulating the levels of transcription factors in neural progenitors. β-catenin is degraded via GSK-3 phosphorylation-dependent ubiquitin-protease machinery in the resting state. However, inhibition of GSK-3 by Wnt allows β-catenin accumulation. GSK-3 is also critical in the control of c-Myc levels. c-Myc is phosphorylated at T58 and recruited by ubiquitin-protease for degradation. FGF inhibits GSK-3 via AKT-mediated phosphorylation at S9 (beta) or S21 (alpha) and leads to increase in c-Myc levels. Inhibition of GSK-3 increases Notch intracellular domain (NICD) and Gli proteins in neural progenitors. Thus, GSK-3 activity is critical in the control of transcription factors in multiple signal pathways, which eventually regulates neural progenitors during development. **(B)** A schematic model of the GSK-3 function in apical–basal polarity and neurogenesis. GSK-3 is required for polarity establishment and possibly for neurogenic division of progenitors. Polarity-associated molecules including β-catenin, atypical PKC, APC, and cadherin are localized in the apical membrane. When progenitors divide, daughter cells that inherit the polarity molecules are thought to become progenitors. Without the polarity protein, cells become neurons. However, in GSK-3-deleted neural progenitors, polarity molecules are distributed throughout the cell. Thus, wherever the cleavage plane is positioned, daughter cells have almost same composition of the molecules. This may be an important factor that leads constant self-renewal of progenitors in GSK-3-deleted brains.

2009). However, stabilization of β-catenin alone may not have fully accounted for the effects of progenitor hyperproliferation in GSK-3 double knockout brains. Although it is difficult to compare studies directly, it appears that the enlarged brain observed upon overexpression of a stabilized β-catenin is associated with a more normal pattern of neuronal differentiation than GSK-3 double knockout brains (Chenn and Walsh, 2002, 2003; Kim et al., 2009). Further, elimination of β-catenin in neural progenitors reduces but does not eliminate progenitor proliferation *in vivo* (Machon et al., 2003; Zechner et al., 2003; Gulacsi and Anderson, 2008). Finally, overexpression of dominant-negative TCF only partially inhibited proliferation in GSK-3 knockout neural progenitors *in vitro* (Kim et al., 2009). Thus, GSK-3 may regulate self-renewal of neural progenitors by mechanisms in addition to β-catenin signaling.

Notch signaling and GSK-3

Notch signaling is critical to the maintenance and the identity of radial glial cells during development (Gaiano et al., 2000; reviewed in Ohtsuka et al., 2001; Hatakeyama et al., 2004; Yoon and Gaiano, 2005; Mizutani et al., 2007; Shimojo et al., 2008). There are several mechanisms by which GSK-3 may regulate Notch signaling both directly and indirectly (Foltz et al., 2002; Espinosa et al., 2003; Uemura et al., 2007; Hayward et al., 2008; Shimizu et al., 2008; Jin et al., 2009a). For example, GSK-3β is able to bind and phosphorylate Notch2 *in vitro* and *in vivo* (Espinosa et al., 2003). The GSK-3α isoform likely has the same function, because the phosphorylation was inhibited by the treatment of Wnt (Espinosa et al., 2003). Thereby, GSK-3 inhibits the transcriptional activation of Notch target genes. Also, β-catenin reportedly enhances the level and transcription activity of Notch1 (Shimizu et al., 2008; Jin et al., 2009b). Meanwhile, there are conflicting studies with regard to the role of GSK-3 in Notch signaling, in which inhibition of GSK-3 using pharmacological drugs or genetic tools decreased NICD levels in neuroblastoma cells (Foltz et al., 2002) and vascular smooth muscle cells (Guha et al., 2011).

Notch signaling was shown to be increased in GSK-3 double knockout brains (Kim et al., 2009). Levels of Notch intracellular domain were markedly enhanced as were levels of the Notch downstream signaling mediators Hes1 and Hes5. Further, immunohistochemical data suggested that most or all of the extra progenitors in GSK-3 double knockout mice express high levels of Hes1 protein, indicative of active Notch signaling in these cells. Levels of Jagged-1, a Notch agonist, were increased in GSK-3-deleted brain lysates, suggesting that the activation of Notch pathway upon GSK-3-deletion requires a Notch agonist. This is different from the activation of β-catenin signal, which is induced in GSK-3-deleted cells in the absence of Wnt ligands (Doble et al., 2007). Overexpression of a dominant-negative Hes1 and pharmacological inhibition of Notch signaling partially inhibited proliferation in GSK-3-deleted progenitors (Kim et al., 2009). Interestingly, Notch signaling levels distinguish radial progenitors from intermediate neural progenitors that have attenuated canonical Notch signaling (Mizutani et al., 2007). Therefore, high levels of Notch signaling in GSK-3 double knockout cells may have suppressed neurogenesis at the stage of radial progenitors such that few cells progress to the stage of Tbr2-positive intermediate neural progenitors. However, whether GSK-3 regulated Notch signaling directly or indirectly via regulation of β-catenin or some other mechanism was not established.

GSK-3 regulation of Myc

GSK-3 is a downstream mediator of FGF/PI3K signaling in neural progenitors (Shimizu et al., 2008). Several recent studies have demonstrated that GSK-3 regulates the stability of Myc family proteins via the PI3K pathway (reviewed in Knoepfler and Kenney, 2006; Bechard and Dalton, 2009; reviewed in Doble and Woodgett, 2009; Otto et al., 2009). It is important to emphasize that GSK-3 can directly regulate the stability of Myc family proteins, independently of β-catenin effects on Myc transcription (Bechard

and Dalton, 2009; reviewed in Doble and Woodgett, 2009; Otto et al., 2009). Interestingly, these effects are thought to be downstream of RTK/PI3K rather than Wnt/Frizzled signaling (reviewed in Knoepfler and Kenney, 2006). Myc family proteins are key elements in the maintenance of neural progenitors by inhibiting differentiation (Knoepfler et al., 2002; Wey et al., 2010). Phosphorylation of c-Myc at T58 thought to be a key to its stability is regulated by GSK-3. The GSK-3-mediated phosphorylation at T58 plays an important role in inducing cell differentiation, as inhibition of the phosphorylation maintains embryonic stem cell pluripotency (Cartwright et al., 2005). This phosphorylation was shown to be eliminated in GSK-3 double knockout brains and the level of c-Myc was increased (Kim et al., 2009). Given the short half-life cycle of c-Myc (∼20 min; reviewed in Eilers and Eisenman, 2008), control of c-Myc stability via T58 phosphorylation may be a critical step in GSK-3 regulation of neural progenitor self-renewal.

Myc binds to promoters of many genes including cyclins and regulates their transcription. This traditional role as a transcription factor has been primarily considered in maintaining self-renewal and in inhibiting differentiation of stem cells. But several recent reports suggest that Myc contributes to stem cell maintenance in other ways by globally maintaining the histone acetylation and methylation euchromatin state (Lin et al., 2009; reviewed in Varlakhanova and Knoepfler, 2009; Araki et al., 2011). Whether Myc elicits changes in histone methylation patterns of promoters in neural progenitors and whether GSK-3 is associated with this process remain to be elucidated. Further characterization of functional genomic analysis will likely elucidate Myc functions as epigenetic transcriptional regulator in neural progenitors.

Apical–basal polarity and GSK-3

Cell polarity is a fundamental property of all cells including neural progenitors. GSK-3 is thought to be a key regulator of cell polarity and microtubule-cytoskeleton regulation based on *in vitro* studies (Etienne-Manneville and Hall, 2003; reviewed in Buttrick and Wakefield, 2008). GSK-3 directly binds to polarity-associated proteins including APC (Adenomatous polyposis coli), EB1 (End binding 1), and CLASPs (Cytoplasmic linker-associated proteins) and regulates their activities by phosphorylation. For example, APC and CLASPs promote microtubule stability and their phosphorylation by GSK-3 inhibits microtubule binding, thus destabilizing microtubules (Akhmanova et al., 2001; Zumbrunn et al., 2001). Asymmetric cell division results from polarized deposition of polarity proteins and it is required for neurogenic division of radial neural progenitors (Gotz and Huttner, 2005). Apical–basal polarity indicated by the distribution patterns of cell polarity/adhesion proteins including APC, EB1, cadherin, and aPKC was disrupted in GSK-3-deleted developing brains (Kim et al., 2009). Thus, the expansion of the radial progenitor pool and inhibition of neurogenesis in GSK-3-deficient brains may be attributed in part to the dysregulation of polarity-associated proteins (**Figure 2B**). Localized distribution patterns of apical molecules may be coordinated by GSK-3 regulation of the microtubule apparatus or motor proteins. In this regard, it is notable that elimination of polarity-associated molecules, Lgl1 and αE-catenin results in a major hyperproliferation of neural progenitors (Klezovitch et al., 2004; Lien et al., 2006).

It has also been demonstrated that GSK-3 regulates centrosome reorientation and microtubule spindle formation during cell division (Wakefield et al., 2003; Cheng et al., 2008; Izumi et al., 2008). Dysregulation of centrosome positioning and cleavage planes may favor divisions that result in additional progenitors rather than post-mitotic neurons (reviewed in Higginbotham and Gleeson, 2007). An important implication of these findings is that apical–basal polarity and centrosome positioning may be among of the mechanisms responsible for GSK-3-mediated neural progenitor homeostasis.

DISC1 and GSK-3 signaling

A recent study has shown that GSK-3 binds to DISC1 and that pharmacological inhibition of GSK-3 can mitigate effects of DISC1 shRNA knockdown on neural progenitor proliferation *in vitro* (Mao et al., 2009). DISC1 protein does not have enzymatic activity, rather it functions as a physiological scaffold by interacting with multiple proteins to modulate their functional activities. For example, DISC1 binds to NUDEL (Duan et al., 2007) and girdin (Enomoto et al., 2009), and modulates the activities of these proteins in adult neural progenitors and their progeny. Likewise, DISC1 may serve as an important scaffold to bridge GSK-3 and its regulators in neural progenitors during development.

HOMEOSTATIC CONTROLS IN NEURAL PROGENITORS

During nervous system development, an over-abundance of cells is produced presumably to accommodate the significant loss of cells that fail to achieve functionality. This overproduction allows for remarkable plasticity in nervous system development but requires exquisite inductive coordination. Temporal and spatial regulation of GSK-3 activity during nervous system development must be achieved in order to terminate neural progenitor proliferation and allow differentiation at just the right moment to yield the correct number of neurons. It is remarkable that regulation of the activity of a single kinase provides a powerful and simple mechanism to control neural progenitor homeostasis.

The functions of GSK-3 in radial neural progenitors have been discussed above. However, whether GSK-3 has significant roles in intermediate progenitors and oRGs remains to be elucidated. It will be important to see if GSK-3-deletion also results in self-renewal of these progenitors.

GSK-3 SIGNALING IN NEURONAL MIGRATION

After progenitor proliferation and neurogenesis, post-mitotic neurons migrate to final locations which in the cortex results in proper lamination. There is increasing evidence that GSK-3 may be important in regulating neuronal migration.

Wnt SIGNAL AND NEURON MIGRATION IN *C. ELEGANS*

Recent studies have shown that Wnt family molecules play important roles in controlling anterior/posterior (AP)-directed neuronal cell migration and polarity in *C. elegans* (reviewed in Silhankova and Korswagen, 2007). Multiple Wnt proteins function in parallel as instructive cues or permissive signals to control neuronal migration along the AP axis. EGL-20/Wnt is one of the five Wnt proteins of *C. elegans* and specifies posterior direction of Q neuroblast migration (Harris et al., 1996; Maloof et al., 1999). This protein activates a canonical Wnt pathway (β-catenin-dependent)

in the Q neuroblast (Salser and Kenyon, 1992; reviewed in Korswagen, 2002). Accordingly, overexpression of SGG-1, the *C. elegans* GSK-3β ortholog induced defects in Q cell migration far toward anterior region (Korswagen et al., 2002). Together, these suggest that GSK-3 signaling plays an important role in *C. elegans* neuron migration by negatively regulating Wnt signaling.

Wnt SIGNALING MOLECULES AND NEURONAL MIGRATION IN MICE

Considerable evidence both direct and indirect now implicates GSK-3 in regulation of neuronal migration in mice. Some evidence has come from functional studies of the GSK-3 target and destruction complex component, APC. In addition to its role in the destruction complex, APC is a microtubule-associated protein and participates in microtubule-based cytoskeleton dynamics (Barth et al., 2008). GSK-3 directly binds to APC and phosphorylates it in order to regulate its activity. APC phosphorylation by GSK-3 dissociates APC from microtubules. A recent study has demonstrated that APC serves an essential function in the maintenance of the polarized radial glial scaffold during mouse brain development (Yokota et al., 2009). Elimination of APC in radial progenitors by conditional gene targeting lead to striking defects in cortical lamination, suggesting an important role for APC in neuronal migration. However, it is not clear whether migration defects were entirely due to disruption of the radial glia scaffold or whether APC regulation of microtubules in neurons also contributed. It will be important to settles this issue with conditional elimination of APC specifically in early post-mitotic cortical neurons.

GSK-3 may also affect neuronal migration via regulation of β-catenin. Several studies have shown that genetic manipulation of β-catenin by either deletion or overexpression of a stabilized form in mice leads to the disruption of brain and spinal cord formation (Chenn and Walsh, 2002, 2003; Zechner et al., 2003). The malformation is mostly attributed to abnormal neural progenitor development. However, there were also abnormally localized neurons found in the mutant nervous system. Further, a separate study has demonstrated that β-catenin levels in progenitors influenced cortical neuron positioning (Mutch et al., 2009). A caveat of this interpretation is that the abnormal neuronal positioning may be due to a delay in progenitor fate determination. However, the β-catenin mutation studies again strongly suggest potential roles for GSK-3 in neuronal migration.

DISC1 regulates neuronal migration during brain development (Kamiya et al., 2005) and is strongly associated with GSK-3. DISC1 binds to GSK-3 and the interaction interferes with GSK-3 inhibition of the β-catenin signal in neural progenitors, resulting in progenitor proliferation (Mao et al., 2009). Recently, the interaction between DISC1 and GSK-3 has been implicated in control of cortical neuronal migration (Ishizuka et al., 2011). DISC1 bound to GSK-3 tightly during mid-embryonic stage (E14) in which neural progenitor proliferation is active (**Figure 3A**). But, DISC1 was dissociated with GSK-3 during later embryonic stages (E18) when neuronal migration and neuronal differentiation become dominant. These findings suggest that the DISC1/GSK-3 interaction determines transition of neural progenitor self-renewal to neuronal migration in the developing brain.

Additional relevant evidence about the role of GSK-3 in neuron migration has been recently reported. Radial migration and

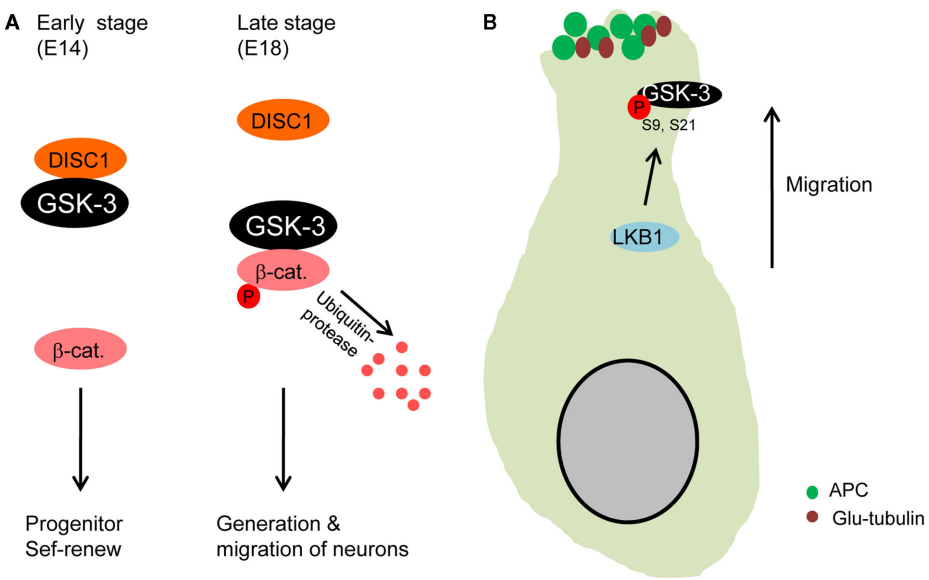

FIGURE 3 | Glycogen synthase kinase-3 in neuronal migration. (A) GSK-3 is required for neuronal migration. During early stages of development, DISC1 that is an important regulator of neuron migration binds to GSK-3, resulting in dissociation of GSK-3 from β-catenin. The increased β-catenin level directs progenitors to self-renew. However, the binding activity of DISC1 decreases at later stages of embryogenesis, which induces reduction in β-catenin level via GSK-3 phosphorylation. Progenitors then produce more neurons that subsequently migrate from the ventricular zone (Ishizuka et al., 2011). **(B)** Microtubules are stabilized at the leading edge of migrating neurons by GSK-3 inactivation. LKB1 inactivates GSK-3 by the phosphorylation at S9. APC is dissociated from GSK-3 and binds to microtubules at the leading edge of migrating neurons (Asada and Sanada, 2010), which stabilizes microtubules (glu-tubulin).

placement of cortical neurons were aberrant in GSK-3/nestin-cre mutant mice (Yokota et al., 2010). This study showed that, compared to control brains, fewer deeper layer neurons (Tbr1-positive) were found in the cortical plate of GSK-3 double knockout brains. Substantial numbers of Tbr1 neurons were trapped within the intermediate zone. Yet, GSK-3/nestin-cre mutant brains also show disrupted radial glial development (Yokota et al., 2010). The disruption of radial glial platform may indirectly create radial neuron migration defects in GSK-3/nestin-cre brains. Therefore, elimination of GSK-3 only in neuronal population needs to be done to establish whether or not GSK-3 has a direct role in neuronal migration in developing mammalian brains.

A separate study has recently shown that *in utero* electroporation with active GSK-3β plasmid into cortical ventricular zone elicits neuronal migration defects (Asada and Sanada, 2010). LKB1 that is an important regulator of neuronal migration in the developing cortex (Asada et al., 2007) mediated GSK-3β phosphorylation at Serine 9 residue. The phosphorylation induced high levels of APC and Glu-tubulin (stabilized microtubule) at the leading process of neurons, thereby directing centrosomal movement. However, inhibition of the Serine 9 phosphorylation by overexpressing active GSK-3β (S9A) resulted in very low levels of APC and Glu-tubulin at the leading process of migrating neurons. These results suggest that GSK-3 inactivation via Serine 9 phosphorylation is required for directional migration of cortical neurons by rearranging APC and stabilizing microtubules at the leading process (**Figure 3B**). An important point of this study is that only sub-population of cortical ventricular cells are manipulated by the gene transfection, leaving surrounding environment including radial glial platform mostly intact.

Although this initial work outlined above has provided valuable information on GSK-3 functions in neuronal migration, it is important now to define the molecular and cellular mechanisms of GSK-3 regulation. For example, GSK-3 may regulate neuronal migration during development by directing gene transcription and/or by rearranging the intracellular cytoskeleton. Results from β-catenin and active GSK-3 overexpression studies strongly suggest that both mechanisms may be significant to GSK-3 regulation in neuronal migration. Defining GSK-3 functions and associated mechanisms has important clinical implications since defects in neuron migration are implicated in a range of neurodevelopmental disorders. Mutations in DISC1 are causative in some cases of schizophrenia.

NEURONAL MORPHOGENESIS

After migration, GSK-3 is critically involved in neuronal morphological development including neurite outgrowth and axon/dendritic specification. The roles of GSK-3 on axon/dendrite development are described well in the article in the same issue from Zhou and Snider.

GLIAL DEVELOPMENT

Although not yet tested, it can be anticipated that GSK-3 will also have important functions related to glial development. IGF1 and FGF2 pathways coordinately control the cell cycle progression of oligodendrocyte progenitors via cyclin D1 expression and localization (Frederick and Wood, 2004). The turnover of this protein is regulated by GSK-3-dependent proteasome degradation (Diehl et al., 1998). GSK-3β is phosphorylated in the presence of IGF1 downstream of PI3K/AKT signaling in oligodendrocyte progenitors (Frederick et al., 2007). This suggests that GSK-3 activity is inhibited by IGF1 in oligodendrocyte progenitors, which then enhances the stability and nuclear localization of cyclin D1 protein. Furthermore, inhibition of GSK-3 in postnatal mice by injecting pharmacological inhibitors increased oligodendrocyte differentiation and myelination (Azim and Butt, 2011). This effect was via canonical Wnt signaling stimulating β-catenin. Thus, these findings suggest that GSK-3 may be a crucial to oligodendrocyte differentiation.

Activation of PI3K and inhibition of GSK-3 using lithium promote Schwann cell differentiation (Ogata et al., 2004). This finding suggests that GSK-3 as a downstream of PI3K–AKT pathway is crucial for initiation of myelination during development. It will be important in future work to define aspects of GSK-3 signaling in gliogenesis during neural development that might have additional implications in the context of glial tumors and demyelinating diseases.

SYNAPTIC DEVELOPMENT

Synapses connect neurons to transmit signals and synapse formation is an important step in later stages of development. Recent studies have suggested important functions for GSK-3 in synaptic development. Shaggy, the fly homolog of GSK-3, is present at synapses and negatively influences AP-1-dependent synaptic growth, by modulating the Jun-N-terminal kinase pathway, and regulates presynaptic neurotransmitter release at the larval neuromuscular junction (Franciscovich et al., 2008). In fractionation studies, GSK-3β is readily detected within the synaptosomal fraction (Hooper et al., 2007). Activation of GSK-3 by transfecting GSK-3 genes in cultured neurons reduces expression and clustering of a synaptic protein Synapsin I (Zhu et al., 2007). In contrast, pharmacological inhibition of GSK-3 activity induces Synapsin I clustering in developing neurons (Hall et al., 2002). Further, GSK-3 activation reduces presynaptic glutamate release by inhibiting the synaptic vesicle exocytosis in response to membrane depolarization (Zhu et al., 2010). GSK-3β negatively regulates synaptic vesicle fusion events via interfering with Ca^{2+}-dependent SNARE complex formation. Finally, in rat, there is strong GSK-3 expression in dendritic spines of cultured hippocampal neurons (Peineau et al., 2007).

Glycogen synthase kinase-3 activity is also implicated in regulation of adult synapses. GSK-3 plays a role in amyloid-induced synaptic alternation and dendritic spine shortening (reviewed in Knobloch and Mansuy, 2008). All these demonstrate that GSK-3 signaling is likely critical to synaptic development, but the precise functions regulated by GSK-3 will require further study.

A recent study has reported functional roles of GSK-3 in the regulation of *N*-Methyl-D-aspartic acid (NMDA) receptor-dependent synaptic plasticity (Peineau et al., 2008). Activation of GSK-3 mediates NMDA-dependent long term depression (LTD) induction. In contrast, inhibition of GSK-3 activity prevents LTD induction and participates in long term potentiation (LTP)

induction (Hooper et al., 2007). For further information on the roles of GSK-3 in synaptic plasticity and LTP/LTD formation, please refer the review from Collingridge group in this issue.

GSK-3 IN TUMORIGENESIS

DUAL FUNCTIONS OF GSK-3 IN TUMORIGENESIS

Glycogen synthase kinase-3 is associated with neoplastic transformation and tumor development (reviewed in Ougolkov and Billadeau, 2006; reviewed in Luo, 2009). Since GSK-3 negatively regulates oncoproteins such as Myc and β-catenin, it may act as a suppressor of tumorigenesis. Indeed, downregulation of GSK-3β and upregulation of phospho-GSK-3β (inactive form) have been found in skin (Leis et al., 2002; Ding et al., 2007; Ma et al., 2007) and mammary tumors (Farago et al., 2005) in mice and humans. Furthermore, overexpression of wild type or constitutively active GSK-3β inhibits the progression of skin tumors in mice (Ma et al., 2007), while mice overexpressing dominant-negative GSK-3 develop mammary tumors (Farago et al., 2005). The promotion of mammary tumorigenesis by GSK-3 inactivation is accompanied by the accumulation of β-catenin and cyclin D1, suggesting the dysregulation of the Wnt pathway. These findings demonstrate the suppressive role of GSK-3 in tumorigenesis. However, some studies have shown the contrary role of GSK-3, that it may positively regulate tumorigenesis in other types of cancers. Increased GSK-3 levels have been found in human ovarian, colon, liver, thyroid, and pancreatic carcinomas (reviewed in Ougolkov and Billadeau, 2006; reviewed in Luo, 2009). Inhibition of GSK-3 activity by pharmacological inhibitors or RNA interference suppresses proliferation of the human ovarian cancer (Cao et al., 2006), colon cancer (Shakoori et al., 2007), pancreatic cancer (Ougolkov et al., 2005), and prostate cancer cells (Zhu et al., 2011). Conversely, overexpressing constitutively active GSK-3β induces cell cycle progression of ovarian cancer cells (Cao et al., 2006). Therefore, GSK-3 also functions as a tumor promoter in certain types of tumors. In this case, inhibition of GSK-3 could be an attractive therapeutic strategy for the treatment of ovarian, colon, and pancreatic cancers. Taken together, GSK-3 plays opposing roles as a tumor suppressor or a promoter, depending on cell-types and cellular contexts.

TUMORIGENESIS IN THE NERVOUS SYSTEM

Recent studies suggest important roles of GSK-3 in the formation and maintenance of tumors in the nervous system. Pharmacological inhibition of GSK-3 using the indirubin family reduces growth and invasion of human glioma cells both *in vitro* and in mice (Nowicki et al., 2008; Williams et al., 2011). β-catenin signaling plays an important role in mediating these effects. Another study using a different GSK-3 inhibitor, TDZD-8, has also shown that inhibition of GSK-3 activity suppresses proliferation of mouse glioma cells and induces apoptosis *in vitro* (Aguilar-Morante et al., 2010). The inhibitory effects of TDZD-8 in culture were confirmed in mice, which showed delay in tumor growth after glioma cell injection, and an increase in survival (Aguilar-Morante et al., 2010). Additionally, GSK-3 is involved in the differentiation of human glioma cells (Korur et al., 2009). Interference of GSK-3 activity with siRNA or pharmacological inhibitors induces differentiation of glioma cells. The capacity of GSK-3 in inducing glioma cell differentiation could be considered for the differentiation therapy that has emerged as a promising treatment tool for brain tumors. However, progression of this idea needs careful assessments, as contrary effects of GSK-3 on glioma differentiation are also reported (Li et al., 2010). GSK-3 also plays a promoting role in the majority of medulloblastoma, the most common pediatric malignant tumor of the central nervous system. Lithium, a clinically available GSK-3 inhibitor, suppresses proliferation of medulloblastoma cells and induces non-apoptotic cell death (Ronchi et al., 2010). Interestingly, the inhibitory effect of GSK-3 is independent of changes in intracellular levels of β-catenin. Together, GSK-3 signaling plays important roles in tumorigenesis and differentiation in the nervous system, and may be a promising target in the establishment of therapeutic strategies. However, before considering clinical application, it is necessary to determine the suppressive and promoting functions of GSK-3 in each tumor type, as both activities have been evident in non-nervous system tumors. This dual role of GSK-3 in tumorigenesis may not be surprising since GSK-3 is involved in multiple pathways and regulates many substrates. Different environments and/or epigenetic programs around different tissues/cells may regulate GSK-3 in different ways, which subsequently influences distinct downstream targets. In this regard, it is important to define and compare GSK-3 signaling mechanisms in each cell-type. This will delineate how the suppressive and promoting activities of GSK-3 are counterbalanced and help to develop therapeutic potentials.

IMPLICATIONS IN NEURODEVELOPMENTAL DISORDERS

Recent studies suggest that changes in GSK-3 activity may be an important aspect of pathophysiologies of neurodevelopmental disorders, including autism spectrum disorders (ASDs), schizophrenia, mental retardation, and bipolar disorders. There are two types of key observations in animal models of neurodevelopmental disorders: (1) The levels and activity of GSK-3 are altered in the brain samples. (2) Pharmacological or genetic inhibition of GSK-3 activity reduces the pathological symptoms. In humans, lithium, a GSK-3 inhibitor is used for controlling abnormal mood changes in patients with bipolar disorders.

ASDs are a spectrum of psychological conditions characterized by abnormalities in social interactions and communication as well as stereotypic behaviors and motor dysfunctions. Neurodevelopmental abnormalities are found in ASD brains. Studies using magnetic resonance imaging and postmortem brains show abnormalities in cortical minicolumn organization and neuron migration in patients with ASDs (Piven et al., 1990; Casanova et al., 2003, 2006). Etiology of autism has yet to be determined. However, recent studies suggest that genetic factors may be the most significant cause for ASDs. Gene linkage studies and array analyses have found multiple ASD-associated genes that encode FMR1 (fragile X mental retardation 1), PTEN (phosphatase and tensin homolog), APC, DISC1, SHANK3 (SH3 and multiple ankyrin repeat domains 3), TSC (tuberous sclerosis), MeCP2 (methyl CpG binding protein 2), and Reelin (reviewed in Abrahams and Geschwind, 2008). All these genes play important roles in functional and anatomical development of the nervous system.

Importantly, GSK-3, a critical regulator in broad aspects of neural development, is associated with many ASD-related genes mentioned above. GSK-3 activity measured by the levels of phospho-GSK-3 is increased in the brains of FMR1-deleted mice (Min et al., 2009; Yuskaitis et al., 2010). Inhibition of GSK-3 activity by treatment with lithium reduces abnormal social behaviors and seizures in the FMR1 mice. Additionally, GSK-3 knock-in mice, in which inhibitory serine 9 (β) and 21 (α) residues are substituted by alanine (McManus et al., 2005), show abnormal social behaviors similar to those of FMR1-deleted mice (Mines et al., 2010). Mice with conditional PTEN deletion in the cortex and hippocampus show macrocephaly and abnormal social interaction (Kwon et al., 2006). The levels of inactive GSK-3β (phospho-GSK-3β) are elevated in the brains. Furthermore, recent studies suggest that GSK-3 is a critical downstream mediator of the DISC1 signal. DISC1 regulates proliferation of neural progenitors by modulating GSK-3 activity (Mao et al., 2009). DISC1 directly binds to GSK-3 and participates in the regulation of β-catenin stability. Inhibition of GSK-3 with lithium or overexpression of a stable β-catenin rescues DISC1-deletion effects in neural progenitors. However, whether DISC1 directly controls GSK-3/β-catenin or serves as a scaffold remains unknown. Together, GSK-3 is associated with genes that are mutated in neurodevelopmental disorders and may play a critical role in the pathogenesis of the diseases. The association of GSK-3 with these genes suggests a therapeutic intervention point in the development of tools for neurodevelopmental disorders.

GSK-3 IN EPIGENETIC CONTROL

Epigenetic control has become an important cell-intrinsic program in the regulation of various processes during neural development. DNA methylation and histone modification (i.e., phosphorylation, acetylation, and methylation) change chromatin structures for transcription factors to gain access to gene promoters (Fischle et al., 2003). Non-coding RNAs also functionally participate in neural development. For example, microRNAs regulate neural progenitor proliferation and differentiation (Aranha et al., 2011; Chen et al., 2011), neural migration (Delaloy et al., 2010), oligodendrocyte differentiation and myelination (Dugas et al., 2010), hippocampal axon growth (Sanuki et al., 2011), and dendritic spine morphogenesis (Lippi et al., 2011).

The role of GSK-3 in epigenetic control has recently been evident. GSK-3 modulates the activity of epigenetic factors. GSK-3 directly phosphorylates histone deacetylase 3 that promotes the death of cortical neurons (Bardai and D'Mello, 2011), suggesting that the neuronal death-promoting activity of GSK-3 could be mediated via an epigenetic mechanism. Pharmacological inhibition of GSK-3 increases methylation at several sites on histone H3 and histone H4 bound to the promoters of the NFκB target genes Bcl-2 and XIAP (Ougolkov et al., 2007). This leads to the suppression of NFkB binding to its target promoters and thereby epigenetic silencing of XIAP and Bcl-2. Additionally, a recent study demonstrates that GSK-3 also participates in epigenetic control by regulating the levels of a DNA methyltransferase. Genetic deletion of both GSK-3α and GSK-3β in mouse embryonic stem cells reduces expression of DNA methyltransferase 3a2, which results in hypomethylation and misexpression of the imprinted genes Igf2, H19, and Igf2r (Popkie et al., 2010). Our understanding of this subject is at an initial stage, but the information thus far strongly suggests important roles of GSK-3 in epigenetic control. This field will be expanded by identifying more epigenetic molecules that are regulated by GSK-3.

CONCLUSION

All of the studies discussed here implicate GSK-3 as a multifunctional regulator of neural development. At early stages, GSK-3 serves as a master switch regulating neural progenitor homeostasis and appears to act via regulation of β-catenin as well as other key signaling pathways. It now seems quite clear that after progenitors differentiate into neurons, GSK-3 signaling is also key to neuronal migration and lamination. Although some evidence implicates β-catenin in these processes, GSK-3 regulation of the cytoskeleton via APC and other microtubule-associated proteins may be particularly important. Finally, functions of GSK-3 in neuronal morphogenesis, synaptogenesis, and behavior are only now being defined. Future studies defining cell-type specific effects *in vivo* with a variety of Cre drivers will rapidly enhance our understanding of GSK-3 functions.

A key regulator of GSK-3 function in neural development is an important protein in genetic causes of schizophrenia. Details of GSK-3 regulation by DISC1 have already provided key insights into regulation of key developmental events with important implications to the pathogenesis of some cases of schizophrenia.

Finally, our current understanding of GSK-3 in neural development largely reflects phenotypic characterization of the loss of the protein. It will be important to learn more cellular and molecular mechanisms of GSK-3 signaling related to these striking phenotypes. Further works should distinguish GSK-3 functions between gene induction activity and cytoskeleton regulation and how these processes are linked in various neurodevelopmental events. Also, key to defining GSK-3 functions will be to understand how GSK-3 activity is regulated. Understanding regulation of GSK-3 activity will be a particularly important goal, as we can expect that even relative small changes might have a large impact on important developmental outcomes such as determination of numbers of neurons in the brain.

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GSK3 as a sensor determining cell fate in the brain

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Glycogen synthase kinase 3 (GSK3) is an unusual serine/threonine kinase that controls many neuronal functions, including neurite outgrowth, synapse formation, neurotransmission, and neurogenesis. It mediates these functions by phosphorylating a wide range of substrates involved in gene transcription, metabolism, apoptosis, cytoskeletal dynamics, signal transduction, lipid membrane dynamics, and trafficking, amongst others. This complicated list of diverse substrates generally follow a more simple pattern: substrates negatively regulated by GSK3-mediated phosphorylation favor a proliferative/survival state, while substrates positively regulated by GSK3 favor a more differentiated/functional state. Accordingly, GSK3 activity is higher in differentiated cells than undifferentiated cells and physiological (Wnt, growth factors) and pharmacological inhibitors of GSK3 promote the proliferative capacity of embryonic stem cells. In the brain, the level of GSK3 activity influences neural progenitor cell proliferation/differentiation in neuroplasticity and repair, as well as efficient neurotransmission in differentiated adult neurons. While defects in GSK3 activity are unlikely to be the primary cause of neurodegenerative diseases, therapeutic regulation of its activity to promote a proliferative/survival versus differentiated/mature functional environment in the brain could be a powerful strategy for treatment of neurodegenerative and other mental disorders.

Keywords: GSK3, phosphorylation, kinase, substrate, proliferation, differentiation, neural progenitor, Alzheimer's disease

GLYCOGEN SYNTHASE KINASE 3

Glycogen synthase kinase 3 (GSK3) is a Ser/Thr protein kinase that is expressed in all mammalian tissues and subcellular organelles, but particularly highly in the brain. There are two isoforms encoded by separate genes (GSK3α and GSK3β), although their functions are often indistinguishable. GSK3 is critical for normal function of the central nervous system, where it regulates a variety of neuronal functions, including neurotransmission, neurite outgrowth, growth-cone dynamics, cytoskeletal dynamics, synaptic plasticity, endocytosis, apoptosis, and neurogenesis. Interestingly, it is one of the most unusual kinases in the human genome for three main reasons; (1) Most (if not all) substrates require prior phosphorylation by another kinase before they can be efficiently phosphorylated at Ser/Thr residues by GSK3. This process is known as "priming" and occurs four or five residues C-terminal to the GSK3 target site. (2) GSK3 is highly active in cells under basal conditions. This is partly due to constitutive phosphorylation of a conserved tyrosine residue on the activation loop of the kinase domain (Tyr279 in GSK3α, Tyr216 in GSK3β) that is absolutely required for kinase activity (Cole et al., 2004a). (3) Phosphorylation of GSK3 at an N-terminal serine residue inhibits its kinase activity (Ser21 in GSK3α, Ser9 in GSK3β). This phosphoserine acts as a pseudo-substrate and binds to the phosphate-binding pocket on GSK3, preventing interaction with primed substrates (Frame et al., 2001). Phosphorylation at this site is mediated by members of the AGC family of kinases (e.g., Akt) and commonly occurs downstream of insulin, growth factor, and PI3K signaling. Activation of the canonical Wnt signaling pathway also inhibits GSK3 activity, preventing phosphorylation of β-catenin, although this is not mediated by N-terminal phosphorylation, but by protein–protein interactions (Thomas et al., 1999; Ding et al., 2000).

GSK3 AND ALZHEIMER'S DISEASE

Glycogen synthase kinase 3 is implicated in the development of Alzheimer's disease (AD), principally because it phosphorylates Tau and increases its propensity to aggregate into neurofibrillary tangles, which together with β-amyloid plaques are a characteristic lesion of the disease. Transgenic mice displaying increased GSK3 activity develop hyperphosphorylated Tau and other neurological defects (Lucas et al., 2001), while treatment of transgenic mice with a GSK3 inhibitor (lithium) reduces the number of tangles in their brains (Noble et al., 2005). A similar microtubule-binding protein called CRMP2 is also hyperphosphorylated by GSK3 in the brains of AD patients (Cole et al., 2007; Williamson et al., 2011). The amyloid precursor protein has been reported to be a GSK3 substrate (Aplin et al., 1996), while Aβ peptide production is reported to be regulated by GSK3 (Sun et al., 2002; Li et al., 2003; Phiel et al., 2003; Ryder et al., 2003; Su et al., 2004; Sereno et al., 2009), although these observations have been recently disputed (Jaworski et al., 2011). Thus, GSK3 has been implicated in many pathologic processes leading to AD. However, it is unlikely that defects in GSK3 *per se* are a direct cause of AD, since no mutations, polymorphisms, or dramatic biochemical changes have been consistently detected in

Abbreviations: AD, Alzheimer's disease; ES, embryonic stem (cell); GSK3, glycogen synthase kinase 3; NPC, neural progenitor cell.

AD patients, nor any other types of neurodegenerative, developmental, or psychiatric disorders. Instead, a key function of GSK3 is to act as an "environmental sensor," by relaying signals from extracellular stimuli (e.g., growth factors, insulin, Wnt) to signaling and transcriptional machinery inside the cell to influence cell fate. This implies that pharmacological manipulation of GSK3 in the brain could be used to selectively promote survival, proliferation, differentiation, neurogenesis, or neuroplasticity in diseased brains. This type of therapy could be used to artificially create an environment in the brain that delays/prevents disease development, or promotes neurogenesis and neuroplasticity to compensate for specific insults. Indeed, encouraging data is now emerging showing chronic lithium treatment improves cognitive function in human patients and mouse models of neurodegeneration and ischemic stroke (for a review, see Chiu and Chuang, 2010). Although GSK3 is not the only *in vivo* target of lithium (e.g., phosphoinositol phosphatases), these effects are consistent with the known actions of GSK3. It remains to be seen what benefits more selective and potent GSK3 inhibitors might provide.

GSK3 SUBSTRATES

In order to fully understand the function of GSK3 in the brain, it is essential to characterize its substrates, since this is the primary function of a kinase and it is the substrates that mediate the functional effects directed by GSK3. Ultimately, all physiological substrates of GSK3 should be cataloged and assigned to particular functions regulated by GSK3 (e.g., neurogenesis, neurite outgrowth, neurotransmission, cytoskeletal regulation). This exercise would delineate the mechanisms by which GSK3 maintains healthy brain function. Importantly, it could identify new therapeutic targets downstream of GSK3 that could be exploited for the treatment of mental and neurodegenerative diseases. Theoretically, these could be more specific with less side effects than targeting GSK3, which is a pleiotropic kinase with many different substrates involved in diverse cellular functions.

So far, over 70 substrates have been identified for GSK3, although caution should be taken since many substrates have been reported with various levels of confidence/evidence (for a full review, see Sutherland, 2011). Reported substrates include a number of cytoskeletal, signaling, and DNA-binding proteins. Interestingly, a pattern emerges whereby many substrates that are negatively regulated by GSK3 are involved in proliferation/survival of cells, whereas substrates that are positively regulated by GSK3 are predominantly expressed and function in mature, differentiated cells. Key substrates that contribute to cellular proliferation, differentiation, and survival are listed in **Tables 1** and **2** and discussed below.

GSK3 AND PROLIFERATION

For some time, it has been known that pharmacological inhibition of GSK3 activity maintains the proliferative state of embryonic stem (ES) cells (Sato et al., 2004; Ying et al., 2008). The GSK3 substrates c-myc (Hall et al., 2009) and Klf5 (Jiang et al., 2008) are among several transcription factors that have been used to induce pluripotency (iPS system). GSK3 has also been implicated as a key regulator of adult neurogenesis (generation and incorporation of new neurons into existing circuits of adult brains). Genetic (Eom and Jope, 2009; Kim et al., 2009; Mao et al., 2009) and pharmacological (Sato et al., 2004; Ying et al., 2008; Bone et al., 2009) inhibition of GSK3 activity increases proliferation of neural progenitor cells (NPC's), but decreases differentiation and incorporation of newborn neurons into the adult brain. Together, these observations demonstrate that low levels of GSK3 activity promote proliferation in ES cells and NPC's. This correlates with signaling pathways upstream of GSK3 that inhibit GSK3 activity and promote proliferation (e.g., Wnt, growth factors).

Several transcription factors are directly phosphorylated by GSK3 within an [ST]PPx[ST]P or [ST]PxL[ST]P motif. Following priming by another kinase (often a Cdk or MAPK), phosphorylation by GSK3 creates a binding site for E3 ubiquitin ligases that ubiquitinate the protein and target it for proteasome-mediated degradation. Many of these transcription factors have short half-lives, largely due to the actions of GSK3, which is highly active under basal conditions in differentiated cells, including post-mitotic neurons. However, GSK3 activity levels are comparatively lower in ES cells and NPC's, induced by persistent growth factor and Wnt signaling to maintain the proliferative capacity of these cells (Cartwright et al., 2005). Here, phosphorylation and ubiquitination of transcription factors by GSK3 is reduced, thus stabilizing the proteins (prolonging their half-lives) and contributing to stem/precursor cell proliferation. Such GSK3 targets include well-known proliferative factors, such as c-myc, c-jun, β-catenin, cyclin E1, and Klf5 (**Tables 1** and **2**; **Figure 1**). Recent studies suggest that attenuating GSK3-mediated degradation of β-catenin, a key effector of the Wnt signaling pathway, is vital for maintaining ES proliferation and pluripotency (Mao et al., 2009; Kelly et al., 2011; Wray et al., 2011). Interestingly, a viral oncogenic form of c-jun (v-jun) is mutated at the GSK3 target site (Ser239). This prevents phosphorylation by GSK3 and subsequent ubiquitination, thus stabilizing the protein and driving uncontrolled proliferation in tumourigenesis (Wei et al., 2005). Similarly, the GSK3 phosphosite (Thr58) is mutated in the viral oncogenic form of c-myc (v-myc; Pulverer et al., 1994). While it is established that low GSK3 activity levels are required for maintaining the proliferative capacity of ES cells and NPC's, there are many DNA-binding substrates of GSK3 implicated in this process and their precise roles and relative importance are only beginning to be clarified.

GSK3 AND DIFFERENTIATION

Not only does low GSK3 activity promote proliferation, it also prevents differentiation. GSK3α/β double knockout ES cells are severely compromised in their ability to differentiate, largely due to hyperactivation of the Wnt signaling pathway (Doble et al., 2007), while conditional deletion of both isoforms in NPC's in mice suppressed the generation of post-mitotic neurons (Kim et al., 2009). Also, expression of mutant GSK3 and RNAi-mediated knockdown impairs neuronal polarization in cultured primary neurons (Jiang et al., 2005; Yoshimura et al., 2005; Kim et al., 2009). GSK3 knockin mice expressing GSK3α/β (Ser21/9Ala) that are insensitive to growth factor-induced inhibition exhibited reduced neurogenesis and behavioral defects, despite normal NPC proliferation (Eom and Jope, 2009; Ackermann et al., 2010), suggesting defective differentiation/maturation of NPC's. In contrast, mice expressing mutant DISC1 (mutated in schizophrenia patients)

Table 1 | Substrates involved in proliferation/survival that are negatively regulated by GSK3.

Substrate	Function	Effect of GSK3-mediated phosphorylation	Reference
c-myc	Transcription factor and oncogene – promotes proliferation	Promotes degradation of the protein via Fbw7-mediated ubiquitination and the proteosome	Moberg et al. (2004), Welcker et al. (2004), Yada et al. (2004)
c-jun	Transcription factor and oncogene – promotes proliferation	Promotes degradation of the protein via Fbw7-mediated ubiquitination and the proteosome	Wei et al. (2005)
β-Catenin	Transcription factor and oncogene – promotes proliferation	Promotes degradation of the protein	Ikeda et al. (1998)
HIF1α	Transcription factor induced by hypoxia. Activates transcription of genes promoting adaptation/survival	Promotes degradation of the protein	Mottet et al. (2003)
HSF1	Transcription factor that promotes expression heat shock factors to protect cells from environmental stress	Reduces DNA-binding and transcriptional activity	Chu et al. (1998)
Klf5	Transcription factor that promotes cell proliferation	Promotes degradation of the protein via Fbw7-mediated ubiquitination and the proteosome	Liu et al. (2010), Zhao et al. (2010)
CyclinE1	Activating cofactor for Cdk2, promoting cell cycle progression	Promotes degradation of the protein via Fbw7-mediated ubiquitination and the proteosome	Welcker et al. (2003)
Mef2D	Transcription factor that promotes survival and activity-dependent synapse formation	Inhibits its transcriptional activity, antagonizing neuronal survival but antagonizing neuronal differentiation.	Wang et al. (2009)
Gli3 (Ci155)	Target of the hedgehog signaling pathway that is important for patterning during development. Full-length Gli3 (Ci155) is a transcriptional activator, while the truncated form is a transcriptional repressor.	Promotes β-TrCP-mediated ubiquitination and proteolytic processing	Jia et al. (2002), Price and Kalderon (2002), Pan et al. (2006), Tempe et al. (2006), Wang and Li (2006)
Snail	Transcription factor that regulates E-cadherin expression during epithelial–mesenchymal transitions (development)	Promotes β-TrCP-mediated ubiquitination and degradation, also promotes translocation from the nucleus to the cytoplasm	Zhou et al. (2004)
NDRG1	Regulated by the cell cycle and cell differentiation, although cellular function is not yet clear	Unknown	Murray et al. (2004)
BCL3	Transcription factor and oncoprotein that regulates NFkB signaling	Promotes ubiquitin and proteasome-mediated degradation	Viatour et al. (2004)
MCL1	Pro-survival member of the Bcl2 family of proteins controlling apoptosis. Overexpressed in some cancer types.	Promotes degradation of the protein via Fbw7-mediated ubiquitination and the proteosome	Maurer et al. (2006)
RBL2	Involved in heterochromatin formation and structure. A key regulator of entry into the cell cycle	Not yet clear	Litovchick et al. (2004)
Smad1	Transcription factor and key mediator of BMP signaling in embryogenesis and tissue homeostasis	Promotes ubiquitination by Smurf1 and proteasome-mediated degradation	Fuentealba et al. (2007), Sapkota et al. (2007)
eIF-2B	Activates initiation of protein translation from mRNA transcripts	Phosphorylation inhibits eIF-2B activity, reducing protein translation	Welsh and Proud (1993)
Myocardin	Muscle-specific transcription factor and SRF-dependent cofactor that promotes expression of contraction-related genes	Inhibits its transcriptional activity and promotes CHIP or UBR5-mediated ubiquitination and degradation by the proteasome	Badorff et al. (2005), Xie et al. (2009), Hu et al. (2010)
VDAC1	Voltage-dependent anion channel in the mitochondrial outer membrane. Mediates cytochrome *c* release from mitochondria during apoptosis	Reduces binding to hexokinase 1, which is overexpressed in many transformed cells, thereby reducing aerobic glycolysis and ATP production in tumor cells	Pastorino et al. (2005)
IRS1	Adaptor protein that mediates signaling downstream of insulin and growth factor receptors	Reduces tyrosine phosphorylation of IRS1, attenuating insulin, and growth factor signaling	Eldar-Finkelman and Krebs (1997), Liberman and Eldar-Finkelman (2005)

(Continued)

Table 1 | Continued

Substrate	Function	Effect of GSK3-mediated phosphorylation	Reference
Bax	Pro-apoptotic member of the Bcl2 family that oligomerizes at the mitochondrial outer membrane, forming a pore to release cytochrome *c*	Promotes translocation to the mitochondria to induce apoptosis	Linseman et al. (2004)
Sufu (exception to the pattern)	Negative regulator of sonic hedgehog pathway, which regulates animal development and cell fate determination. In adults, it maintains the proliferative state of stem cells	Stabilizes Sufu by preventing its degradation and promotes localization in the primary cilium	Chen et al. (2011)
PTEN (exception to the pattern)	Lipid phosphatase and commonly mutated tumor suppressor in human cancers	Phosphorylation stabilizes the protein by reducing degradation	Al-Khouri et al. (2005), Maccario et al. (2007)

Table 2 | Substrates predominantly expressed and functional in mature differentiated cells that are positively regulated by GSK3.

Substrate	Function	Effect of GSK3-mediated phosphorylation	Reference
Polycystin-2 (PKD2)	Non-selective calcium permeable cation channel and part of the TRP channel family, which are broad cellular sensors for multiple stimuli	Promotes translocation to the cell membrane	Streets et al. (2006)
CRMP2	Binds to tubulin heterodimers to promote polymerization of microtubules. Also involved in kinesin-mediated transport and receptor trafficking	Regulates neurite outgrowth and neuronal polarity	Brown et al. (2004), Cole et al. (2004b), Uchida et al. (2005), Yoshimura et al. (2005)
MAP1B	Cytoskeletal component of the developing nervous system with important functions in migrating and differentiating neurons	Unclear, but may destabilize microtubules, making them more dynamic	Goold et al. (1999)
MAP2C	Abundant cytoskeletal components predominantly expressed in neurons	Promotes dissociation from the cytoskeleton, destabilizing microtubules	Sanchez et al. (2000)
Tau	Tubulin-binding protein that stabilizes microtubule structures. Primary constituent of neurofibrillary tangles generated in brains of Alzheimer's Disease and other dementia patients	Reduces binding to tubulin, destabilizing microtubules, making them more dynamic. Promotes aggregation of tau, forming neurofibrillary tangles	Hanger et al. (1992)
β-Adducin	Cytoskeletal-associated protein that links the actin and spectrin networks	Promotes neurite outgrowth	Farghaian et al. (2011)
Dynamin1	GTPase protein that regulates vesicular trafficking processes. Contributes to efficient neurotransmitter release at the pre-synapse	Promotes activity-dependent bulk endocytosis at the pre-synapse, facilitating efficient neurotransmission	Clayton et al. (2010)
CLASP2	Microtubule plus-end tracking protein that promotes the stabilization of dynamic microtubules	Causes dissociation from the plus end of microtubules and other MT-associated proteins	Wittmann and Waterman-Storer (2005), Watanabe et al. (2009)
CaMKKβ	Calcium/CaM dependent protein kinase that regulates learning, memory, migration, neurite outgrowth, and synaptogenesis	Stabilizes newly synthesized protein, decreases calcium/CaM autonomous activity	Green et al. (2011)
Glycogen synthase (exception to the pattern)	Enzyme involved in converting glucose to glycogen for storage	Reduces its enzymatic activity, thus reducing glycogen synthesis and storage	Rylatt et al. (1980)
FAK (exception to the pattern)	Plasma membrane protein and tyrosine kinase involved in cell–cell adhesion	Reduces FAK kinase activity, reducing cell migration	Bianchi et al. (2005)
pVHL (exception to the pattern)	Tumor suppressor that binds and stabilizes microtubules. Important in primary cilium. Component of an E3 ubiquitin ligase complex. Antagonizes cell cycle progression.	Phosphorylation negatively regulates stability (but not binding) of microtubules	Hergovich et al. (2006)

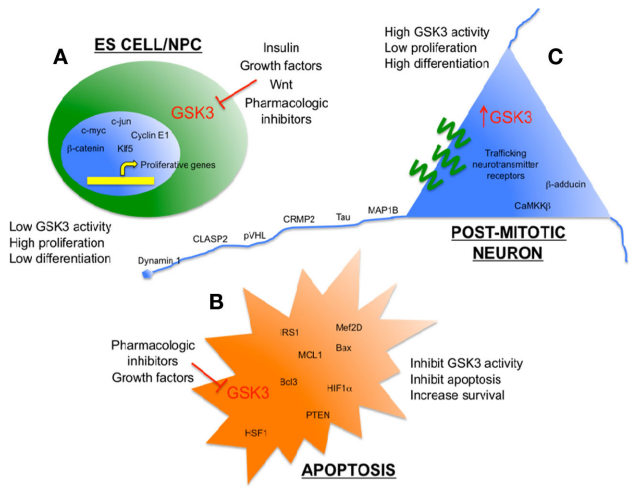

FIGURE 1 | Glycogen synthase kinase 3 as an enzymatic sensor for determining cell fate in the brain. (A) Low levels of GSK3 activity in ES cells/NPC's are maintained by persistent growth factor/Wnt signaling to promote proliferation. Some pro-proliferation transcription factors that are direct targets of GSK3 are shown. **(B)** Low levels of GSK3 activity inhibit apoptosis and promote survival when cells are exposed to toxic stimuli, such as hypoxia and amyloid peptides. Some pro-survival substrates of GSK3 are shown. **(C)** Relatively high levels of GSK3 activity help to promote differentiation and efficient function of mature, post-mitotic neurons, including several cytoskeleton-associated proteins that maintain neuronal morphology and neurotransmission.

caused increased GSK3 activity, inhibition of the Wnt signaling pathway, and decreased NPC proliferation (Mao et al., 2009). This suggests that inhibition of GSK3 by the Wnt signaling pathway promotes NPC proliferation, while inhibition of GSK3 by growth factor signaling promotes differentiation of NPC's into post-mitotic neurons.

Candidate substrates for promoting differentiation include the zinc-finger transcriptional regulator Gli3 (mammalian homolog of Ci155 in the fly), an effector of the hedgehog pathway that is critical for efficient embryo patterning and neural tube formation. In the absence of hedgehog, Gli3 is phosphorylated by GSK3 and CK1 (following priming by PKA), which targets it for ubiquitination and proteolysis, generating a truncated repressor form lacking the C-terminal activation domains (Jia et al., 2002; Price and Kalderon, 2002; Pan et al., 2006; Tempe et al., 2006; Wang and Li, 2006). But in the presence of hedgehog, phosphorylation and processing of Gli3 is inhibited, leading to transactivation by the full-length protein. It has recently been shown that the truncated repressor form of Gli3 is important for differentiation of cortical neurons from neural progenitors, while the full-length active form of Gli3 helps to maintain progenitors in the cell cycle (Wang et al., 2011). This correlates with GSK3 activity, with low GSK3 activity in undifferentiated ES cells increasing the amount of active, full-length Gli3 for maintaining proliferation in ES cells, and high GSK3 activity promoting proteolysis of Gli3 and formation of the truncated repressor form, facilitating differentiation into mature neurons.

Another zinc-finger transcriptional repressor called snail regulates transition of epithelial cells into fibroblast-like mesenchymal cells during development and tumor metastasis, essentially a form of "de-differentiation." Snail suppresses the expression of E-cadherin, a cell–cell adhesion molecule that is critical for maintaining epithelial cell identity. Relatively high activity of GSK3 in epithelial cells promotes phosphorylation and ubiquitin/proteasome-mediated degradation of snail (Zhou et al., 2004). However, in fibroblast/mesenchymal-like cells of human breast tumors where GSK3 activity is lower, snail is stabilized and suppresses E-cadherin expression (Zhou et al., 2004; Yook et al., 2006). Pharmacological inhibition of GSK3 activity in epithelial cells reduces E-cadherin expression and induces a more-mesenchymal-like morphology via increased snail activity (Bachelder et al., 2005). These observations demonstrate that Snail is an example of a GSK3 substrate controlling cellular differentiation. It does not appear to regulate neuronal differentiation (Murray and Gridley, 2006), although it has been shown to regulate cell fate in glioblastoma cells (Han et al., 2011).

Myocardin is a transcription factor that is required for cardiac and skeletal muscle cell differentiation during development. Again, it is not expressed in neurons or glial cells, but interestingly, it is upregulated in vascular smooth muscle cells in the brains of AD patients, where it enhances accumulation of Aβ in blood vessel walls (Bell et al., 2009). Myocardin is phosphorylated by GSK3, targeting it for ubiquitin, and proteasome-mediated degradation (Badorff et al., 2005; Xie et al., 2009; Hu et al., 2010), however it is not yet clear if upregulation of myocardin levels is due to reduced GSK3-mediated phosphorylation and degradation. In summary, several substrates of GSK3 regulating cell differentiation have been identified, although mostly in non-neural cell types and neuron-specific differentiation factors await identification.

GSK3 AND SURVIVAL

Glycogen synthase kinase 3 promotes intrinsic apoptotic signaling in neurons, and overexpression of GSK3 is sufficient to induce apoptosis in cultured cells (Pap and Cooper, 1998; Bijur et al., 2000) and in mouse brain (Lucas et al., 2001). Deletion of the GSK3β isoform in mice causes severe liver degeneration during mid-gestation due to excessive tumor necrosis factor-induced apoptosis (Hoeflich et al., 2000). In contrast, numerous studies have demonstrated that genetic or pharmacologic inhibition of GSK3 protects neurons from a wide range of environmental stresses, including hypoxia and amyloid toxicity, which may be relevant for treatment of stroke and AD patients, respectively (for a review, see Mines et al., 2011).

Several GSK3 substrates have been implicated in regulation of apoptosis. Bax is a pro-apoptotic member of the Bcl2 family that oligomerizes at the mitochondrial outer membrane, forming a pore to release cytochrome *c*, inducing cell death. Phosphorylation of Bax at Ser163 by GSK3 promotes translocation to the mitochondria, whereas inhibition of phosphorylation using lithium reduced Bax translocation and cytochrome *c* release, thus antagonizing apoptosis (Somervaille et al., 2001; Linseman et al., 2004). VDAC1 is a voltage-dependent anion channel in the mitochondrial outer membrane that also mediates cytochrome *c* release during apoptosis and is a direct substrate of GSK3 (Pastorino et al., 2005), although the effect of phosphorylation on cytochrome *c* release from the mitochondria and apoptosis is not yet clear. In

contrast, MCL1 is an anti-apoptotic, pro-survival member of the Bcl2 family, and phosphorylation by GSK3 targets it for degradation by the ubiquitin–proteasome-mediated pathway (Maurer et al., 2006). Thus, low GSK3 activity would reduce phosphorylation and degradation of MCL1, favoring cell survival. Several transcription factor substrates of GSK3 have also been implicated in the balance between apoptosis and cell survival by regulating transcription of pro-apoptotic or pro-survival genes, including the pro-survival factors HIF1α, HSF1, Mef2D, and BCL3. GSK3 phosphorylation of each of these substrates targets them for ubiquitin and proteasome-mediated degradation. In summary, many apoptosis-related GSK3 substrates identified so far are pro-survival, and when GSK3 activity is low (e.g., undifferentiated or pharmacologically treated cells), reduced phosphorylation of substrates protects them against ubiquitin and proteasome-mediated degradation, promoting survival of the cell.

GSK3 AND NEURONAL MORPHOLOGY

GSK3 is an important regulator of neuronal morphology and synapse formation in mature, post-mitotic neurons. Pharmacologic inhibition of GSK3 activity reduces the rate of axon elongation in hippocampal neurons, increases the size of growth cones (Owen and Gordon-Weeks, 2003), and disturbs polarity, leading to the formation of multiple axon-like processes (Gartner et al., 2006; Garrido et al., 2007). Treatment of cerebellar granule cells with a GSK3 inhibitor increased the number of synapses on mossy fibers (Hall et al., 2000), whereas inactivation of the *Drosophila* homolog of GSK3, *shaggy*, promoted synapse formation at neuromuscular junctions by increasing the number of synaptic boutons (Franco et al., 2004). Assuming these interventions were selective, then taken together they demonstrate that GSK3 regulates synapse formation. Accordingly, neurotrophin and growth factor stimuli (e.g., BDNF, NGF, IGF-1) that inhibit GSK3 activity, promote neurite outgrowth, and synapse formation.

Several groups recently demonstrated that the actin-capping protein β-adducin is critical for synapse stability and turnover, underlying learning and memory in flies and mammals (Bednarek and Caroni, 2011; Pielage et al., 2011; Ruediger et al., 2011). Moreover, dynamic disassembly and reassembly of synapses by β-adducin is regulated by phosphorylation at its C-terminal region by PKC. This domain is also targeted by GSK3 following priming by Cdk5 and phosphorylation by these kinases is necessary for β-adducin's ability to promote neurite outgrowth in cultured primary neurons (Farghaian et al., 2011). Therefore, it will be interesting to see if GSK3-mediated phosphorylation of β-adducin also regulates synapse formation and stability. Several other cytoskeleton-associated proteins are phosphorylated by GSK3 in mature, post-mitotic neurons, in particular the tubulin-binding proteins Tau, MAP1B, MAP2, CRMP2, CLASP2, and pVHL. Phosphorylation of these substrates causes their dissociation from tubulin, destabilizing the microtubule structure. In post-mitotic neurons where GSK3 activity levels are relatively high, this would reduce interactions between GSK3 substrates and tubulin, resulting in destabilization of microtubules. Accordingly, non-phospho-mutant forms of substrates (e.g., CRMP2, MAP1B) increase the stability of microtubules, causing impaired neurite outgrowth, and polarity in cultured primary neurons (Cole et al., 2004b; Trivedi et al., 2005; Yoshimura et al., 2005). In theory, high levels of phosphorylation of these substrates would promote their dissociation from microtubules, favoring dynamic remodeling of the cytoskeleton, and enhancing neuroplasticity, although this is yet to be proven *in vivo*.

GSK3 AND NEUROTRANSMISSION

A systematic screen of Ser/Thr kinases using a panel of pharmacological inhibitors revealed that GSK3 was the only kinase among 58 Ser/Thr kinases that was required for induction of NMDA-induced long-term depression (LTD) in hippocampal CA1 pyramidal neurons (Peineau et al., 2009). LTD increases GSK3 activity via decreased phosphorylation of Ser21/9 at its N-terminus, while NMDA-induced long-term potentiation (LTP) reduces GSK3 activity by increasing Ser21/9 phosphorylation (Hooper et al., 2007; Peineau et al., 2007). Meanwhile, GSK3 inhibitors do not affect baseline synaptic transmission (Peineau et al., 2007; Zhu et al., 2007; Li et al., 2009). GSK3 regulates transmission at both the pre- and post-synapse. For example, high GSK3 activity reduces glutamate release from the pre-synapse, inhibiting LTP (Hooper et al., 2007; Zhu et al., 2007, 2010), while retrieval of synaptic vesicles at the pre-synapse by endocytosis requires GSK3 (Clayton et al., 2010). Dynamin 1 is a large GTPase that regulates vesicle endocytosis at the pre-synapse. Phosphorylation by GSK3 at Ser774 is required for re-uptake of neurotransmitters during times of elevated neuronal activity (Clayton et al., 2010). Thus, relatively high GSK3 activity in differentiated neurons would be expected to activate Dynamin 1 and facilitate efficient recycling of neurotransmitters at the synapse. At the post-synapse, pharmacological inhibition of GSK3 decreases surface expression of NMDA and AMPA receptors (Chen et al., 2007; Wei et al., 2010). CRMP2 is a GSK3 substrate that has been implicated in trafficking of transmembrane proteins to the cell surface (Nishimura et al., 2003; Brittain et al., 2011), although the effect that phosphorylation has on this process has not yet been determined. Together, these observations demonstrate a clear requirement for GSK3 at the synapse, although the synaptic substrates that mediate these effects remain to be fully uncovered.

CONCLUSION

When analyzing the substrates of GSK3, a pattern emerges whereby those that are negatively regulated by GSK3 are commonly involved in promoting proliferation and/or survival, while substrates that are positively regulated by phosphorylation are predominantly expressed in differentiated post-mitotic neurons and are required for efficient function of mature neurons. The former substrates include pro-proliferation transcription factors or pro-survival proteins targeted for ubiquitin-mediated degradation by GSK3, while the latter are often cytoskeleton-associated proteins. Thus, low GSK3 activity levels are conducive to proliferative ES cells and NPC's, while higher GSK3 activity is required for efficient function of differentiated neurons. This pattern implies that pharmacologic manipulation of GSK3 activity can be used to influence cell fate between proliferative/undifferentiated and mature/differentiated states, as has already been successfully demonstrated for ES cells. In the brain, inhibition of GSK3 would promote proliferation of NPC's, while high levels of GSK3 would

promote neuronal differentiation and efficient function of post-mitotic neurons. Also, it is possible that high GSK3 activity in post-mitotic neurons could promote neuroplasticity, learning, and memory via increased dynamics of the cytoskeleton. Manipulation of GSK3 activity may be of great therapeutic benefit for neurodegenerative and other mental disorders. In AD, the use of pharmacologic inhibitors of GSK3 has been proposed to decrease phosphorylation of Tau, reducing its aggregation and formation of neurofibrillary tangles. This strategy has shown some success in mouse models of AD (Perez et al., 2003; Nakashima et al., 2005; Noble et al., 2005; Leroy et al., 2010). In elderly humans and AD patients, chronic (but not acute) treatment with GSK3 inhibitors reduced decline in cognitive and memory function (Nunes et al., 2007; Chiu and Chuang, 2010; Kessing et al., 2010; Forlenza et al., 2011). These studies have been performed using lithium, a relatively weak and non-specific inhibitor of GSK3, so it is necessary to advance these studies using more potent and specific inhibitors of GSK3.

Another exciting potential therapeutic use of GSK3 inhibitors in the clinic is to maintain neuron survival under stressful conditions, including neurodegenerative diseases and acute injuries, such as stroke. Since GSK3 inhibitors are such effective inhibitors of neuronal apoptosis (at least *in vitro*), rapid administration of these drugs could help to prevent neuronal loss during the immediate period following injury. By keeping these neurons alive, one might expect an improved prognosis for functional recovery. It might also promote proliferation of NPC's that could later be induced to differentiate into functional post-mitotic neurons to compensate for damages incurred at the site of injury. So far, several groups have elegantly demonstrated that lithium treatment effectively protects neurons and even promotes migration of stem cells to affected regions (Chiu and Chuang, 2010; Tsai et al., 2011).

Of course, there is the danger that inhibition of GSK3 activity could impede the basic function of post-mitotic neurons. However, it should be remembered that very few drugs inhibit kinases 100%, therefore any treatments are likely to reduce GSK3 activity, not completely inhibit it. Also, GSK3 substrates that are relatively resistant to phosphatases are beginning to be discovered [e.g., β-adducin (Farghaian et al., 2011), CRMP2 (Cole et al., 2008)] and moderate reduction of GSK3 activity is unlikely to affect the stoichiometry of phosphorylation of these substrates. This provides another good reason for identifying and characterizing each individual substrate of GSK3 in the brain. Importantly, downstream targets of GSK3 that are specifically involved in a particular neuronal process (e.g., neurogenesis, neurotransmission) may prove to be better therapeutic targets than GSK3, being more potent and selective with fewer side effects. Therefore, the full catalog of GSK3 substrates and their physiological functions needs to be completed.

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Role of GSK3 signaling in neuronal morphogenesis

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Glycogen synthase kinase 3 (GSK3) is emerging as a key regulator of several aspects of neuronal morphogenesis including neuronal polarization, axon growth, and axon branching. Multiple signaling pathways have been identified that control neuronal polarization, including PI3K, Rho-GTPases, Par3/6, TSC–mTOR, and PKA–LKB1. However, how these pathways are coordinated is not clear. As GSK3 signaling exhibits crosstalk with each of these pathways it has the potential to integrate these polarity signals in the control neuronal polarization. After neurons establish polarity, GSK3 acts as an important signaling mediator in the regulation of axon extension and axon branching by transducing upstream signaling to reorganization of the axonal cytoskeleton, especially microtubules. Here we review the roles of GSK3 signaling in neuronal morphogenesis and discuss the underlying molecular mechanisms.

Keywords: glycogen synthase kinase 3, neuronal polarity, axon growth, axon branching, microtubules, growth cone

During neural development, differentiated neurons undergo multiple steps of morphogenesis, including directed neuronal migration, neuronal polarization, axon outgrowth, axon guidance, and axon branching, to form the architectural basis of neural circuits. A common cellular process underlying these morphogenetic events is reorganization of the neuronal cytoskeleton directed by extracellular cues. Therefore, understanding how extracellular signals are translated into cytoskeletal reorganization is a key question in study of neuronal morphogenesis. Glycogen synthase kinase 3s (GSK3s), including GSK3α and GSK3β, are key components of the canonical Wnt signaling pathway. Recent studies indicate that GSK3s are also key upstream regulators of neuronal microtubules, as a number of microtubule-binding proteins (MBPs) are GSK3 substrates. Further, in addition to canonical Wnt ligands GSK3 activity is modulated by many other extracellular factors and their associated downstream pathways. Therefore, GSK3 is emerging as a major signaling mediator that functions to orchestrate each step of neuronal morphogenesis. In this review, we summarize and evaluate studies that show the involvement of GSK3 signaling in neuronal polarization, axon growth, and axon branching. We also discuss the potential molecular and cellular mechanisms by which GSK3 signaling regulates these morphogenetic processes.

GSK3 SIGNALING IN NEURONAL POLARIZATION

The development of axon/dendrite polarity forms the structural basis for directional transmission of neural activities between neurons. In the past decade, the cellular and molecular mechanisms by which neuronal polarity is regulated have been extensively studied (Barnes and Polleux, 2009). Based on observations in a seminal *in vitro* model (Craig and Banker, 1994), we know that neuronal polarization involves multiple cellular steps. First, an intracellular signal is locally activated in one of the unpolarized neurites that breaks the cell symmetry and initiates the polarization process. Additional polarity determinants, likely downstream of the initiation signal, are then asymmetrically accumulated in the same neurite, which later becomes the axon. Second, such asymmetric signaling then triggers reorganization of cytoskeletal elements (actin filaments and microtubules) in the future axon and leads to its preferential growth. Lastly, sustained activation of multiple cellular processes specifically in the axon, including polarized protein/membrane transport and local protein translation/degradation, act to maintain the established asymmetry and axon extension. At the same time, complementary signaling occurs in the dendrites to suppress neurite extension. During the past decade, many intracellular signaling pathways and molecules have been identified that control these polarization steps. These include the PI3K pathway, the Rho-GTPase pathways, the Par3/6 pathway, the TSC–mTOR pathway, and the PKA–LKB1 pathway (Shi et al., 2003; Nishimura et al., 2004, 2005; Schwamborn and Puschel, 2004; Barnes et al., 2007; Shelly et al., 2007; Choi et al., 2008; Li et al., 2008). However, how these pathways regulate neuronal polarization in a coordinated manner is not clear. GSK3 signaling has been shown to crosstalk with each of these pathways, suggesting that it may function to coordinate and integrate polarity signaling. Here, we review the roles of GSK3 signaling and its potential link to established polarity pathways during each step of neuronal polarization (**Figure 1**).

BREAKING THE SYMMETRY TO ESTABLISH NEURONAL POLARITY

Before polarization, intracellular signaling molecules that control polarization are distributed evenly among unpolarized neurites. The breaking of cell symmetry is initiated by local activation of intracellular signals in one of the neurites, which later develops into the axon. One such symmetry-breaking signaling molecule is PI3K (Shi et al., 2003), which in neurons is a key downstream signaling mediator of receptor tyrosine kinases (RTKs; Reichardt, 2006). Activated PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to produce

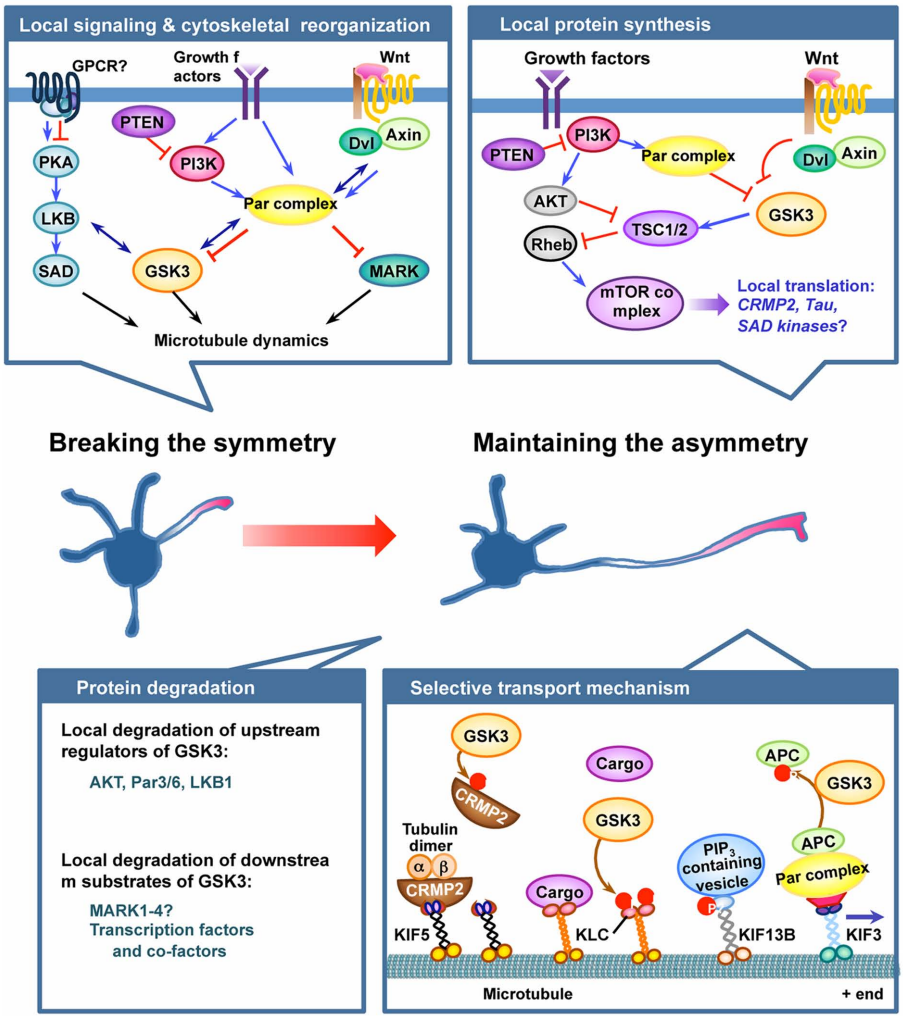

FIGURE 1 | GSK3 plays pivotal roles in the establishment and maintenance of neuronal polarization. Neuronal polarization process begins by breaking cell symmetry, which is thought to be initiated by local activation of intracellular signaling in the nascent axon. Symmetry-breaking signals might involve confined activation of cell surface receptors, such as G protein-coupled receptors (GPCRs) or receptor tyrosine kinases, in the growth cone of the future axon. Several lines of evidence suggest GSK3 as a crucial node molecule of the initial symmetry-breaking pathways, such as the protein kinase A (PKA)–LKB1 pathway, the PI3K–partition defective protein (Par) pathway, and the Wnt–disheveled (Dvl) pathway. Local activation of symmetry-breaking signals leads to the reorganization of cytoskeletal elements, thereby establishing neuronal polarity. GSK3 might serve as a key molecule in the coordination of cytoskeletal elements by controlling microtubule dynamics and assembly via regulation of several microtubule-binding proteins (MBPs). Microtubule affinity-regulating kinase (MARK) has also been suggested to function downstream of the Par complex to control neuronal polarization by regulating MBPs. Once the symmetry is broken, maintaining the selective extension one neurite – the future axon – can be controlled by multiple mechanisms, including local protein synthesis and degradation, and selective transport mechanisms. GSK3 has been implicated in the local protein synthesis pathway by controlling the activity of the mammalian target of rapamycin (mTOR) complex via phosphorylating tuberous sclerosis 2 (TSC2), but the exact roles of the players involved and their regulation in the context of axon growth await to be determined. Local protein degradation through activation of the ubiquitin–proteasome system (UPS) is another possible mechanism to maintain asymmetrical accumulation of polarity proteins. Molecules that control GSK3 activity, such as Akt, PAR3/6, and LKB1, have been suggested as targets of the local degradation process. In addition, accumulating evidence suggests that protein levels of multiple downstream substrates of GSK3 are controlled by phosphorylation-dependent proteolytic degradation. Future studies are required to determine whether GSK3 activation in dendrites plays a part in maintaining the polarized growth of the axon and restricting the growth of the dendrites by controlling local protein degradation. Finally, selective delivery of membrane and cytoskeletal components and signaling proteins into defined compartments is an important mechanism to maintain neuronal polarity. GSK3 phosphorylates several motor proteins and polarity proteins to control their selective transport and localization. See text for further details for each of the mechanism depicted in the figure.

phosphatidylinositol-3,4,5-trisphosphate (PIP3) in the cell membrane, which then activates its downstream targets, such as AKT and Rho-GTPases (Cantley, 2002). Local activation of PI3K and the subsequent accumulation of PIP3 in the neurite that becomes the future axon have been shown to be early markers of neuronal polarization (Menager et al., 2004). Several lines of evidence

suggest that GSK3 inactivation functions downstream of the PI3K–PIP3 pathway to control neuronal polarization. Thus, global inhibition of GSK3 with pharmacological inhibitors induces multiple axon formation, whereas overexpression of an active mutant of GSK3β (GSK3β-S9A) prevents axon formation (Jiang et al., 2005; Yoshimura et al., 2005). Importantly, overexpression of PTEN (a phosphatase that dephosphorylates PIP3) blocks neuronal polarization, which can be rescued by direct inhibition of GSK3 with pharmacological inhibitors (Jiang et al., 2005). Conversely, knocking down of PTEN induces multiple axon formation, which can be antagonized by overexpression of the active GSK3 mutant. However, how PI3K inactivates GSK3 to control neuronal polarization is still not clear.

One way that PI3K inactivates GSK3 is through AKT-mediated phosphorylation of GSK3 at an N-terminal domain serine residue (Cross et al., 1995). In support of the importance of Akt regulation, phosphorylated GSK3β co-localizes with phosphorylated AKT in the axon (Zhou et al., 2004; Jiang et al., 2005). Moreover, overexpression of a myristoylated active form of AKT leads to multiple axon formation which is antagonized by GSK3β-S9A expression (Jiang et al., 2005). However, neurons from the GSK3α-S21A/GSK3β-S9A double knockin mice, in which GSK3 cannot be phosphorylated by AKT, show no defect in neuronal polarization (Gartner et al., 2006), arguing against the idea that PI3K controls neuronal polarization via AKT-mediated GSK3 inactivation. PI3K has also been shown to regulate neuronal polarization through the Rho-GTPase Cdc42 and the subsequent activation of the Par3/6 cell polarity pathway (Shi et al., 2003). Par3/6 are mammalian homologs of the partition defective protein 3/6 of *C. elegans*. Interestingly, during directed cell migration locally activated Cdc42 at the cell leading edge can recruit the Par3/6 complex, which subsequently interacts with GSK3 and leads to GSK3 inactivation (Etienne-Manneville and Hall, 2003). Therefore, an alternative way that PI3K might inactivate GSK3 during neuronal polarization is via the Cdc42–Par3/6 pathway (**Figure 1**; Hur and Zhou, 2010). Further studies are required to test this hypothesis in neurons during polarization. How Par3/6 regulates GSK3 activity is unclear. A recent study showed that GSK3 activity could be regulated by DISC1 through protein–protein interactions independent of GSK3 serine phosphorylation (Mao et al., 2009). It is possible that Par3/6 also regulate GSK3 activity via the similar mechanism (Hur and Zhou, 2010). Interestingly, Par3 can directly bind to PI3K and enhance its activity (Itoh et al., 2010), suggesting that increased serine phosphorylation of GSK3 by Akt might be downstream of GSK3 inactivation by Par3. As a result, the levels of GSK3 serine phosphorylation can still be used as an indicator of GSK3 activity, even though GSK3 phosphorylation may not be a major mechanism by which GSK3 activity is inactivated (Hur and Zhou, 2010).

In addition to PI3K, another signaling molecule also activated downstream of RTKs or GPCRs that may act to break symmetry and initiate polarization is PKA. A recent study of neuronal polarization using live cell imaging of a cAMP biosensor suggests that local elevation of cAMP and activation of PKA in one undifferentiated neurite is an early signal to establish neuronal polarity (Shelly et al., 2010). The downstream mediator of PKA in regulation of neuronal polarization is the serine/threonine kinase LKB1 (**Figure 1**), which is the homolog of the partition defective protein 4 (Par4) of *C. elegans* (Barnes et al., 2007; Shelly et al., 2007). Activated PKA phosphorylates LKB1 and elevates its protein level. LKB1 then phosphorylates and activates SAD kinases, which have a similar kinase domain to that of partition defective protein 1 (Par1) and its mammalian homologs, MARK1–MARK4 (Kishi et al., 2005). Activated SAD kinases are believed to regulate neuronal polarization via the microtubule-binding protein Tau (Kishi et al., 2005). Interestingly, activation of PKA in neurons induces increased phosphorylation of both LKB1 and GSK3 (Shelly et al., 2010). Moreover, depletion of LKB1 abolishes GSK3 phosphorylation in all unpolarized neurites (Asada et al., 2007). These results suggest that GSK3 acts downstream of the PKA–LKB1 pathway to control neuronal polarization. Consistent with this idea, the *Xenopus* homolog of LKB1, XEEK1, is able to directly interact with GSK3β *in vivo* (Ossipova et al., 2003; **Figure 1**). In addition, a recent study showed that LKB1 inactivates GSK3 in mammalian neurons to control neuronal migration (Asada and Sanada, 2010). Taken together, these results suggest that GSK3 may act downstream of PI3K and PKA to establish neuronal polarity via direct interaction with Par3/6 and LKB1, respectively. While the cAMP–PKA pathway promotes axon formation during the symmetry-breaking process, reciprocal activation of the cGMP pathway acts to promote dendrite formation. A new study showed that semaphorin 3A acts upstream of cGMP to induce dendrite formation (Shelly et al., 2011). In contrast to the cAMP–PKA pathway that leads to GSK3 inactivation, semaphorin 3A has been shown to activate GSK3 (Eickholt et al., 2002). Consistent with this observation, semaphorin 3A treatment antagonizes PKA-induced GSK3 phosphorylation (Shelly et al., 2011). Together, these studies indicate that reciprocal regulation of cAMP/cGMP signaling controls axon/dendrite formation via opposite modulation of GSK3 activity.

POLARIZED CYTOSKELETAL REORGANIZATION PRECEDING ASYMMETRICAL AXON EXTENSION

After polarity signals are accumulated in the future axon but before asymmetric axon extension occurs, the cytoskeletal elements in the future axon reorganize to complete the establishment of neuronal polarity (Witte and Bradke, 2008). Specifically, the actin filaments (F-actin) in the growth cone of the future axon become more dynamic than those in growth cones of other neurites (Bradke and Dotti, 1999). In addition, the microtubules in the future axon are preferentially stabilized (Witte et al., 2008). Importantly, these polarized reorganizations of F-actin and microtubules *per se* are sufficient to establish neuronal polarity, indicating that they play instructive roles in establishing neuronal polarization, rather than simply supporting axon extension (which will be discussed later in this review). However, how such polarized cytoskeletal rearrangement is regulated by upstream polarity signals remains unclear.

Among all the polarity pathways, GSK3 signaling is the best candidate for transducing the polarity signal into cytoskeletal reorganization, in particular microtubule stabilization (Zhou and Snider, 2005; Hur and Zhou, 2010). Many MBPs involved in neuronal polarization are GSK3 substrates, such as collapsin response-associated protein 2 (CRMP2; Cole et al., 2004), adenomatous

polyposis coli (APC), Tau, and MAP1b. The function of these MBPs is to promote microtubule assembly and stability, and phosphorylation of them by GSK3 abolishes their ability to interact with microtubules. As a result, polarity signals can stabilize microtubules in the future axon by inactivation of GSK3. For instance, inhibition of GSK3 enhances the interactions between CRMP2 and tubulin dimers, which subsequently promotes microtubule assembly and stability. Overexpression of CRMP2 is sufficient to induce the formation of multiple axons (Yoshimura et al., 2005). Moreover, knocking down of CRMP2 antagonizes multiple axon formation induced by global GSK3 inactivation, indicating the CRMP2 acts downstream of GSK3 inactivation (Yoshimura et al., 2005). The microtubule plus end tracking protein (+TIP) APC localizes at the tips of all unpolarized neurites before neuronal polarization, and later becomes enriched in the growth cone of the future axon (Votin et al., 2005). Binding of APC to the microtubule plus ends functions to stabilize microtubules, a process negatively regulated by GSK3-mediated phosphorylation (Zumbrunn et al., 2001; Zhou et al., 2004). Therefore, local GSK3 inactivation in the future axon promotes the binding of APC to the microtubule plus ends, leading to asymmetrical microtubule stabilization in the future axon. However, neurons from APC knockout mice do not show polarization defects (Yokota et al., 2009), suggesting compensatory effect of its homolog APC-L (Nakagawa et al., 1998) or redundant function of other microtubule +TIPs.

How polarized cytoskeletal reorganization directs neuronal polarization is unknown. One potential mechanism is that it may affect post-translational modification of microtubules, which then facilitate the binding of motor proteins to the microtubules and the subsequent directed transport (see detailed discussion below). Indeed, activated kinesin-1 (or KIF5) is preferentially accumulated in the future axon before polarized axon extension, and global application of taxol or GSK3 inhibitors disrupts the polarized targeting of kinesin-1 (Hammond et al., 2010).

MAINTAINING ASYMMETRICAL AXON EXTENSION

After the establishment of initial polarity, neurons require sustained asymmetrical axon extension to complete the polarization process. Many cellular processes have been suggested to maintain such polarized axon extension, including polarized transport of proteins and membrane, local protein translation, and local protein degradation. GSK3 signaling has been shown to be involved in all of these processes.

Polarized transport of polarity determinants is important for their sustained accumulation in the axon to direct asymmetrical axon extension (**Figure 1**). Studies have shown that PI3K, PIP3, and Par3 are all specifically transported to the axon by kinesins, and that disruption of such transport blocks neuronal polarization (Shi et al., 2004; Horiguchi et al., 2006; Amato et al., 2011). GSK3 can directly phosphorylate several kinesin light chains and thus regulate the interactions between kinesins and their transported cargoes (Morfini et al., 2002, 2004; Szebenyi et al., 2002). In addition, many of the kinesin superfamily proteins have conserved GSK3 consensus sites (Taelman et al., 2010). Thus GSK3 may be a major regulatory factor of kinesin function. In support of this idea, Par3 is specifically delivered to the axon by forming a protein complex with APC and KIF3A, and such polarized transport requires local inactivation of GSK3 in the axon (Shi et al., 2004). Moreover, a recent study demonstrated that activation of the energy-sensing adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) suppressed neuronal polarization by phosphorylating the kinesin light chain (KCL2) of the KIF5 protein, which functions to target PI3K to the axon (Amato et al., 2011). Because GSK3 also phosphorylates KCL2 and negatively regulates its function (Morfini et al., 2002, 2004), it is possible that GSK3 inactivation promotes PI3K transport into the axon, thus forming a positive feedback loop to control neuronal polarization. Finally, PIP3 is specifically transported to the axon by KIF13B, which is also a potential GSK3 substrate (Horiguchi et al., 2006). In addition to regulating transport of proteins, GSK3 also regulates polarized axonal transport of vesicles containing membrane components (Morfini et al., 2004; Gartner et al., 2006). GSK3β is specifically accumulated in the Golgi region of hippocampal neurons in a membrane-bound form. Global inhibition of GSK3 with inhibitors disrupts preferential traffic of membrane containing vesicles to the axon, and directs them symmetrically toward all neurites (Gartner et al., 2006). However, how GSK3 regulates membrane transport is unknown. Together, these studies suggest that GSK3 functions to maintain asymmetrical axon extension via regulation of directed axonal transport.

Local and asymmetrical protein translation of polarity molecules has been reported to control neuronal polarization (**Figure 1**). One important pathway that regulates protein translation is the TSC–mTOR pathway (Ma and Blenis, 2009). Activation of AKT activates Rheb and the subsequent mammalian target of rapamycin (mTOR) kinase by inactivating their negative regulators TSC1/2. Activated mTOR then phosphorylates multiple translation regulators, such as ribosomal S6 kinase (S6K) and translation initiation factor 4E binding protein (4EBP), to initiate protein translation. During neuronal polarization phosphorylated S6K and 4EBP are enriched in the axon (Morita and Sobue, 2009), indicating local activation of mTOR signaling. Further, overexpression of constitutively activated Rheb and S6K induces multiple axon formation, whereas dominant negative Rheb and S6K block neuronal polarization (Morita and Sobue, 2009). Importantly, genetic knockout of TSC1 or TSC2 leads to the formation of multiple axons *in vitro* and *in vivo* (Choi et al., 2008). Evidence showing direct involvement of GSK3 signaling in the TSC–mTOR pathway during neuronal polarization is lacking. However, one study has shown that GSK3 directly phosphorylates TSC2 after AMPK-mediated priming, leading to inhibition of the mTOR pathway (Inoki et al., 2006). Conversely, inactivation of GSK3 is sufficient to activate mTOR-dependent protein translation. Therefore, it is possible that local inactivation of GSK3 in the axon plays a role in regulation of local translation through the mTOR pathway. Several polarity-related proteins are locally translated in the axon, including SAD kinase, CRMP2, Tau, and Par3 (Inoki et al., 2006; Choi et al., 2008; Hengst et al., 2009). Very interestingly, as mentioned earlier CRMP2 and Tau are GSK3 substrates, whereas Par3 acts upstream of GSK3, indicating additional examples of positive feedback loops involving GSK3 signaling through local protein translation.

Several studies have shown that local protein degradation via the ubiquitin–proteasome system (UPS) also controls neuronal

polarization (**Figure 1**). Local degradation of polarity determinants in minor neurites is necessary to maintain their asymmetrical accumulation in the axon. In support, UPS has been shown to control the local degradation of AKT, Par3/6, and LKB1 in minor neurites during neuronal polarization, and global inhibition of UPS results in the formation of multiple axons (Yan et al., 2006; Cheng et al., 2011). Conversely, local degradation of RhoA (a suppressor of axon extension) is also required to maintain asymmetrical axon extension (Cheng et al., 2011). Although the GSK3 level in neurons is not regulated by UPS-mediated protein degradation, inhibition of UPS increases the level of phosphorylated GSK3β (Yan et al., 2006), suggesting that GSK3 inactivation might be involved in multiple axon formation induced by UPS inhibition. In support of this idea, the best studied function of GSK3 is regulation of the degradation of β-catenin in the canonical Wnt pathway via UPS (Hur and Zhou, 2010). A recent study demonstrates that GSK3 regulates UPS-mediated degradation of a much broader list of proteins (Kim et al., 2009a; Xu et al., 2009), raising the possibility that GSK3 may regulate the degradation of polarity determinants. For instance, Par3 and the aforementioned Par1 homologs, MARK1-4, contain three and six consecutive GSK3 phosphorylation sites (Taelman et al., 2010), respectively. It will be extremely interesting in the future to investigate whether these proteins can be regulated by GSK3-dependent, UPS-mediated protein degradation.

In summary, evidence is emerging that GSK3 is a key signal-integrating molecule that coordinates neuronal polarization by transducing early symmetry-breaking signaling, controlling cytoskeletal reorganization, and modulating polarized protein distribution. How GSK3 is asymmetrically regulated during neuronal polarization is not fully understood. One possible mechanism is that upstream regulators of GSK3 is locally distributed or activated. Another potential mechanism is local sequestration of GSK3 by scaffolding proteins. For instance, GSK3 has been shown to bind to the A-kinase anchoring proteins 220 (AKAP220), which mediates localized interaction between GSK3 and PKA (Tanji et al., 2002). In addition, a recent study showed that a GSK3 interacting protein could also function as an AKAP (Hundsrucker et al., 2010).

GSK3 SIGNALING IN AXON GROWTH

After the neuron polarizes, the axon may extend a long distance to reach its targets and form synaptic connections. Axon growth is supported by extracellular factors, which transduce their signals into coordinated gene expression in the neuronal soma and local assembly of cytoskeleton and membrane in the growth cone (Zhou and Snider, 2006). GSK3 is a key mediator that translates extracellular signals into controlled axon assembly. Here we summarize how extracellular factors regulate GSK3 activity and discuss how GSK3 controls cytoskeletal reorganization in the growth cone during axon elongation.

GSK3 IS REGULATED BY MULTIPLE EXTRACELLULAR FACTORS THAT CONTROL AXON GROWTH

The most studied extracellular factors that support axon growth are growth factors, such as neurotrophins, IGF-1, and GDNF (Zhou and Snider, 2006). All these factors initiate their intracellular signals through RTKs. One of the major downstream mediators of RTKs is PI3K which plays a key role in controlling axon growth and is the best-known regulator of GSK3 signaling. Treatment of embryonic dorsal root ganglion (DRG) neurons with nerve growth factor (NGF) leads to PI3K activation and increased phosphorylation of GSK3β at its N-terminal serine-9 (Zhou et al., 2004). Functionally, overexpression of the active mutant of GSK3β (GSK3β-S9A) blocks NGF-induced axon growth. These results indicate that NGF inactivates GSK3 downstream of PI3K to promote axon growth. However, other studies have shown that NGF treatment leads to increased phosphorylation of MAP1b at its GSK3 sites, indicating that NGF also activates GSK3 (Goold and Gordon-Weeks, 2003; Trivedi et al., 2005). In contrast to GSK3β inactivation downstream of PI3K, GSK3 activation, and the subsequent phosphorylation of MAP1b is regulated by ERK1/2 (Goold and Gordon-Weeks, 2005), another major signaling mediator downstream of RTKs. Global application of netrin, a well-studied attractive axon guidance cue, also promotes axon growth through its receptor deleted in colon cancer (DCC) and the activation of the ERK1/2 pathway (Forcet et al., 2002). Similar to NGF, treatment of cultured neurons with netrin increased the level of phosphorylated MAP1b at its GSK3 site (Del Rio et al., 2004). Moreover, netrin treatment led to increased levels of GSK3β phosphorylation at both tyrosine-216, which is often associated with GSK3 activation, and serine-9, which indicates GSK3 inactivation (Del Rio et al., 2004). Thus, both NGF and netrin seem to inactivate and activate GSK3 in same cells downstream of PI3K and ERK1/2, respectively. One potential explanation is that regulation of GSK3 by NGF or netrin in neurons is segregated spatially or temporary allowing inactivation and activation at different times or places inside cell. Another possible explanation is that phosphorylation of GSK3β, either at serine-9 or at tyrosine-216, may not be a reliable indicator of GSK3 activity (Hur and Zhou, 2010). Future studies to directly examine GSK3 activity in live cells are required to test these hypotheses. Finally, one study has shown that Wnt3a can promote axon growth of mouse embryonic DRG neurons through its atypical receptor Ryk and the canonical Wnt pathway (Lu et al., 2004), suggesting the involvement of GSK3. However, the role of GSK3 in Wnt3a-induced axon growth has not been directly tested.

GSK3 CONTROLS AXON ELONGATION VIA REGULATION OF THE GROWTH CONE MACHINERY

Axon elongation requires continuous addition of microtubules to the growing end, which is controlled by coordinated regulation of microtubule dynamics and actin reorganization in the growth cone (Zhou and Snider, 2006). Actin filaments are enriched in the peripheral domain (P-domain) of the growth cone, and are organized into actin bundles and actin meshwork. Microtubules project from the axon shaft to the central region (C-domain) of the growth cone in a bundled form. At the end of the microtubule bundles, individual microtubules splay apart and a subset of microtubules invade the actin-rich P-domain. Axon elongation is ultimately achieved through microtubule assembly at the nerve growth cones, in which a key step is microtubule extension toward the growth cone leading edge. Several factors have been shown to control microtubule extension in the growth cone. First, microtubules undergo cycles of polymerization and depolymerization, a

process called dynamic instability (Burbank and Mitchison, 2006). There is an extensive list of microtubule or tubulin-binding proteins that act to regulate this process (Poulain and Sobel, 2010). For instance, some proteins can bind to the tubulin dimers and control tubulin polymerization (e.g., CRMP2) or depolymerization (e.g., stathmin). In addition, classic microtubule associated proteins (MAPs, e.g., Tau and MAP1b) bind along the microtubule lattices, whereas microtubule +TIPs (e.g., APC, EB1/3, and CLASP1/2) specifically interact with the plus ends, both of which function to stabilize polymerized microtubules. Lastly, there are proteins that function to disassemble microtubules, including microtubule severing proteins (e.g., katanin and spastin) and microtubule depolymerases (e.g., MCAK/KIF2c). Second, microtubule extension in the growth cone can be regulated by motor protein-mediated transport of microtubule polymers. The plus end directed motor kinesin-5, when anchored on the membrane, has been shown to transport microtubules away from the leading edge (Myers and Baas, 2007). Conversely, the minus end directed motor dynein, when immobilized at the growth cone leading edge, can pull the microtubules toward the leading edge (Myers et al., 2006). Third, dynamic interactions between actin filaments and microtubules in the growth cone are key processes that control the microtubule extension. Actin bundles function to guide microtubule extension toward the leading edge, whereas retrograde flow of actin meshwork powered by myosin II carries microtubules rearward, thus acting as a dynamic barrier for microtubule extension (Schaefer et al., 2002; Zhou et al., 2002; Medeiros et al., 2006).

Many of these cytoskeletal proteins are established GSK3 substrates, including CRMP2, APC, CLASP1/2, MAP1b, Tau, stathmin, and myosin II (Moreno and Avila, 1998; Kim et al., 2009a; Hur and Zhou, 2010), or contain conserved GSK3 sites, such as Katanin and KIF2c (Taelman et al., 2010). Thus, GSK3 is considered to be a major signaling pathway that regulates the growth cone machinery to drive axon elongation (**Figure 2**). However, because these molecules play different, and sometimes opposing roles in controlling cytoskeletal reorganization, the role of GSK3 signaling in regulation of axon elongation is complex. Expression of the active mutant of GSK3 (GSK3β-S9A) blocks axon growth (Zhou et al., 2004, 2006), indicating that inactivation of GSK3 is necessary for efficient axon elongation. Surprisingly, inhibition of GSK3 has been shown to either promote or impede axon growth depending on the extent of inhibition. Moderate GSK3 inhibition locally at the growth cone is required for efficient axon growth, whereas more complete inhibition of GSK3 impedes axon growth (Kim et al., 2006). One potential explanation for such pleiotropic effects is that different degrees of GSK3 inhibition differentially affect different GSK3 substrates. In support of this idea, phosphorylation of CRMP2 by GSK3 is more sensitive to GSK3 inhibition than phosphorylation of MAP1b (Kim et al., 2006). The identities of GSK3 substrates and the molecular mechanisms by which they specifically mediate axon growth promotion or axon growth inhibition upon GSK3 inactivation are being actively studied. Several molecules have been identified that promote axon growth downstream of GSK3 inactivation, including CRMP2 and APC. As mentioned earlier, inhibition of GSK3 facilitates the binding of CRMP2 to tubulin dimers and thus promotes axon growth by enhancing microtubule polymerization in the growth cone (Yoshimura et al., 2005). Similarly, dephosphorylated APC specifically bind to the microtubule plus ends and function to stabilize polymerizing microtubules, leading to increased microtubule extension in the growth cone (Zhou et al., 2004).

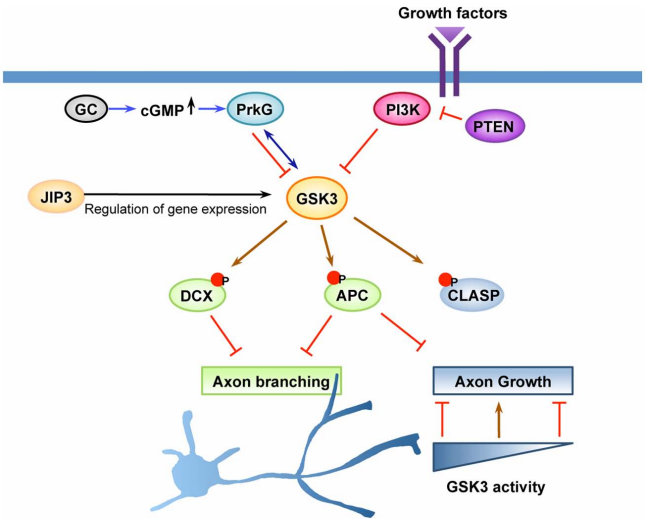

FIGURE 2 | GSK3 is a key regulator of axon growth and branching. Axon growth is thought to be initiated by activation of cell surface receptors upon recognition of extrinsic cues, such as growth factors or other ligands. GSK3 has been suggested as a key molecule downstream of the PI3K pathway and the cyclic guanosine monophosphate (cGMP) pathway to control axon growth and/or branching. Upon activation of PI3K, GSK3 becomes inactivated, leading to dephosphorylation of GSK3 substrates that control the stability and dynamics of axonal microtubules. Dephosphorylation and subsequent binding of adenomatous polyposis coli (APC) on microtubule plus ends at certain locations along the axon enhances axonal branching, whereas its enrichment at the growth cone leads to the promotion of axon growth. CLASP, another substrate of GSK3 that binds to microtubules in response to GSK3 inactivation, plays a role to regulate axon growth downstream of GSK3. CLASP can both promote and inhibit axon growth depending on its microtubule-binding activity, a property which is tightly controlled by GSK3 activity. The cGMP pathway is another pathway that has been shown to control axonal branching. In response to elevation of cGMP by activation of guanylyl cyclase (GC), cGMP-dependent protein kinase G (PrkG) becomes activated and functions to increase axon branching by inactivating its substrate GSK3. In addition to the direct regulation of the kinase activity, GSK3 activity can be controlled by other mechanisms, such as regulation of its expression level. JNK-interacting protein (JIP) 3 has been suggested to control the expression level of GSK3 at the transcription level and/or by regulating mRNA stability. Down-regulation of JIP3, which decreases GSK3 level, increases axonal branching by controlling the phosphorylation status of doublecortin (DCX), a substrate of GSK3. Coordinated regulation of multiple GSK3 substrates likely defines the extent of axon growth and the locations at which axonal branching occurs.

Which substrates mediate impaired axon growth upon GSK3 inhibition to a greater extent? A new study has identified CLASP as a mediator to block axon growth upon greater GSK3 inhibition (Hur et al., 2011). Specifically, the study showed that in the absence of CLASP2, knocking down of GSK3 was unable to block axon growth. The study also revealed that in nerve growth cones CLASP could either track the microtubule plus ends or bind along the microtubule lattice depending on its phosphorylation states

mediated by GSK3. Interestingly, these two microtubule-binding activities of CLASP regulate axon growth in an opposing manner: the microtubule lattice-binding activity mediates axon growth inhibition via preventing microtubule protrusion into the growth cone periphery, whereas the microtubule plus end-binding property supports axon extension via stabilizing the growing ends of axonal microtubules. As a result, when GSK3 activity becomes strongly suppressed the binding of CLASP along the microtubule lattice is increased, which subsequently induces microtubule looping in the growth cone and blocks axon growth. In addition to CLASP, other GSK3 substrates may also contribute to impaired axon growth upon greater GSK3 inhibition. For instance, the regulatory light chain of myosin II (MLC) was recently identified to be a GSK3 substrate, and phosphorylation of MLC by GSK3 promotes its degradation through UPS (Kim et al., 2009a). Moreover, both myosin IIA and IIB contain conserved GSK3 sites (Taelman et al., 2010), suggesting GSK3 as an important regulator of myosin II function. As a result, inhibition of GSK3 could block axon growth by enhancing myosin II activity in the growth cone. Indeed, similar to that of CLASP2, knocking down of GSK3 was unable to block axon growth when myosin II activity was inhibited (Hur et al., 2011). Further study is needed in the future to show directly that GSK3 phosphorylates myosin II and regulates its function in the growth cone. Another potential substrate is MAP1b, which plays a central role in maintaining microtubule dynamics in the growth cone when phosphorylated by GSK3 (Goold et al., 1999). Because microtubule dynamics are necessary for efficient axon growth, dephosphorylation of MAP1b upon greater inhibition of GSK3 may impede axon growth by reducing microtubule dynamics in the growth cone (Owen and Gordon-Weeks, 2003). In support of this idea, neurons from the MAP1b knockout mice show axon growth defects and exhibit markedly reduced microtubule dynamics in their growth cones (Gonzalez-Billault et al., 2002). Lastly, a recent study has provided some evidence that CRMP4 might act downstream of GSK3 inhibition to block axon growth (Alabed et al., 2010). Dephosphorylation of CRMP4 increases its binding to RhoA, a known negative regulator of axon growth.

In summary, abundant evidence indicates that an optimal level of GSK3 activity is required for efficient axon elongation (**Figure 2**). Under such conditions, some substrates are dephosphorylated (e.g., CRMP2, APC) to promote microtubule extension, whereas others are phosphorylated (e.g., CLASP, MAP1b) to maintain microtubule dynamics. Therefore, a delicate balance between activation and inactivation of GSK3 acts to support the most efficient axon elongation by coordinating the functions of multiple GSK3 substrates in the growth cone.

GSK3 SIGNALING IN AXON BRANCHING

When axons arrive at their destination, they usually form branches to innervate multiple targets. As with axon elongation, axon branching is tightly controlled by extracellular cues and their downstream intracellular signaling pathways that mediate reorganization of the axonal cytoskeleton to form axon branches. Based on morphology, axon branching can be grouped into arborization, bifurcation, and interstitial branching (Gibson and Ma, 2011). Time-lapse studies of live cells demonstrate that axon arborization and bifurcation usually occur at the distal axon through growth cone splitting, whereas interstitial branching forms *de novo* along the axon shaft (Zhou et al., 2002; Portera-Cailliau et al., 2005; Schmidt et al., 2007). Increasing evidence points to GSK3 as a converging signaling mediator that transduces branching signals into regulated cytoskeletal rearrangement at the growth cone or along the axon shaft. Here we review previous studies that show the involvement of GSK3 in axon branching and discuss the potential cellular mechanisms by which GSK3 signaling controls axon branching (**Figure 2**).

GSK3 functions have been carefully studied in sensory axons of DRG neurons that bifurcate at the dorsal root entry zone (DREZ) when they enter the spinal cord during development. Recent studies have identified C-type natriuretic peptide (CNP; also known as NPPC) as the extracellular factor that controls DRG axon bifurcation (Schmidt et al., 2009; Zhao and Ma, 2009). Downstream of CNP, cGMP, and cGMP-dependent protein kinase G1 (PrkG1) act to control axon branching via GSK3 inactivation (Zhao et al., 2009). Specifically, axon branching of cultured embryonic DRG neurons was substantially increased upon treatment of CNP or cGMP (8-Br-cGMP), and the effect was abolished in neurons from the PrkG1 knockout mice (Zhao and Ma, 2009). Moreover, PrkG1 inhibits GSK3β by binding and directly phosphorylating GSK3β at its serine-9 residue (Zhao et al., 2009). Importantly, cGMP-induced axon branching can be antagonized by overexpression of the active mutant of GSK3β (GSK3β-S9A), confirming that GSK3 inactivation is necessary for the cGMP–PrkG1 pathway to induce axon branching. As discussed earlier, serine phosphorylation may not be a major mechanism by which GSK3 is inactivated. Thus, it will be interested to examine if DRG axon bifurcation in the spinal cord during development is normal in the GSK3α-S21A/GSK3β-S9A double knockin mice, or to determine if PrkG1 is able to inactivate GSK3 via alternative mechanisms. After entering the spinal cord, axons of the NT3-responsive neurons in the DRG project ventrally and form terminal arborizations upon contact with the motor neurons, their synaptic targets. Wnt3 secreted by the motor neurons has been shown to cause such axon branching, suggesting that inhibition of GSK3 through the canonical Wnt pathway might be involved (Krylova et al., 2002). Indeed, inhibition of GSK3 with pharmacological inhibitors induced similar axonal arborization, mimicking the effect of Wnt3 (Krylova et al., 2002). In addition to the central axons in the spinal cord, the peripheral axons of sensory neurons also form terminal branches within their targeting areas (e.g., skin). The axon guidance cue Slit has been shown to play an important role in regulation of such peripheral axon branching of sensory neurons (Ma and Tessier-Lavigne, 2007). Whether GSK3 signaling is involved in Slit-induced axon branching is unknown. However, regulated GSK3 inactivation is involved in Slit-mediated neuronal migration (Higginbotham et al., 2006). In addition, the GSK3 substrate CLASP has been shown to act downstream of Slit to mediate axon repulsion (Lee et al., 2004). These results raise the possibility that Slit regulates sensory axon branching via GSK3 signaling.

In addition to extracellular cues, recent studies have provided evidence that intracellular molecules regulate axon branching via GSK3 signaling. One study (Bilimoria et al., 2010) showed that JNK-interacting protein 3 (JIP3) functioned to restrict axon branching of mouse cerebellar neurons via GSK3 and its substrate

doublecortin (DCX). Knocking down of JIP3 resulted in significantly increased axon branching and a reduced level of GSK3β, protein suggesting that down-regulation of GSK3β mediated axon branching-induced by JIP3 depletion (**Figure 2**). Consistent with this idea, knocking down of GSK3β led to increased axon branching in a similar manner. Moreover, knocking down both JIP3 and GSK3β did not show an additive effect on axon branching, confirming that they share the same pathway. Lastly, DCX was identified as a substrate of GSK3β acting downstream of JIP3 and GSK3β to restrict axon branching. Finally, A few studies have demonstrated that PTEN deletion promotes axon branching (Kwon et al., 2006; Drinjakovic et al., 2010). As down-regulation of PTEN leads to increased activity of PI3K signaling, it is possible that axon branching-induced by loss of PTEN is through GSK3 inactivation. Together, these studies indicate that GSK3 inactivation is a common mechanism by which both extracellular cues and intracellular signals induce axon branching.

How GSK3 inactivation is translated into axon branching is not fully understood. High-resolution time-lapse studies of F-actin and microtubule dynamics indicate that coordinated regulation of F-actin reorganization and microtubule extension controls branch formation, similar to what occurs in the growth cone to drive axon elongation (Kalil et al., 2000; Dent and Kalil, 2001). At the growth cone, GSK3 inactivation is known to induce growth cone enlargement (Owen and Gordon-Weeks, 2003; Kim et al., 2006), presumably by promoting microtubule protrusion. Increased growth cone size with enhanced microtubule protrusion may therefore result in increased growth cone splitting to form axon branches. Along the axon shaft, axon branch formation requires coordinated cytoskeletal reorganization, including microtubule de-bundling and fragmentation, actin filopodia formation followed by microtubule extension (Dent and Kalil, 2001). Local inactivation of GSK3 along the axon shaft may promote these cytoskeletal changes through various GSK3-regulated cytoskeletal proteins. For instance, inactivation of GSK3 may modulate the function of DCX (Bilimoria et al., 2010), which is known to regulate microtubule organization along the axon shaft (Bielas et al., 2007; Tint et al., 2009; **Figure 2**). The microtubule severing protein Katanin is involved in microtubule fragmentation, a required process for initiating the branch formation (Qiang et al., 2010). The microtubule depolymerase KIF2a functions to impair microtubule polymerization and thus limiting the extension of axon branches (Homma et al., 2003). Interestingly, both Katanin and KIF2a are potential GSK3 substrates (Taelman et al., 2010), suggesting that inactivation of GSK3 may regulate their functions to control axon branching. Moreover, local inactivation of GSK3 along the axonal shaft has been shown to induce local accumulation of APC (Kim et al., 2006), which may stabilize polymerized microtubules to promote axon branching.

CONCLUSION AND FUTURE DIRECTIONS

GSK3 is now considered to be a key signaling node molecule that acts to regulate multiple steps of neuronal morphogenesis during development by controlling a broad range of biological processes, such as cellular transport, protein translation and degradation, and cytoskeletal reorganization. Functions of GSK3 are achieved through its large number of substrates and multiple modes of regulation. Unlike most other kinases that are controlled by a simple activation/inactivation switch, it appears that regulation of GSK3 is more complex. Different levels of GSK3 activities regulated by upstream signals may affect different substrates, thus leading to distinct functions. Therefore, an important future direction is to identify and validate additional GSK3 substrates to understand how specific functions of GSK3 signaling are achieved via specific substrates. Moreover, the phosphorylation of GSK3 substrates is also regulated by various phosphatases, providing another level of regulation of GSK3 signaling.

As neuronal morphogenesis is a dynamic process, the ability to detect GSK3 activity at higher spatiotemporal resolution in live cells is necessary to fully understand the roles of GSK3 signaling. In addition to neuronal polarization, axon growth and axon branching, GSK3 signaling is also involved in other neuronal morphogenesis processes, such as directed neuronal migration, axon guidance, dendrite development, and synaptogenesis. Futures studies are necessary to establish the roles and the molecular mechanisms by which GSK3 signaling control these developmental processes. Finally, a number of neurological disorders, such as schizophrenia and autism, are associated with abnormal regulation of GSK3 signaling. It is therefore important in the future to identify the potential links between the functions of GSK3 signaling in neural development and the etiologies of these diseases.

The studies discussed in this review are mainly based on *in vitro* experiments. Most *in vitro* preparations are highly reduced and may not clearly reflect GSK3 functions in neurons *in vivo*, where a myriad of surface molecules and growth factors are present and a multiplicity of signaling pathways are active. Therefore, it is likely that the details of the developmental regulation by GSK3 signaling may not be entirely predictable from the *in vitro* studies that have appeared to date. Recently, decisive mouse genetic models (see other chapters) that enable cell-type specific GSK3 deletion have been generated. The first clear finding from these mice is that the two family members GSK3α and GSK3β can compensate for each other during brain development. Thus, when either GSK3α or GSK3β alone is deleted, gross morphological development proceeds. However, deletion of both family members results in dramatic neurodevelopmental abnormalities (Kim et al., 2006). The second clear finding is that GSK3 plays a major role in regulation of neural progenitors (Kim et al., 2009b). The neural progenitor cells of GSK3 deleted mice show striking disruption of apical–basal polarity, suggesting that GSK3 may also regulate cytoskeletal reorganization of neural progenitors. Work on GSK3 regulation of postmitotic neurons *in vivo* is just beginning. Experiments introducing constitutively activated GSK3 constructs via *in utero* electroporation strongly suggest a key role of GSK3 signaling in regulation of migration and cortical lamination (Asada and Sanada, 2010). It can be expected that further work with these mice will generate a decisive view of *in vivo* GSK3 functions in developmental processes discussed here, including neuronal polarization, axon extension and branching, as well as other key developmental processes, such as axon guidance, dendritic arborization, and synaptogenesis. However, it is important to keep in mind that because the regulation of GSK3 is complex, knockout studies, although very informative, may not fully reveal the molecular mechanisms by which GSK3 regulates neural development.

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Glycogen synthase kinase-3 is an intermediate modulator of serotonin neurotransmission

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Serotonin is a neurotransmitter with broad functions in brain development, neuronal activity, and behaviors; and serotonin is the prominent drug target in several major neuropsychiatric diseases. The multiple actions of serotonin are mediated by diverse serotonin receptor subtypes and associated signaling pathways. However, the key signaling components that mediate specific function of serotonin neurotransmission have not been fully identified. This review will provide evidence from biochemical, pharmacological, and animal behavioral studies showing that serotonin regulates the activation states of brain glycogen synthase kinase-3 (GSK3) via type 1 and type 2 serotonin receptors. In return, GSK3 directly interacts with serotonin receptors in a highly selective manner, with a prominent effect on modulating serotonin 1B receptor activity. Therefore, GSK3 acts as an intermediate modulator in the serotonin neurotransmission system, and balanced GSK3 activity is essential for serotonin-regulated brain function and behaviors. Particularly important, several classes of serotonin-modulating drugs, such as antidepressants and atypical antipsychotics, regulate GSK3 by inhibiting its activity in brain, which reinforces the importance of GSK3 as a potential therapeutic target in neuropsychiatric diseases associated with abnormal serotonin function.

Keywords: serotonin, GSK3, 5-HT1A, 5-HT1B, 5-HT2A, antidepressants

SEROTONIN NEUROTRANSMISSION

SEROTONIN

Serotonin (5-hydroxytryptamine, 5-HT) is a monoaminergic neurotransmitter that is synthesized from the amino acid tryptophan. Serotonergic neurons arise from the raphe nucleus of the brain stem, and they project upward to most areas of the brain and downward to peripheral nerve terminals. Upon release, 5-HT in the synapse is recycled by reuptake through the 5-HT transporter (5-HTT) and is catabolized by monoamine oxidase (MAO). There are at least 7 families and 14 subtypes of 5-HT receptors that are classified by their sequence homology and associated type of G-proteins and signal transduction pathways (Hoyer and Martin, 1997; Bockaert et al., 2006; **Table 1**). Among these 5-HT receptors, the type 1 (5-HT1) and the type 2 (5-HT2) receptors are the most studied, with rich literatures showing their roles in brain function and diseases (Hannon and Hoyer, 2008).

SEROTONIN TYPE 1A (5-HT1A) RECEPTORS

5-HT1A receptors are the prototypical 5-HT1 receptors. In addition to coupling to the inhibitory G-protein (Gi) that inhibits adenylyl cyclase, decreases cyclic AMP (cAMP) production, and inactivates protein kinase A (PKA; De Vivo and Maayani, 1986), studies in neurons reveal that 5-HT1A receptors also regulate other protein kinases, such as growth factor-associated Akt and extracellular signal-regulated kinases (Erk; Cowen et al., 1996, 2005). 5-HT1A receptors are widely distributed in major brain areas including the dorsal raphe nucleus, cerebral cortex, hippocampus, striatum, and nucleus accumbens (Varnas et al., 2004). In 5-HT neurons, 5-HT1A autoreceptors are located in the cell bodies and somatodendrites, and activation of these receptors reduces neuron firing and suppresses 5-HT release (Riad et al., 2000). In non-serotonergic neurons, 5-HT1A heteroreceptors are found in both pre- and post-synaptic locations (Hoyer et al., 2002). Depending on the location and associated neurons, activation of 5-HT1A heteroreceptors modulates neurotransmission of glutamate, GABA, norepinephrine, and acetylcholine (Fink and Gothert, 2007), generally causing inhibition of long-term potentiation (Edagawa et al., 1998; Tachibana et al., 2004), increased axonal and dendritic branching (Yan et al., 1997), and postnatal neurogenesis (Banasr et al., 2004). 5-HT1A receptors mediate many behavioral effects of 5-HT, particularly those related to mood and anxiety (Kennett et al., 1987; Parks et al., 1998).

SEROTONIN TYPE 1B (5-HT1B) RECEPTORS

The rodent 5-HT1B receptors and its human homolog 5-HT1D receptors share high protein sequence homology and signal transduction processes with 5-HT1A receptors, and 5-HT1B receptors also act as both autoreceptors and heteroreceptors in the brain (Bouhelal et al., 1988; Hoyer and Martin, 1997; Leone et al., 2000; Bockaert et al., 2006). Different from 5-HT1A autoreceptors, 5-HT1B autoreceptors are primarily located on the 5-HT neuron axon terminals that extend to other brain regions. Upon activation, 5-HT1B autoreceptors cause a strong feedback inhibition of 5-HT release (Riad et al., 2000). 5-HT1B receptors also act as heteroreceptors in several brain regions to control release of other neurotransmitters (Sari, 2004). Both 5-HT1B receptor agonists and antagonists may have antidepressant effects; blocking autoreceptors increases extracellular 5-HT which enhances the effect of

Table 1 | Serotonin receptors.

Family	Subtypes		Classical signal transduction
5-HT1	1A, 1B, 1D, 1E, 1F	G_i/G_o-protein coupled	Inhibit AC, reduce cAMP, inhibit PKA
5-HT2	2A, 2B, 2C	G_q/G_{11}-protein coupled	Increase IP3, increase intracellular calcium, increase DAG, activate PKC
5-HT3		Ligand-gated Na^+ and K^+ cation channel	Membrane depolarization
5-HT4		G_s-protein coupled	Activate AC, increase cAMP, activate PKA
5-HT5	5A, 5B	G_i/G_o-protein coupled	Inhibit AC, reduce cAMP, inhibit PKA
5-HT6		G_s-protein coupled	Activate AC, increase cAMP, activate PKA
5-HT7		G_s-protein coupled	Activate AC, increase cAMP, activate PKA

serotonin-reuptake inhibitors (Dawson et al., 2006), whereas activation of heteroreceptors contributes to an antidepressant effect, potentially through their effects on dopaminergic neurotransmission (Chenu et al., 2008). Recently, 5-HT1B receptors have been found to interact with an intracellular adaptor protein p11 (Svenningsson et al., 2006), which regulates 5-HT1BR activity and stability, and deletion of which causes depression-like behaviors in animals (Svenningsson and Greengard, 2007).

SEROTONIN TYPE 2A (5-HT2A) RECEPTORS

5-HT2A receptors are the prototypical type 2 5-HT receptors that couple to Gq protein to activate phospholipase C (PLC) and its down-stream targets such as protein kinase C (PKC; Conn and Sanders-Bush, 1984; Roth et al., 1986; Takuwa et al., 1989). 5-HT2A receptors have also been reported to activate Akt and Erk (Watts, 1998; Johnson-Farley et al., 2005; Zhong et al., 2008), although the receptor-coupling mechanism of this regulation is not completely understood. 5-HT2A receptors also couple to the intracellular scaffolding protein β-arrestin2, which may have unique functions to initiate desensitization of 5-HT2A receptors and to direct ligand-selective signal transduction processes (Schmid et al., 2008; Schmid and Bohn, 2010). 5-HT2A receptors are expressed throughout the brain, most prominently in the cerebral cortex, striatum, and hippocampus (Bockaert et al., 2006), where they are located on soma, dendrites, and axons of pyramidal neurons, interneurons, and monoaminergic neurons (Jakab and Goldman-Rakic, 1998; Cornea-Hebert et al., 1999; Hoyer et al., 2002; Miner et al., 2003). Activation of 5-HT2A receptors modulates levels of other neurotransmitters, such as causing inhibitory control over dopamine release (Schmidt and Fadayel, 1995). In many studies, 5-HT2A receptors are found to counteract the physiological and behavioral effects of 5-HT1A receptors (Marek et al., 2003). For example, Yuen et al. (2008) reported that activation of 5-HT2A/C receptors in prefrontal cortical neurons significantly attenuates the effect of 5-HT1A receptors on NMDA currents and microtubule depolymerization. 5-HT2A receptors have a different behavioral profile as 5-HT1A receptors. Most notably, a group of 5-HT2A receptor agonists are hallucinogens (Nichols, 2004), but 5-HT2A receptors also have significant effects in regulating other neuropsychiatric behaviors, such as mood, cognition, and sleep (Landolt and Wehrle, 2009).

THERAPEUTIC IMPLICATIONS OF 5-HT MODULATORS

For decades, modulation of 5-HT neurotransmission has been the primary pharmacological target for the treatment of major neuropsychiatric diseases, particularly depression and anxiety. The monoamine oxidase inhibitors (MAOI) enhance 5-HT neurotransmission by blocking 5-HT metabolism (Youdim and Bakhle, 2006), while tricyclic antidepressants (TCA; Klerman and Cole, 1965), serotonin-selective reuptake inhibitors (SSRI), and serotonin–norepinephrine reuptake inhibitors (SNRI) all enhance synaptic 5-HT action by blocking reuptake of 5-HT. Global enhancement of serotonin neurotransmission may activate all subtypes of serotonin receptors in brain, while each 5-HT receptor subtype has different and specific functions in defined brain regions. Activation of 5-HT1A receptors is thought to contribute to the effect of antidepressants in regulating mood and anxiety (Parks et al., 1998; Ramboz et al., 1998; Leonardo and Hen, 2008; Akimova et al., 2009; Savitz et al., 2009; Polter and Li, 2010; Price and Drevets, 2010), whereas acute activation-induced down-regulation of 5-HT1A autoreceptors may be necessary for the antidepressant and anxiolytic effects during long-term 5-HT reuptake inhibitor treatment (Blier and Ward, 2003; Albert and Lemonde, 2004). The specific roles of other 5-HT receptor subtype in the antidepressant action remain to be largely unknown.

In addition to enhancing 5-HT neurotransmission for treatment of anxiety and depression, agents with 5-HT2A receptor antagonistic property appear to have a unique role in treating neuropsychiatric diseases. Several antidepressants, including mirtazapine, nefazodone, and trazodone have 5-HT2A receptor antagonistic properties that may contribute to their unique antidepressant effect. Atypical antipsychotics not only are antagonists of dopamine D2 receptors (as with the conventional antipsychotics), but they also block 5-HT2A and 5-HT2C receptors (Meltzer et al., 1989; Markowitz et al., 1999). In addition to treating psychosis, most atypical antipsychotics have indications for bipolar mania, and some have shown efficacy in ameliorating symptoms of depression (Tohen et al., 2003; Calabrese et al., 2005; Berman et al., 2007; Marcus et al., 2008; Bauer et al., 2009). Although the exact mechanism remains to be unknown, facilitating dopamine and norepinephrine neurotransmission (Schmidt and Fadayel, 1995; Zhang et al., 2000) and/or enhancing 5-HT1A receptor action (Marek et al., 2003) may contribute to this therapeutically relevant action of 5-HT2A receptor antagonists.

The brain's serotonergic system is highly complex, in part due to its diffusely distributed multiple receptor subtypes and the signal transduction pathways regulated by these receptors. Therefore, identifying intracellular signaling molecules that direct 5-HT-regulated signals to specific physiological and behavioral effects may facilitate our understanding of brain 5-HT function and its

role in neuropsychiatric diseases. Below we review current findings on the interrelationship between 5-HT and glycogen synthase kinase-3 (GSK3), with an emphasis on the type 1 and type 2 5-HT receptors.

EFFECTS OF 5-HT ON REGULATING GSK3

5-HT REGULATES GSK3 BY PHOSPHORYLATION

5-hydroxytryptamine was first found to regulate GSK3 in mouse brain. When wild type mice received an acute administration of d-fenfluramine, a drug that enhances 5-HT release and blocks 5-HT reuptake, phosphorylation of GSK3β at serine-9 residue was significantly increased in the cerebral cortex, hippocampus, and striatum (Li et al., 2004). Phosphorylation of this residue transforms the N-terminal of GSK3 into a pseudosubstrate which blocks other GSK3 substrates from entering the active site of the enzyme, thus, d-fenfluramine treatment results in inhibition of GSK3β in the brain. However, even in the presence of a MAOI that blocks the metabolism of 5-HT, the effect of a single intraperitoneal injection of d-fenfluramine is transient, peaking at 1 h, and gradually returning to baseline level by 4 h. This could be due to rapid metabolism of the drug in mice, but rapid release of 5-HT may cause negative feedback inhibition of 5-HT release as well as 5-HT receptor desensitization; both may also contribute to the transient effect of d-fenfluramine on GSK3 phosphorylation.

The inhibitory control of GSK3 by 5-HT is further demonstrated by an elegant study (Beaulieu et al., 2008b) in a mouse model where mice carry a mutation in the tryptophan hydroxylase-2 (TPH-2) gene that is equivalent to a rare human variant (R441H) identified in a few individuals with major depressive disorder (Zhang et al., 2005). The homozygous mutant mice, when compared to littermate wild type mice, have significantly lower levels of the 5-HT precursor 5-hydroxytryptophan (5-HTP), 5-HT, and its metabolite 5-Hydroxyindoleacetic acid (5-HIAA) in the frontal cortex, hippocampus, and striatum. Meanwhile, the level of phosphorylated serine-9 of GSK3β is significantly lower, and GSK3 activity is elevated in the same brain regions of the mutant mice (Beaulieu et al., 2008b). Therefore, a sustained deficiency of 5-HT may reset the activity level of GSK3 in brain to higher level, compared to wild type mice where the constitutively active GSK3 is likely under inhibitory regulation (Doble and Woodgett, 2003). The overactive GSK3 in the R441H mutant mice may be involved in the behavioral abnormalities of these mice, since the depressive- and anxiogenic-like behavioral phenotypes of these mice were largely reversed by a GSK3 inhibitor, and by genetically reducing the level of GSK3β.

BEHAVIORAL SIGNIFICANCE OF GSK3 REGULATION BY 5-HT

Regulation of GSK3 by 5-HT may have functional significance in maintaining brain physiology and behaviors that are regulated by serotonergic neurotransmission. The 5-HT reuptake inhibitor antidepressant fluoxetine has been shown to increase phospho-Ser9-GSK3β in the cerebral cortex, hippocampus, striatum, and cerebellum of mouse brain (Li et al., 2004, 2007; Beaulieu et al., 2008b) and phospho-Ser21-GSK3α in the hippocampus (Polter et al., 2011). Several studies also suggest that inhibition of GSK3β is an important intermediate step in the antidepressant effect of fluoxetine. In the TPH-2 mutant mice, abnormal behavior associated with 5-HT deficiency is mediated by GSK3β (Beaulieu et al., 2008b). Additionally, inhibition of GSK3 by small molecule inhibitors or in GSK3β-deficient mice have been shown to reduce immobility in the forced swim test (Gould et al., 2004; Kaidanovich-Beilin et al., 2004; O'Brien et al., 2004; Beaulieu et al., 2008a; Rosa et al., 2008), similarly to the antidepressants fluoxetine (Page et al., 1999; Bianchi et al., 2002). We have recently tested this behavioral effect of fluoxetine in mutant GSK3 knock-in (KI) mice in which serine-9 of GSK3β or serine-21 of GSK3α was substituted with alanine (S9A-GSK3β-KI and S21A-GSK3α-KI; McManus et al., 2005; Polter et al., 2010). These mice have normal levels of GSK3β and GSK3α, but the N-terminal serine cannot be regulated by phosphorylation. In the forced swim test (Porsolt et al., 1977) the baseline immobility (saline treatment) was not significantly different between S9A-GSK3β-KI, S21A-GSK3α-KI, and littermate wild type mice. As expected, wild type mice responded to fluoxetine (20 mg/kg, i.p., 30 min) with a significant 56% reduction in immobility when compared to saline-treated mice. However, the anti-immobility effect of fluoxetine in S9A-GSK3β-KI mice was markedly diminished, with only 17% non-significant reduction of immobility (Polter et al., 2011). Although fluoxetine was able to increase phospho-Ser21-GSK3α in the hippocampus, S21A-GSK3α-KI mice partially responded to fluoxetine in the forced swim test. Therefore, phosphorylation of GSK3β predominantly mediates the acute antidepressant-like effect of fluoxetine, which reinforces the importance of phosphorylation of GSK3β in the therapeutic action of fluoxetine (**Figure 1**).

Since the antidepressant effect of fluoxetine in human only appears after chronic administration, to associate GSK3 regulation by fluoxetine to a therapeutic effect, it is important to determine if chronic fluoxetine treatment also regulates GSK3β. This has recently be reported by Okamoto et al. (2010) who found that chronic fluoxetine administration for 3 weeks significantly increased phospho-Ser9-GSK3β in mouse hippocampus. Although this is the only selective 5-HT reuptake inhibitor thus far shown to increase phospho-Ser9-GSK3β upon chronic treatment, a dual-acting antidepressant venlafaxine (blocks both 5-HT and norepinephrine transporters) has also been shown to increase phospho-Ser9-GSK3β after chronic administration (Okamoto et al., 2010). When investigating the effect of chronic treatment with imipramine, a TCA that inhibits reuptake of both 5-HT and norepinephrine on activation of Akt and down-stream transcription factor FoxO3a (Polter et al., 2009), we also noticed a significant increase in the level of phospho-Ser9-GSK3β in the cerebral cortex, hippocampus, and striatum of mouse brain after 4 weeks of intraperitoneal administration (**Figure 2**). It is not yet verified if the effect of venlafaxine and imipramine on GSK3β is a combined action via both 5-HT and norepinephrine, as regulation of brain GSK3β by selective adrenergic-enhancing drugs has not been studied.

REGULATION OF GSK3 BY 5-HT1A RECEPTORS

ACTIVATION OF 5-HT1A RECEPTORS REGULATES GSK3 BY PHOSPHORYLATION

With many physiological actions of brain 5-HT that are divergently mediated by 5-HT receptor subtypes, it is critically important to

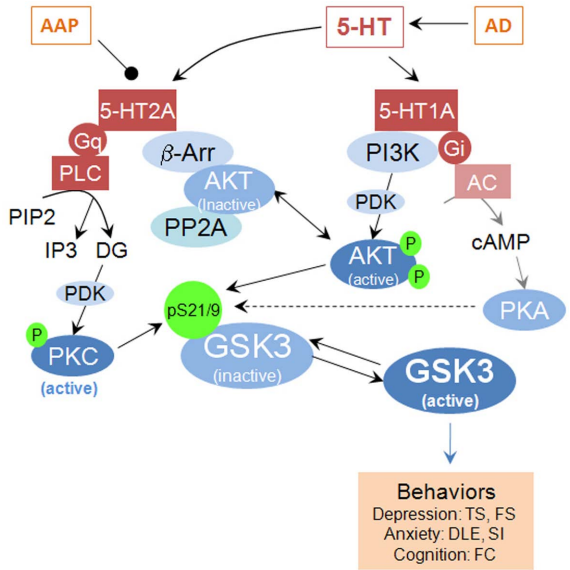

FIGURE 1 | Schematic illustration of GSK3 as an intermediate modulator of 5-HT receptor-mediated signaling pathways and physiological functions. AAP, atypical antipsychotic; α, β, γ, G-protein subunits; AC, adenylyl cyclase; AD, antidepressant; β-arr, β-arrestin; DG, diacylglycerol; Gi/Gq, G-protein; GSK3, glycogen synthase kinase-3; 5HT, serotonin; 5-HT1A and 5-HT2A, serotonin receptor subtypes; P, phosphorylated; PDK1, phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol-3-kinase; PP1/PP2A: protein phosphatase 2A; PKA, protein kinase A; PKC, protein kinase C. TS, tail suspension; FS, forced swim; DLE, dark-light emergence; SI, social interaction; FC, fear conditioning.

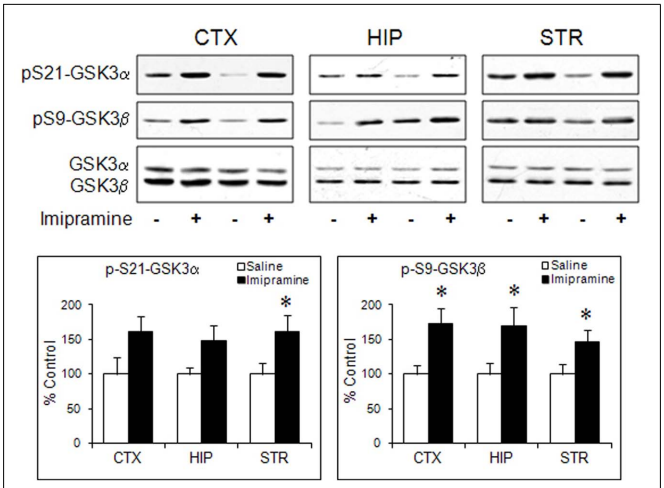

FIGURE 2 | Regulation of phospho-Ser-GSK3 by chronic imipramine treatment in mouse brain. C57BL/6 mice were treated with imipramine (20 mg/kg/d, i.p.) for 28 days before brain dissection. Homogenates of the cerebral cortex (CTX), hippocampus (HIP), and striatum (STR) were immunoblotted for phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, total GSK3α and total GSK3β, and data are quantified by densitometry. Protein levels in imipramine-treated samples are calculated as percentage control (saline treatment). Data are mean ± SEM. $*p < 0.05$ ($n = 7$–9 per group) when values are compared to saline treatment in Student's *t*-test.

determine the receptor subtypes that transduce 5-HT signal to inhibition of GSK3. In mice treated with d-fenfluramine, the 5-HT1A receptor antagonist WAY100635 was able to block over 60% of d-fenfluramine-induced increase in phospho-Ser9-GSK3β, suggesting that 5-HT1A receptors have a major role in mediating the GSK3-regulating effect of 5-HT. Indeed, when mice receive a single systemic administration of 5-HT1A receptor agonist 8-hydroxy-*N*,*N*-dipropyl-2-aminotetralin (8-OH-DPAT), the level of phospho-Ser9-GSK3β, but not total GSK3β, is significantly increased in the cerebral cortex, hippocampus, and striatum (Li et al., 2004). 8-OH-DPAT is also able to increase the level of phospho-Ser21-GSK3α in the hippocampus, but the effect is less robust (Polter et al., 2011).

In the hippocampus, a brain area that is enriched in 5-HT1A receptors (Hoyer et al., 2002), GSK3β is ubiquitously expressed with high level of immunoreactivity in neuronal cell bodies and dendritic processes (Peineau et al., 2007; Perez-Costas et al., 2010). 8-OH-DPAT-induced increase in phospho-Ser9-GSK3β is particularly prominent in the dendrites and cell bodies of CA3, and is observable in the cell bodies and projections of dentate granule cells and the dendrites of CA1 (Polter et al., 2011). The quantified ratio of phospho-Ser9-GSK3β to nuclear marker in the subfields of the hippocampus reveals significant increases in the stratum pyramidale and the stratum radiatum/stratum lucidum of CA3 and in the hilus of the dentate gyrus. Therefore, the response of GSK3β to systemic administration of 8-OH-DPAT in the hippocampus is preferentially in the pyramidal glutamatergic neurons and their dendrites, suggesting that regulation of GSK3β by 5-HT1A receptors may have functional impact on the glutamatergic circuits of the hippocampus. GSK3β has been shown to be a mediator of glutamate receptor activity and synaptic plasticity in the hippocampus (Hooper et al., 2007; Peineau et al., 2007; Du et al., 2010), thus regulation of GSK3β by 5-HT1A receptors in the hippocampus may further link this 5-HT signaling mechanism to hippocampal regulation of learning and memory.

THE ROLE OF GSK3 IN 5-HT1A RECEPTOR-REGULATED BEHAVIORS AND OTHER PHYSIOLOGICAL FUNCTIONS

Among 5-HT1A receptor-regulated behaviors, inhibition of contextual fear conditioning, a form of associative learning (Kim and Jung, 2006), is a hippocampus-dependent function (Stiedl et al., 2000; Tsetsenis et al., 2007; Ogren et al., 2008). To determine the role of GSK3β in 5-HT1A receptor-regulated fear conditioning, we tested the expression of contextual and cued fear responses in S9A-GSK3β-KI, S21A-GSK3α-KI, and littermate wild type mice. In wild type mice, 8-OH-DPAT (1 mg/kg, i.p.) injected 30 min prior to contextual test completely suppressed a standard fear conditioning training-induced contextual freezing (Polter et al., 2011). Compared to wild type mice, S9A-GSK3β-KI mice also exhibited increased freezing in contextual tests, but 8-OH-DPAT had no significant effect in reducing the context freezing. In contrast to S9A-GSK3β-KI mice, S21A-GSK3α-KI mice responded to 8-OH-DPAT in contextual freezing similarly as wild type mice. Thus, inhibition of GSK3β, but not GSK3α, via 5-HT1A receptors is a necessary intermediate process for 5-HT1A receptor-regulated inhibition of contextual fear learning. This function of GSK3β is

selective to the contextual content since the response of cued fear learning to 8-OH-DPAT was similar in S9A-GSK3β-KI mice and wild type mice.

The functional significance of GSK3β in 5-HT1A receptor-mediated physiological functions is not limited to contextual fear learning. In cultured rat hippocampal neurons, 5-HT1A receptor activation by 8-OH-DPAT increases phospho-Ser9-GSK3β and stimulates mitochondrial movements in the axons, an effect mimicked by a GSK3 inhibitor (Chen et al., 2007). This finding suggests the importance of GSK3β in 5-HT1A receptor-mediated regulation of energy distribution in neurons. In *Drosophila*, overexpressing the mammalian 5-HT1A receptor ortholog d5-HT1B receptor increases the level of phosphorylated serine of SHAGGY (SGG), the *Drosophila* GSK3β (Yuan et al., 2005). This inhibitory regulation of SGG by d5-HT1B receptor prevents SGG from phosphorylating timeless (TIM) protein for light-induced degradation. Therefore, d5-HT1B receptor reduces behavioral phase shifts in *Drosophila* by increasing phospho-Ser-SGG. The role of GSK3 in other 5-HT1A receptor-mediated functions remains to be elucidated, but this could be an exciting area in therapeutic drug development, as GSK3 inhibitors, when applied appropriately, may rescue abnormal physiology and behaviors due to functional deficiency of 5-HT1A receptors in brain.

SIGNALING MECHANISMS MEDIATING THE EFFECT OF 5-HT1A RECEPTORS ON GSK3

5-HT1A receptors activate Gi-coupled signal pathways. In a recent study, Talbot et al. (2010) found that mice expressing regulators of G protein signaling (RGS)-insensitive Giα2 have increased sensitivity to 8-OH-DPAT-induced activation, and exhibit elevated levels of cortical and hippocampal phospho-Ser9-GSK3β. This effect of RGS-insensitive active Giα2 was blocked by the 5-HT1A receptor antagonist WAY100635. This finding suggests that regulation of GSK3β by 5-HT1A receptors is mediated by a Gi-coupled signaling pathway (**Figure 1**). However, activation of Giα2 results in inhibition of adenylyl cyclase and inactivation of PKA. Although PKA is one of the several protein kinases that phosphorylate GSK3β on the serine-9 residue (Fang et al., 2000; Li et al., 2000), it is unlikely that this conventional Giα-coupled signaling pathway is responsible for direct phosphorylation of GSK3β. Alternatively, 5-HT1A receptor agonists have consistently shown to increase Akt phosphorylation in neuronal cells, including hippocampal derived HN2-5 cells (Adayev et al., 1999), primary hippocampal neurons (Cowen et al., 2005; Chen et al., 2007), and primary fetal rhombencephalic neurons (Druse et al., 2005). Regulation of Akt by 5-HT1A receptors is mediated by phosphoinositide 3-kinase (PI3K; Cowen et al., 2005; Hsiung et al., 2005, 2008), and is sensitive to inhibition of Giα activity by pertussis toxin (Cowen et al., 2005). Furthermore, activation of Akt by 5-HT1A receptors can be inhibited by cAMP and restored after inactivation of PKA (Hsiung et al., 2008). Therefore, 5-HT1A receptor-induced activation of Akt likely follows 5-HT1A receptor-induced activation of the Giα–adenylyl cyclase–cAMP–PKA signaling pathway. Since Akt is another major protein kinase that regulates phospho-Ser9-GSK3β (Cross et al., 1995), Akt may mediate 5-HT1A receptor-induced GSK3β phosphorylation. Indeed, systemic treatment of mice with the 5-HT1A receptor agonist 8-OH-DPAT significantly increased the active phospho-Thr308-Akt in the hippocampus, and intrahippocampal infusion of the PI3K inhibitor LY294002 blocked both phospho-Thr308-Akt and phospho-Ser9-GSK3β in response to 8-OH-DPAT (Polter et al., 2011).

SELECTIVITY OF GSK3 REGULATION BY 5-HT1A RECEPTORS

As discussed above, activation of 5-HT1A receptors increases both phospho-Ser9-GSK3β and phospho-Ser21-GSK3α in the hippocampus, however, the magnitude of response in GSK3α is smaller than GSK3β (Polter et al., 2011). Additionally, regulation of GSK3α phosphorylation by 5-HT1A receptors has less impact in fear conditioning (Polter et al., 2011). These pilot studies suggest different roles of GSK3 isoforms in mediating the physiological and behavioral functions of 5-HT1A receptors. Additional studies are needed to differentiate the response of GSK3α and GSK3β to 5-HT1A receptor agonists in different brain regions, and to compare the impact of each GSK3 isoform in other 5-HT1A receptor-regulated behaviors, which may provide valuable information on the physiological and behavioral impacts of the two GSK3 isoforms in 5-HT neurotransmission.

A caveat of studying 5-HT1A receptor-regulated signaling in brain is that the differential functions of 5-HT1A autoreceptors and heteroreceptors in different brain regions have divergent functions. Thus, systemic treatment of animals with 5-HT1A receptor agonists can activate 5-HT1A autoreceptors to reduce firing of raphe 5-HT neurons projected to other brain regions, but simultaneously activate 5-HT1A heteroreceptors in those brain regions, such as the hippocampus. Therefore, the effect seen after global activation of 5-HT1A receptors may involve indirect response of GSK3 to activation or inhibition of other neurotransmitters. Therefore, additional studies of GSK3 regulation by systemically and regionally applied 5-HT1A receptor agonists in specific neuron populations in combination with studies in isolated primary neuron cultures will further elucidate the sophisticated mechanisms underlying the GSK3-regulating effect of 5-HT1A receptors. Nevertheless, the effect of global activation of 5-HT1A receptors should be appreciated since systemic drug treatment is likely more relevant to therapeutic implications.

REGULATION OF GSK3 BY 5-HT2A RECEPTORS

THE PARADOXICAL EFFECTS OF 5-HT2A RECEPTOR AGONISTS AND ANTAGONISTS ON GSK3

Although 5-HT1A receptors have a prominent regulatory effect on GSK3, more than one 5-HT receptor subtype should be activated upon elevated brain 5-HT. Among them, 5-HT2A receptors have been found to regulate GSK3 with rather sophisticated and yet unidentified mechanisms. We previously reported that activation of 5-HT2A receptors by systemic administration of 1-(2,5-dimethoxy-4-iodophenyl)-propan-2-amine (DOI) for 1 h (a time point that maximally increases phospho-Ser9-GSK3β by d-fenfluramine, fluoxetine, and 8-OH-DPAT), had little effect on phospho-Ser9-GSK3β in the cerebral cortex, hippocampus, or striatum (Li et al., 2004). However, Abbas et al. (2009) later reported that a brief 15-min DOI treatment caused an increase in phospho-Ser9-GSK3β in the mouse brain, although this report did not specify the brain region in which this effect was seen. If the time length of treatment is the major difference of the discrepant

findings, one possibility is that 1 h treatment might have caused desensitization of 5-HT2A receptors. Therefore a thorough kinetic study of 5-HT2A receptor agonists may further clarify their effect on GSK3, especially the effects in different brain regions.

Since activation of 5-HT2A receptors by the hallucinogen DOI is somewhat different from 5-HT-induced activation of 5-HT2A receptors (Schmid et al., 2008), the exact action of 5-HT2A receptor activation on GSK3 phosphorylation should be further elucidated using endogenous 5-HT2A receptor agonists and selective 5-HT2A receptor antagonists. However, this has been difficult to study with 5-HT2A receptors, because it is already known that endogenous 5-HT increases phospho-Ser-GSK3 by activating other 5-HT receptors, particularly 5-HT1A receptors (Li et al., 2004); whereas selective blocking 5-HT2A receptors with their antagonists, one of the commonly used manipulations to determine a receptor-selective effect, paradoxically elicit robust increase in the level of phospho-Ser-GSK3 in mouse brain. This phenomenon was first observed with the non-selective 5-HT2 receptor antagonist LY53857 (Li et al., 2004). Systemic LY53857 treatment not only caused a prolonged elevation of phospho-Ser9-GSK3β, but it also potentiated the GSK3β-regulating effect of d-fenfluramine and 8-OH-DPAT. This effect has also been observed using the 5-HT2A receptor-selective antagonist MDL11939, which elevates both phospho-Ser21-GSK3α and phospho-Ser9-GSK3β in mouse brain, with the effect more prominent in the cerebral cortex and striatum and less in the hippocampus (**Figure 3A**).

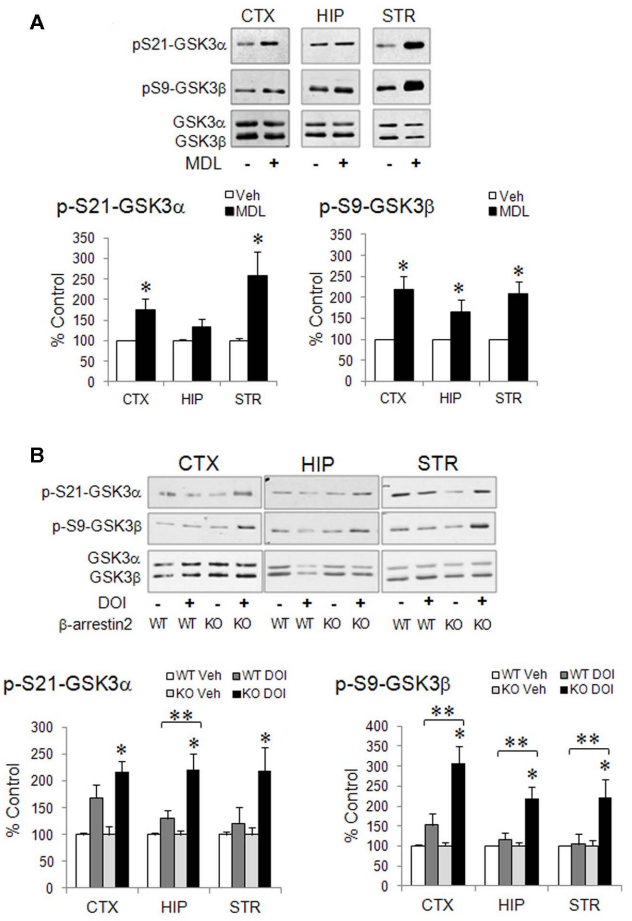

FIGURE 3 | Regulation of phospho-Ser-GSK3 by 5-HT2A receptor ligands in mouse brain. (A) Wild type mice were treated with saline (Veh) or the 5-HT2A receptor antagonist MDL11939 (MDL, 4 mg/kg, i.p., 1 h). Immunoblots of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, total GSK3α, and total GSK3β in the cerebral cortex, hippocampus, and striatum are quantified by densitometry. Mean ± SEM, $*p < 0.05$ when values are compared to saline treatment in Student's *t*-test, $n = 4$–13. **(B)** Wild type (WT) and β-arrestin2-knockout (KO) mice were treated with saline (Veh) or DOI (20 mg/kg, i.p.) for 1 h. Immunoblots of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, total GSK3α, and total GSK3β in the cerebral cortex, hippocampus, and striatum are quantified by densitometry. Mean ± SEM, $*p < 0.05$ between saline and DOI treatment within the same genotype, Student's *t*-test; $**p < 0.05$, two-way ANOVA followed by Holm–Sidak comparison, $n = 10$–12.

THE EFFECT OF ATYPICAL ANTIPSYCHOTICS ON GSK3

Despite the paradoxical nature of 5-HT2A receptors in regulating GSK3, the effect of 5-HT2A receptor antagonists may have clinical significance. Several independent studies have found that antipsychotic drugs, including both conventional and atypical antipsychotics, regulate GSK3 in animal brain. The conventional antipsychotic haloperidol was reported to either alter the phosphorylation state or increase the protein level of GSK3 (Alimohamad et al., 2005; Kozlovsky et al., 2006; Roh et al., 2007). A group of atypical antipsychotics, including risperidone, olanzapine, clozapine, quetiapine, and ziprasidone, were consistently found to increase phospho-Ser-GSK3 in mouse brain (Alimohamad et al., 2005; Li et al., 2007; Roh et al., 2007). Although the dopamine D2 receptor-blocking property of these agents may be involved in their GSK3-regulating effect (Beaulieu et al., 2009), one of the major pharmacological difference between conventional antipsychotics and atypical antipsychotics is that the latter group has a dual antagonistic action on both 5-HT2 receptors and dopamine D2 receptors (Schotte et al., 1995). In our study, a low dose haloperidol that binds to D2 receptors but not 5-HT2A receptors had no effect on mouse brain GSK3 (Li et al., 2007). In contrast, systemic administered risperidone increases brain phospho-Ser9-GSK3β at a dose as low as 0.1 mg/kg (Li et al., 2007). As the average dose of risperidone used to attain a 50% *in vivo* blockade of D2 receptors in rats is about 0.3 mg/kg (Kapur et al., 2003), and the binding affinity of risperidone to 5-HT2A receptors is at least 3 times higher than to D2 receptors (Weiner et al., 2001), it is likely that the increase of phospho-Ser9-GSK3β by the low dose risperidone involves blocking 5-HT2A receptors. It remains to be determined if putative 5-HT2A receptor antagonists have similar clinical implications as atypical antipsychotics, and if such effects are dependent on regulation of GSK3.

Clinically, atypical antipsychotics have indications in both psychotic disorders and mood disorders (Derry and Moore, 2007; Philip et al., 2008). Some atypical antipsychotics, either as monotherapy or augmentation, have shown efficacy in ameliorating symptoms of depression (Tohen et al., 2003; Calabrese et al., 2005; Berman et al., 2007; Marcus et al., 2008; Bauer et al., 2009). Interestingly, a combination treatment with risperidone and fluoxetine enhance the effect of either agent alone on phosphorylation of GSK3β in the rodent brain (Li et al., 2007). This effect agrees

with the finding that the 5-HT2 receptor antagonist LY53857 potentiated the effect of d-fenfluramine and 8-OH-DPAT on GSK3 phosphorylation. However, the role of GSK3 in this clinically relevant combination effect between antidepressants and atypical antipsychotics remains to be determined.

SIGNALING MECHANISMS INVOLVED IN REGULATION OF GSK3 BY 5-HT2A RECEPTORS

Few studies have investigated the signaling mechanisms that mediate the effects of either 5-HT2A receptor agonists or antagonists on GSK3 phosphorylation. Classically, the 5-HT2A receptors couple to Gq protein that activates PLC and its down-stream PKC (Carr et al., 2002; **Figure 1**). PKC was reported to mediate DOI-induced Erk phosphorylation in the brain (Schmid et al., 2008). Since PKC is one of the protein kinases that regulate GSK3 phosphorylation (Goode et al., 1992), it would be straightforward if the PLC/PKC signaling mechanism is found to mediate 5-HT2A receptor-regulated GSK3. However, this mechanism has not been reported, especially since the kinetic correlation between 5-HT2A receptor activation and phosphorylation of GSK3 has not been characterized. In a study of dopamine D2 receptor-regulated signaling (Beaulieu et al., 2004), the intracellular scaffolding protein β-arrestins were found to negatively affect GSK3 phosphorylation in mouse brain, wherein activation of dopamine D2 receptors reduces phospho-Ser9-GSK3 due to inactivation of Akt by protein phosphatase-2 (PP2A) in a β-arrestin2-driven protein complex (Beaulieu et al., 2005). D2 receptor antagonists block this effect of dopamine and increase phospho-Ser-GSK3 by disrupting the effect of β-arrestins. 5-HT2A receptors also interact with β-arrestins in a cell type- and ligand-selective manner (Gelber et al., 1999; Allen et al., 2008; Schmid et al., 2008; Schmid and Bohn, 2010), but the role of β-arrestins in 5-HT2A receptor-regulated signal pathways is less understood. It should be noted that in a previous study by Schmid et al. (2008), DOI-induced head twitch, receptor internalization, and Erk phosphorylation are not dependent on the presence or absence of β-arrestins. However, we have recently found that DOI treatment for 1 h induced a significant over twofold increase in phospho-Ser21-GSK3α and phospho-Ser9-GSK3β in the cerebral cortex, hippocampus, and striatum in β-arrestin2-knockout mice, an effect that is not seen in wild type mice (**Figure 3B**). This observation suggests that β-arrestin2 may influence how 5-HT2A receptors regulate GSK3. Future studies investigating signal transduction mechanisms mediating the effect of 5-HT2A receptors on GSK3 should include not only PLC/PKC signaling, but also β-arrestin-mediated signaling. Additionally, since 5-HT2A receptors are involved in modulating dopamine neurotransmission (Alex and Pehek, 2007; Di Giovanni et al., 2008; Esposito et al., 2008), a potential indirect effect of 5-HT2A receptors on D2 receptor-regulated GSK3 should also be considered.

GSK3 SELECTIVELY REGULATES 5-HT1B RECEPTOR-MEDIATED SIGNAL TRANSDUCTION AND FUNCTION

Glycogen synthase kinase-3 phosphorylates many protein substrates that distribute in both neural and peripheral tissues (Woodgett, 2001; Jope and Johnson, 2004). As a result, *in vivo* inhibition of GSK3 can have a variety of physiological effects. Thus, identifying GSK3 substrates that have specific functions in brain circuits could be critical in developing GSK3-targeting treatment for therapeutics. With each clearly identified substrate, substrate-targeting inhibition of brain GSK3 activity may lead to selective modulation of physiological function of the brain. Most 5-HT receptor subtypes, except 5-HT3 receptors, are G-protein coupled receptors (GPCRs) that represent the largest group of drug targets. Besides activation by receptor ligands, the activity of GPCRs can be modulated by posttranslational modifications, such as phosphorylation by protein kinases (Tobin, 2008), or interaction with intracellular proteins (Ritter and Hall, 2009; Bockaert et al., 2010). However, little evidence has shown GPCR regulation by GSK3.

DIRECT INTERACTION BETWEEN GSK3β AND 5-HT1B RECEPTORS

In an effort to identify GSK3 substrates within the 5-HT neurotransmission system, we searched GSK3 consensus phosphorylation sequence for primed substrates – pre-phosphorylated by a serine/threonine kinase to allow access of GSK3 to the serine/threonine located four amino acids N-terminal of the primed site (Doble and Woodgett, 2003). Somewhat surprisingly, both human and mouse 5-HT1B receptors were found to contain eight GSK3 consensus phosphorylation sites spreading within intracellular loops 1, 2, and 3 of the receptor. In contrast, the highly homologous 5-HT1A receptors have no GSK3 consensus sites in intracellular loops 1 and 2, and the two sites in intracellular loop-3 of human 5-HT1A receptors are not homologous with mouse 5-HT1A receptors. In cultured heterologous cells expressing 5-HT1B or 5-HT1A receptors, GSK3β was found to directly associate with 5-HT1B receptors (Chen et al., 2009), which was detected using the bioluminescence resonance energy transfer (BRET) assay that measures the proximity of two proteins within a distance of 1–10 nm (Angers et al., 2000), and confirmed by co-immunoprecipitation of 5-HT1B receptors and GSK3β. Intriguingly, this interaction of GSK3β is selective to 5-HT1B receptors, as GSK3β does not interact with 5-HT1A receptors (Chen et al., 2009). Mutation of every potential GSK3 consensus phosphorylation site of 5-HT1B receptors by replacing the serine with alanine further shows that GSK3β associates with 5-HT1B receptors at the [pS(154)AKRpT(158)] sequence located in the i2-loop of 5-HT1B receptors (Chen et al., 2009).

GSK3β DIFFERENTIALLY INFLUENCES 5-HT1B RECEPTOR ACTIVITY AND ASSOCIATED SIGNALINGS

Both 5-HT1B and 5-HT1A receptors couple to Gi-protein, activation of which causes inhibition of adenylyl cyclase and reduction of cAMP. Disrupting the interaction between 5-HT1B receptors and GSK3β by mutating the GSK3β-interactive Ser-154 residue of the receptor (S154A–5-HT1B receptor) effectively abolishes 5-HT-induced conformational change between 5-HT1B receptors and Giα2 (Chen et al., 2011). In accordance with altered activity of Giα2, GSK3β-insensitive S154A–5-HT1B receptor does not respond to 5-HT in assay of 5-HT-induced inhibition of cAMP (Chen et al., 2009). These data highly suggest that GSK3β has a functional impact on 5-HT1B receptor-mediated signaling. Indeed, several small molecule GSK3 inhibitors and the clinically used lithium are able to disrupt 5-HT-induced conformational

change between 5-HT1B receptors and Giα2 and inhibition of cAMP. This is a highly selective function of the GSK3β isoform, as it only occurs in cells with GSK3β-knockdown or over-expressing inactive GSK3β, but GSK3α-knockdown does not affect 5-HT1B receptor-associated Giα–cAMP signaling (Chen et al., 2009, 2011). This is another example showing that despite GSK3α and GSK3β are highly homologous and are regulated via similar upstream mechanisms, the two isoforms of GSK3 have different substrates and mediate different physiological functions (Wang et al., 1994; Liang and Chuang, 2006). In agreement with a selective interaction between GSK3β and 5-HT1B receptors, GSK3 inhibitors and molecular manipulation of intracellular GSK3β do not alter 5-HT-induced 5-HT1A receptor-Giα2 interaction or 5-HT1A receptor-mediated inhibition of cAMP.

Giα-mediated cAMP production is not the only GSK3-dependent signaling pathway of 5-HT1B receptors as activation of Akt by 5-HT or the selective 5-HT1B receptor agonist anpirtoline is also significantly diminished in the mutant S154A–5-HT1B receptor-expressing cells (Chen et al., 2011). The mechanisms of regulating Akt by 5-HT1B receptors are not fully understood, but since both α- and βγ-subunits of G-proteins may be linked to GPCR-induced activation of Akt (DeWire et al., 2007; New et al., 2007; Yang et al., 2009), the effect of GSK3β on 5-HT1B receptor-induced Akt activation could be a consequence of the prominent effect of GSK3β on 5-HT1B receptor-associated Gi-protein. In addition, since Akt is one of the upstream GSK3-regulating protein kinases that inactivates GSK3 (Cross et al., 1995), the GSK3-dependent activation of Akt by 5-HT1B receptors could function as a feedback regulation to prevent prolonged effect of GSK3β on 5-HT1B receptors, but this postulation remains to be examined.

As a GPCR, 5-HT1B receptors are expected to interact with β-arrestins that have been recognized to interact with many GPCRs and play important roles in GPCR internalization and alternative signaling (Lefkowitz and Shenoy, 2005). Indeed, we found that β-arrestin2 can be recruited to 5-HT1B receptors in response to 5-HT. However, in contrast to the prominent effect of GSK3β on 5-HT1B receptor-coupling to Giα2, removing, or inhibiting GSK3β did not affect β-arrestin2 recruitment to 5-HT1B receptors (Chen et al., 2011). As a chaperone protein, recruitment of β-arrestins to GPCRs initiates receptor internalization and desensitization (Bohn et al., 1999, 2000). 5-HT was able to internalize both wild type and S154A–5-HT1B receptors in 5-HT1B receptor-expressing cells (Chen et al., 2009). If this internalization event is directed by β-arrestin2, it is in agreement with our finding that β-arrestin2 recruitment to 5-HT1B receptors is independent of GSK3β. However, the time course of 5-HT1B receptor internalization is drastically different between wild type and the mutant S154A–5-HT1B receptors, wherein wild type receptors rapidly reappear on the cell membranes within 60 min of 5-HT treatment, while mutant GSK3β-insensitive receptors continue to be absent from the cell surface 2 h after 5-HT treatment. This suggests a potentially important function of GSK3β in an unidentified mechanism that is crucial for 5-HT1B receptor synthesis and membrane recycling.

Interestingly, the effect of GSK3β on 5-HT1B receptors appears to facilitate activity and functional recovery, which is opposite from other GPCR-regulating protein kinases (GRKs) that typically facilitate desensitization and termination of GPCR activity (Gainetdinov et al., 2004). The underlying importance of GSK3β on 5-HT1B receptor-mediated Gi signaling and β-arrestin recruitment is that GSK3β does not function as a general 5-HT1B receptor activator, instead, GSK3β selectively modulates signaling-specific actions of 5-HT1B receptors. This effect of GSK3β could be attractive when developing GSK3-targeting therapeutic agents, as inhibition of GSK3β would not make inert 5-HT1B receptors, but to selectively shift 5-HT1B receptor-mediated physiological and behavioral functions toward a Gi signaling-independent direction. This postulation is an important area of future research especially when β-arrestin-associated 5-HT1B receptor signaling pathways are identified.

EFFECT OF GSK3 INHIBITORS ON THE PHYSIOLOGICAL FUNCTIONS OF 5-HT1B RECEPTORS

Noticeably, several earlier studies showed that lithium selectively inhibits 5-HT binding to 5-HT1B receptors, reduces 5-HT1B receptor-induced GTPγs binding, and abolishes 5-HT1B receptor-reduced adenylyl cyclase activity (Massot et al., 1999). Similar results have been observed in human platelets from both healthy and depressed subjects where lithium dose-dependently reverses the inhibitory effect of a 5-HT1BR agonist on adenylyl cyclase (Januel et al., 2002). In animal behavior studies, lithium selectively regulates the behavioral effect of 5-HT1B receptor agonists, but not the effect of 5-HT1A receptor agonists (Redrobe and Bourin, 1999). With lithium being a selective GSK3 inhibitor (Klein and Melton, 1996), it is highly likely that the selective effect of lithium on 5-HT1B receptor function is the result of its inhibition of GSK3. Since lithium is a therapeutic drug that has been found to inhibit GSK3 *in vivo*, perhaps by both its direct and indirect actions on GSK3 (Klein and Melton, 1996; Chalecka-Franaszek and Chuang, 1999; De Sarno et al., 2002), disrupting the interaction between 5-HT1B receptors and GSK3β may have significant impact in the physiological functions of 5-HT1B receptors and relevant therapeutic effects in neuropsychiatric diseases.

The above postulation has been tested now in mouse brain tissues using small GSK3 inhibitors. When mouse cerebral cortical slices are pre-treated with the GSK3 inhibitors 6-bromoindirubin-3′-oxime (BIO) or kenpaullone, both inhibitors concentration-dependently abolish the inhibitory effect of 5-HT1B receptor agonist anpirtoline on forskolin-stimulated cAMP, whereas neither affects the effect of 5-HT1A receptor agonist 8-OH-DPAT on cAMP (Chen et al., 2009). This finding in brain tissues is consistent with findings in cultured cells, where ablation or inhibition of GSK3β selectively abolishes 5-HT1B receptor-mediated Giα–cAMP signaling. Furthermore, pre-treatment of cortical slices with the GSK3 inhibitors AR-A014418 (Bhat et al., 2003) and BIP-135 (Gaisina et al., 2009) completely abolish the inhibitory effect of anpirtoline on potassium-evoked ^{3}H-5-HT release (Chen et al., 2011). Since negatively regulating 5-HT release is a characteristic function of 5-HT1B autoreceptors in the axon terminals of 5-HT neurons (Trillat et al., 1997; Riad et al., 2000; Sari, 2004), this finding suggests that active GSK3 plays a role to rapidly resets the surge of 5-HT to baseline, which may result in termination

of a physiological or behavioral action of 5-HT. In reverse, inhibition of GSK3 may be important in maintaining 5-HT at a sufficient level that allows 5-HT to deliver sustained physiological action. The signaling pathways mediating 5-HT1B autoreceptor-induced inhibition of 5-HT release have never been confirmed. In a study conducted in 5-HT1B receptor-expressing cardiac ventricle myocytes, inhibition of 5-HT release by 5-HT1B receptors was found to be a pertussis toxin-sensitive Gi-mediated effect, where the effect was not mediated by cAMP, but by an inwardly rectifying potassium channel (Ghavami et al., 1997). However, pertussis toxin-dependent 5-HT release by 5-HT1B receptors in the brain has not been confirmed. It should therefore be noted that although GSK3 inhibitors abolish the effect of 5-HT1B receptors on Gi-associated signalings and 5-HT release, the latter is not necessarily due to the impaired 5-HT1B receptor-Giα coupling.

EFFECT OF GSK3 INHIBITORS ON 5-HT1B RECEPTOR-REGULATED BEHAVIORS

If GSK3β differentially affects 5-HT1B receptor-mediated signal transduction and brain physiological function, it would be expected that GSK3 inhibitors may also differentially affect 5-HT1B receptor-regulated behaviors. Indeed, intracerebroventricular infusion of GSK3 inhibitor AR-A014418 or BIP-135 prior to systemic administration of anpirtoline significantly facilitates the anti-immobility effect of the 5-HT1B receptor agonist in the tail suspension test (Chen et al., 2011). In this behavioral test, low doses of GSK3 inhibitors and anpirtoline are used so that GSK3 inhibitors alone has no effect and anpirtoline alone only mildly reduces the immobility, which highlights the combination effect of GSK3 inhibition and 5-HT1B receptor activation. Finding of this study suggests that GSK3 inhibitors facilitate the anti-immobility effect of 5-HT1B heteroreceptors by abolishing 5-HT1B autoreceptor-mediated inhibition of serotonin release. Interestingly, the anpirtoline-induced increase in horizontal locomotor activity was not altered by GSK3 inhibitors, but is largely diminished in β-arrestin2-knockout mice (Chen et al., 2011), which is in agreement with the *in vitro* findings showing that association of β-arrestin2 with 5-HT1B receptors does not depend on GSK3β. Therefore, these initial behavior data further suggest that GSK3 has differential effects in modulating 5-HT1B receptor functions in brain.

Taken together, molecular and *in vivo* studies support a prominent effect of GSK3β to selectively regulate 5-HT1B receptor function (**Figure 4**). The effect of GSK3β is elicited by directly interacting with 5-HT1B receptors at the intracellular loop-2. This interaction functions to facilitate 5-HT1B receptor-regulated Giα-mediated signaling. In the absence of GSK3β, 5-HT1B receptors are dissociated from the conventional Giα-mediated signaling, but still able to respond to agonist-induced recruitment of β-arrestin2 and receptor internalization. Regulation of 5-HT1B receptors by GSK3β has functional significance since it impacts 5-HT1B receptor-dependent 5-HT release. Although this and other physiological functions of the highly selective modulation of 5-HT1B receptors by GSK3β remain to be investigated in further detail, initial evidence supports a significant behavioral impact of this GSK3 action that may be important for therapeutic development. Several important questions remain to be addressed before the importance of this unique effect of GSK3β is completely elucidated. Particularly important is to understand if the effect of GSK3β is selective to 5-HT1B autoreceptors only or both auto- and hetero-receptors, which 5-HT1B receptor-regulated signaling mechanisms in the brain are affected by GSK3β, and if the GSK3β-dependent signaling pathways correlate with the effect of GSK3β on the physiological and behavioral functions of brain 5-HT1B receptors.

SUMMARY

Cumulative evidence from *in vitro* measurements, pharmacological studies, and animal behavioral tests strongly support GSK3 as an integrative mediator of 5-HT neurotransmission. First, GSK3 is an early intracellular responder to a surge of brain 5-HT, which results in GSK3 phosphorylation at an N-terminal serine and inactivation of its constitutive activity. Phosphorylation of GSK3 by increasing brain 5-HT may be therapeutically important as the behavioral effect of fluoxetine is mediated by GSK3 phosphorylation. Second, GSK3 is a down-stream target of 5-HT1A receptor signaling, and enhancing GSK3 phosphorylation by 5-HT1A receptors in the hippocampus is associated with its inhibitory effect on contextual fear learning. Third, 5-HT2A receptors elicit sophisticated regulatory effect on GSK3, as both agonist and antagonist of 5-HT2A receptors may inactivate it, but the effects likely depend on the receptor activation state and the presence of other receptor interactive proteins. Regulation of GSK3 by 5-HT2A receptors may have therapeutic implication as evidenced by the prominent inhibitory effect of atypical antipsychotics on GSK3, presumably related to the 5-HT2A receptor antagonistic effect of these drugs. Finally, GSK3β interacts with and modulates 5-HT1B receptor activity in a receptor subtype- and signaling pathway-selective manner. Although the physiological impact of this action in intact brain remains to be elucidated, it is predicted that this action of GSK3β is to rapidly reset the level of 5-HT in certain brain areas to ameliorate prolonged effect of 5-HT, whereas the therapeutic implication of this unique action of GSK3 remains to be further studied.

The prominent effect of GSK3 on serotonin neurotransmission may partially explain the many findings of behavioral actions of GSK3 as well as it being a converging target of mood stabilizers, antidepressants, and antipsychotics (Li and Jope, 2010). Integrating 5-HT neurotransmission by GSK3 may be particularly indicative in the pathophysiological roles of GSK3 in mood, anxiety, and cognitive disorders that involve dysregulation of 5-HT neurotransmission, and in the therapeutics targeting GSK3 for the treatment of these diseases.

However, our current knowledge on this potentially important function of GSK3 is limited. GSK3 has only been found to associate with 5-HT1A, 5-HT1B, and 5-HT2A receptors. Evidence showing GSK3 as a down-stream target or a modulator of other 5-HT receptor subtypes is still lacking. As discussed in this review, the two isoforms of GSK3 appear to have common and unique relations to different 5-HT receptors, but it is not yet clear if the divergent relationship is receptor subtype- or brain region-specific. Additional investigations are also necessary to fully understand how altered activity of GSK3 affects 5-HT-regulated behaviors, and to determine the mechanisms of how each behavior is affected by

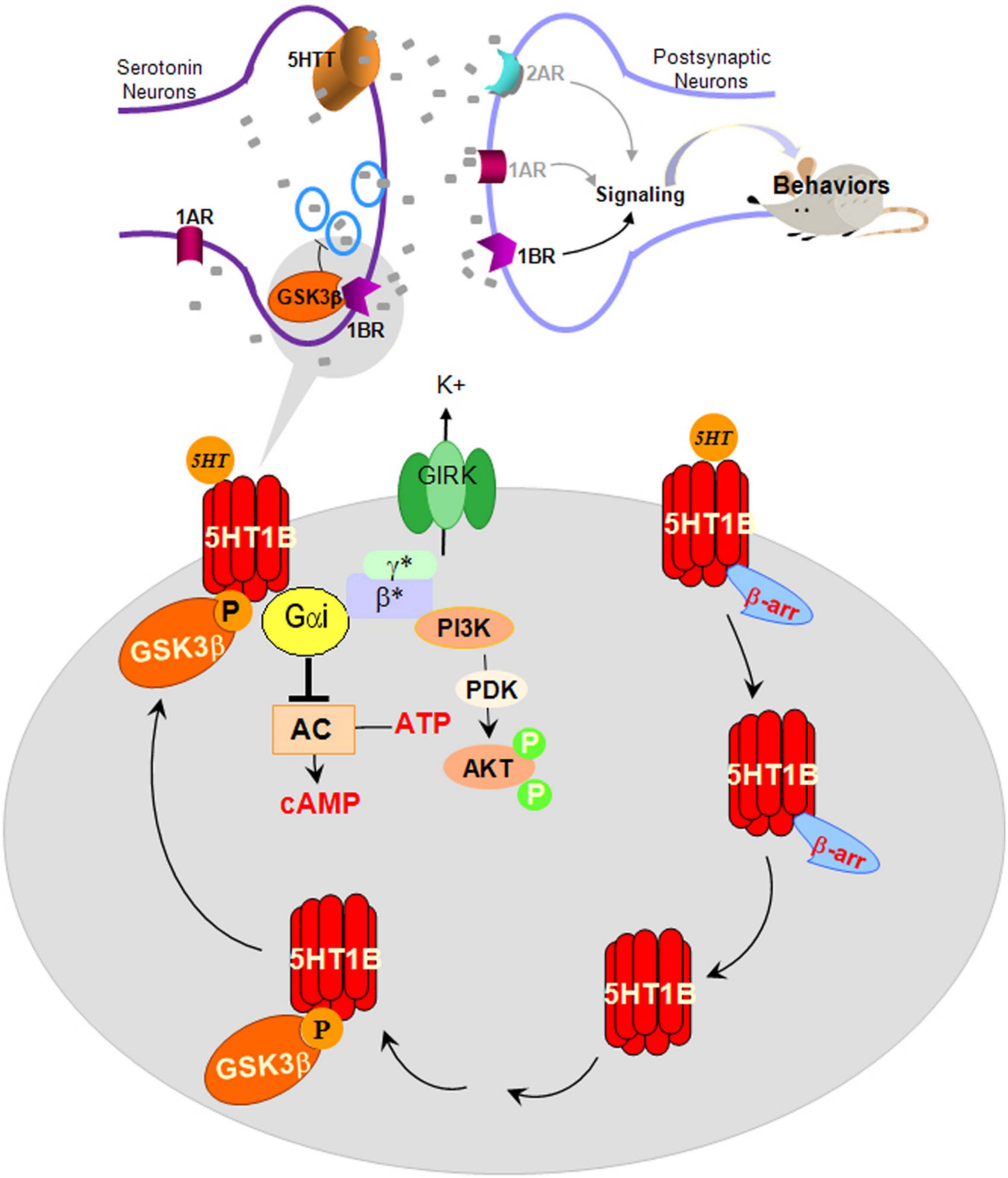

FIGURE 4 | Proposed effects of GSK3β in regulating 5-HT1B receptor-coupled signal transduction, 5-HT1B receptor-regulated 5-HT release, and 5-HT1B receptor-regulated behaviors. α, β, γ, G-protein subunits; AC, adenylyl cyclase; β-arr, β-arrestin; Gi, G-protein; GSK3, glycogen synthase kinase-3; 5HT, serotonin; 1AR, 1BR, 2AR, serotonin receptor subtypes; P, phosphorylated; PDK, phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol-3-kinase.

altered GSK3 activity. Since GSK3 is a protein kinase, an important task is to identify specific protein substrates that mediate the divergent physiological and behavioral effects of GSK3 within and beyond 5-HT neurotransmission. Human studies are needed to determine if clinical findings support the ample preclinical evidence suggesting a role of GSK3 in 5-HT dysregulation and related brain disorders. Finally, all the ongoing and future research tasks should converge to developing GSK3 modulators that are able to substitute, facilitate, or supplement therapeutic drugs that are known to modulate 5-HT neurotransmission.

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Beyond cAMP: the regulation of Akt and GSK3 by dopamine receptors

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Brain dopamine receptors have been preferred targets for numerous pharmacological compounds developed for the treatment of various neuropsychiatric disorders. Recent discovery that D2 dopamine receptors, in addition to cAMP pathways, can engage also in Akt/GSK3 signaling cascade provided a new framework to understand intracellular signaling mechanisms involved in dopamine-related behaviors and pathologies. Here we review a recent progress in understanding the role of Akt, GSK3, and related signaling molecules in dopamine receptor signaling and functions. Particularly, we focus on the molecular mechanisms involved, interacting partners, role of these signaling events in the action of antipsychotics, psychostimulants, and antidepressants as well as involvement in pathophysiology of schizophrenia, bipolar disorder, and Parkinson's disease. Further understanding of the role of Akt/GSK3 signaling in dopamine receptor functions could provide novel targets for pharmacological interventions in dopamine-related disorders.

Keywords: glycogen synthase kinase 3, Akt, protein kinase B, dopamine, catecholamines, arrestin, G protein coupled receptor, receptor tyrosine kinase

INTRODUCTION

Dopamine is a catecholamine neurotransmitter involved in the regulation of multiple functions in the CNS and periphery, including locomotion, cognition, emotional behaviors, and endocrine regulation (Carlsson, 1993; Beaulieu and Gainetdinov, 2011). Consequently, abnormal dopamine signaling could play a role in many neuropsychiatric disorders such as schizophrenia, bipolar disorder, depression, Parkinson's disease, attention deficit hyperactivity disorder (ADHD), Tourette syndrome, and drug abuse. Dopamine exerts its biological functions by activation and signaling through two different groups of G protein-coupled receptors (GPCRs). The first category, the D1 family, comprises the D1- and D5-receptors (D1R and D5R). The second family, named D2-class receptors, is formed by the D3R, D4R along with the short and long splice variants of the D2R (Missale et al., 1998; Beaulieu and Gainetdinov, 2011).

Historically, it was believed that dopamine receptors signal exclusively through G protein-dependent cellular processes. D1R is mostly coupled to Gαs/olf proteins and stimulate the activity of adenylate cyclase and the production of the second messenger cAMP. In contrast, D2R is associated to Gαi/o protein to inhibit the production of cAMP (Spano et al., 1978; Kebabian and Calne, 1979). However, more recent investigations have revealed that dopamine receptors can exert some of their biological effects through alternative signaling pathways which may or may not involve cAMP (Beaulieu et al., 2004, 2005; Hasbi et al., 2009). For instance, there are indications that both D1R and D2R can transactivate the brain derived neurotrophic factor (BDNF) receptor in neurons (Swift et al., 2011). These two dopamine receptors have also been shown to regulate the internalization of calcium channels through direct protein:protein interaction *in vivo* (Kisilevsky and Zamponi, 2008; Kisilevsky et al., 2008). Finally, there is strong evidence that dopamine receptors can signal *in vivo* by activating cAMP-independent mechanisms involving the multifunctional adaptor protein beta-arrestin 2 (βArr2; Beaulieu et al., 2004, 2005). This protein is generally recruited to activated/phosphorylated GPCRs and plays a central role in receptor desensitization through receptor-G protein uncoupling and clathrin-dependent internalization (Lohse et al., 1990; Ferguson et al., 1996). In addition to this well established role, βArr2 can act as a scaffold for kinases and phosphatases by forming a signaling complex that leads to the activation of various G protein-independent intracellular signaling cascades (**Figure 1**) including the Akt/GSK3 pathway (Luttrell et al., 1999; Beaulieu et al., 2004, 2005; Luttrell and Gesty-Palmer, 2010).

The serine/threonine kinase Akt is known to be regulated by phosphoinositide 3-kinase (PI3K) signaling via the activatory phosphorylation of Akt at its threonine (Thr 308) and its

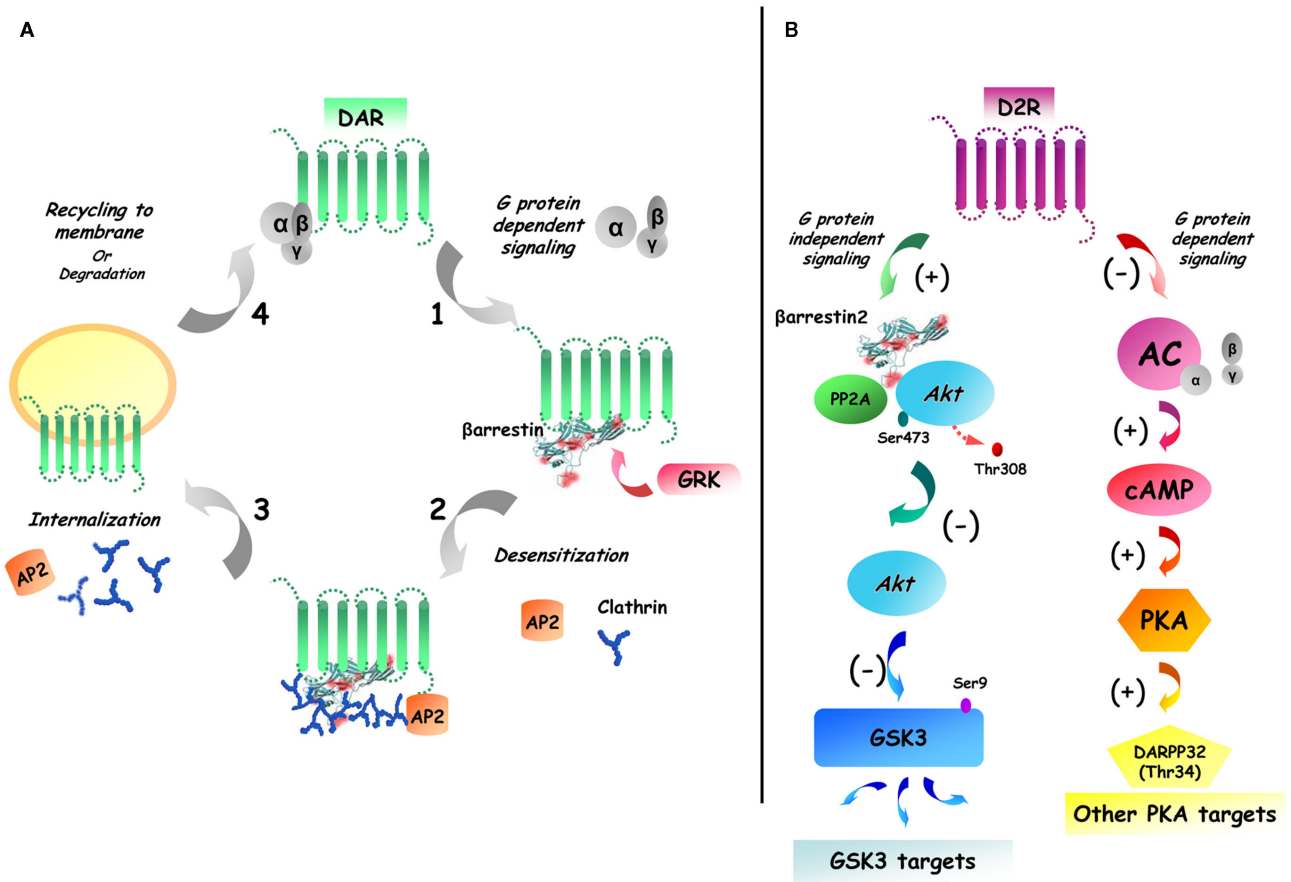

FIGURE 1 | Dual role of beta-arrestin 2 in D2R desensitization and Akt/GSK3 signaling. (A) GPCR activation/desensitization cycle. Following the activation of dopamine receptors (DAR), the receptor is phosphorylated by G protein receptor kinases (GRK), which leads to the recruitment of beta-arrestins. The recruitment of beta-arrestins to the receptors results in clatrin-mediated endocytosis that is followed either by receptor degradation of cell surface recycling. **(B)** G protein-dependent and G protein-independent beta-arrestin-mediated signaling of D2R. Receptor activation leads to both classical G protein mediated signaling and to the formation of complexes of signaling molecules that are associated together by beta-arrestins. In the specific case of striatal D2R beta-arrestin 2 has been shown to enhance the interaction between Akt and protein phosphatase 2A (PP2A) therefore resulting in Akt inactivation and increased activation of GSK3.

serine (Ser 473) residues by two phosphatidyl-dependant kinases, PDK1 and PDK2/rictor-mTOR, respectively (Scheid and Woodgett, 2001; Jacinto et al., 2006). Once activated, Akt in turn phosphorylates GSK3 isoforms at the single regulatory serine residues serine 21 (GSK3α) and serine 9 (GSK3β) that are located in the N-terminal domains of both GSK3α and GSK3β (Stambolic and Woodgett, 1994; Frame and Cohen, 2001) and thereby causing their inactivation. Over the last 7 years, several independent lines of evidence have demonstrated that dopamine receptors can exert at least some of their biological functions by regulating the activity of Akt and GSK3 isoforms.

Here we provide an overview of the molecular mechanisms by which dopamine receptors can regulate the activity of brain Akt and GSK3. In addition, we evaluate the role played by these kinases in the regulation of various behaviors by dopamine, the role of Akt/GSK3 signaling cascade in the action of psychoactive drugs and potential relevance of the abnormalities found in this pathway for the development of dopamine-related neuropsychiatric diseases.

THE REGULATION OF Akt AND GSK3 BY D2 DOPAMINE RECEPTORS

Several lines of evidence have suggested that dopamine may play a role in the regulation of Akt and GSK3 signaling *in vivo*. For instance, as early as in 1971, behavioral observations in rodents have shown that lithium, which is now known to inhibit GSK3, can antagonize locomotor activity induced by drugs elevating dopaminergic tone or by the direct injection of dopamine in the nucleus accumbens (Cox et al., 1971; Barnes et al., 1986; Aylmer et al., 1987). However, these studies were all conducted before the discovery of the effect of lithium on GSK3 (Klein and Melton, 1996; Stambolic et al., 1996), and the actions of lithium in these experimental systems were generally attributed to other mechanisms including changes in dopamine release or dopamine receptor blockade. More recently, independent lines of research have shown that several antipsychotic drugs, known to share a D2R antagonist action, can activate Akt and increase the inhibitory phosphorylation of GSK3 in the rodent brain (Beaulieu et al., 2004; Emamian et al., 2004; Li et al., 2007). However, since these

drugs also have other molecular targets and alternative mechanisms such as an action on serotonin receptors (Beaulieu, 2007; Li et al., 2007; Beaulieu et al., 2008b), these additional mechanisms could also contribute to the effect of these drugs on Akt/GSK3 signaling.

Characterization of cell signaling abnormalities in mice lacking the dopamine transporter (DAT–KO) have provided the first direct *in vivo* evidence for a role of dopamine in the regulation of Akt/GSK3 signaling in the mouse striatum (Beaulieu et al., 2004). The absence of dopamine reuptake by presynaptic dopamine neurons in these mice lead to about fivefold increase in extracellular dopamine concentration in the striatum (Gainetdinov et al., 1999). This persistent hyperdopaminergia results in reduced phosphorylation of Akt on its regulatory threonine 308 residue leading to a reduction of Akt activity and a concomitant activation of both GSK3α and GSK3β due to a reduction in the phosphorylation of their N-terminal domain by Akt (Beaulieu et al., 2004). A similar effect on Akt and GSK3 has been observed following the administration of indirect dopamine agonist amphetamine, that exerts its effects by elevating extracellular dopamine concentrations (Beaulieu et al., 2004; Polter et al., 2010). Furthermore, the non-selective D1R/D2R agonist apomorphine has also been reported to inhibit Akt phosphorylation in the mouse striatum (Beaulieu et al., 2005, 2007b).

A direct contribution of dopamine to the regulation of Akt and GSK3 was established by inhibiting dopamine synthesis in DAT–KO mice using the irreversible tyrosine hydroxylase inhibitor α-methyl-*para*-tyrosine (Beaulieu et al., 2004). It is important to note, that since these mice cannot reuptake and recycle dopamine to presynaptic terminal, inhibition of dopamine synthesis in DAT–KO mice leads to a virtual absence of striatal dopamine within minutes following drug administration (Beaulieu et al., 2004; Sotnikova et al., 2005). Two hours after this treatment, DAT–KO mice showed a restoration of Akt activity and enhanced inhibitory N-terminal phosphorylation of GSK3α and GSK3β.

THE ROLE OF D2-CLASS RECEPTORS

Pharmacological characterization of the receptor involved in the inhibition of Akt and activation of GSK3 by dopamine in DAT–KO mice showed that Akt and GSK3 phosphorylation can be restored in DAT–KO mice by the administration of raclopride, a D2-class receptor antagonist (Beaulieu et al., 2004). A contribution of this family of receptors has later been confirmed by an investigation of dopamine-dependent regulation of Akt and GSK3 phosphorylation in mice lacking different subtypes of dopamine receptors. This study showed that D2R is essential for the inhibition of striatal Akt by amphetamine and apomorphine, while the effect of these drugs remained intact in D1R–KO mice. Interestingly, mice lacking the dopamine D3R showed a reduced sensitivity of Akt-mediated signaling to dopaminergic drugs but retained the action of these drugs on Akt at high dose regimens, suggesting that D3R also participates in the regulation of Akt/GSK3 signaling, potentially by enhancing D2R responses (Beaulieu et al., 2007b).

Since these initial characterizations were performed (Beaulieu et al., 2004), several independent studies using different pharmacological approaches have shown that a regulation of Akt and GSK3 by D2R end D3R is not restricted only to the mouse striatum. Indeed, prolonged elevation of dopaminergic tone (Li et al., 2009) or subchronic treatment with the D2R agonist quinpirole have been reported to inactivate Akt and activate GSK3 in the rat frontal cortex (Sutton and Rushlow, 2011). In contrast, administration of the D2R antagonist raclopride or D3R antagonist nafadotride has the opposite effect on Akt activity in different regions of the rat brain (Sutton and Rushlow, 2011). Finally, a recent report has convincingly shown the inactivation of Akt in response to D2R stimulation in the developing zebrafish brain (Souza et al., 2011), thus demonstrating that regulation of Akt and GSK3 is a shared functional property of D2R across several species of vertebrates.

cAMP-INDEPENDENT DOPAMINE RECEPTOR SIGNALING

Dopamine D2-class receptors are coupled to Gαi/o G protein and therefore inhibit adenylate cyclase and the production of cAMP and the activity of protein kinase A (PKA). Importantly, modulation of cAMP levels does not affect Akt and GSK3 activity in the mouse striatum, therefore indicating that D2R regulate this signaling cascade via a different type of mechanism (Beaulieu et al., 2004). Characterization of cell signaling responses in mice lacking βArr2 has shown that this molecule is essential for the inhibition of Akt and concomitant activation of GSK3 by D2R (Beaulieu et al., 2005; **Figure 1**). Furthermore, the inactivation of Akt by dopamine can also be prevented by inhibiting the serine/threonine protein phosphatase 2A (PP2A), which is known to play a role in mediating Akt inactivation in different biological systems (Ugi et al., 2004; Beaulieu et al., 2005). Further characterizations of the mechanisms through which D2R triggers the inactivation of Akt have shown that dopamine promotes the formation of a signaling complex (**Figure 2**) composed at least of Akt1, βArr2, and PP2A, thereby facilitating the inactivation of Akt by the phosphatase (Beaulieu et al., 2005, 2008a).

It is noteworthy, that the formation of the Akt:βArr2:PP2A signaling complex in response to D2R activation represents a mechanism through which an intercellular messenger molecule (i.e., dopamine) can trigger the inactivation of PI3K/Akt signaling in a regulated fashion. Importantly, the Akt:βArr2:PP2A signaling complex can be dissociated in response to lithium (**Figure 2**), thus providing a likely explanation for early behavioral observations of an antagonistic effect of lithium on dopamine-mediated behaviors as well as a plausible mechanism for the activation of Akt by lithium (Beaulieu et al., 2008a; O'Brien et al., 2011; Pan et al., 2011). The details of the mechanism(s) by which lithium triggers this dissociation are not yet fully understood. Present evidence suggests that lithium may affect the stability of this complex by acting on several of its components, possibly in a synergistic fashion. First, lithium can interfere with the interaction of Akt1 and βArr2 (Beaulieu et al., 2008a). Indeed *in vitro* experiments conducted on recombinant proteins have shown that this interaction is dependent upon the presence of magnesium ions and that addition of an excess of magnesium can prevent the dissociation of Akt and βArr2 by lithium at therapeutic concentrations (1 mM). Second, GSK3β has also been shown to interact with βArr2. Recent evidence obtained from transgenic mice overexpressing frog GSK3β in neurons indicate that activated GSK3 can act as a feed forward mechanism for its own activation by stabilizing the Akt:βArr2:PP2A signaling complex (O'Brien et al.,

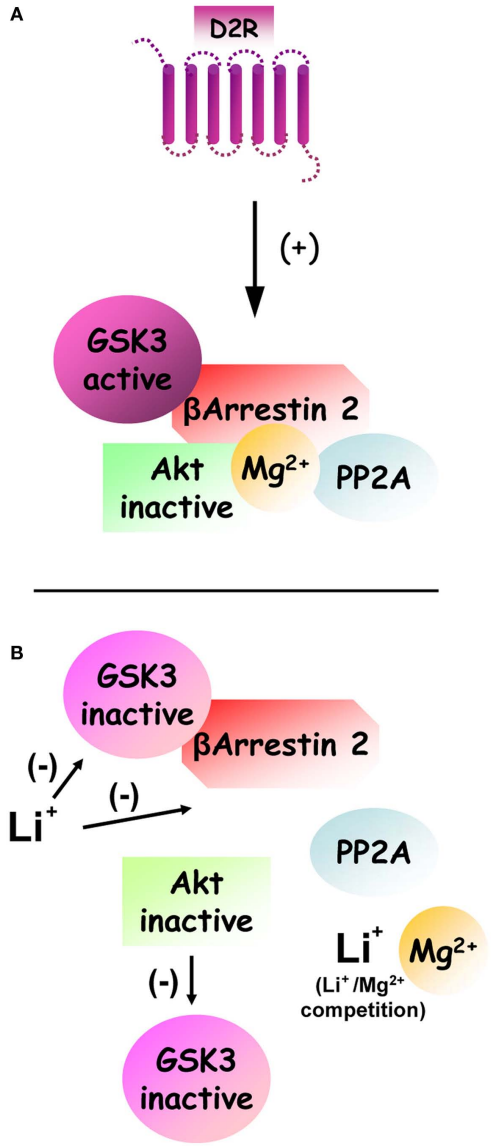

FIGURE 2 | Regulation of dopamine and D2R-dependent Akt:βArr2:PP2A signaling complex by lithium and GSK3. (A) Under basal condition, the activation of D2R stimulates the formation of a signaling complex composed by beta-arrestin 2, PP2A, and Akt. The formation of this complex results in increased inactivation of Akt by PP2A. Furthermore enhanced activation of GSK3 that results from Akt inhibition would act as a positive feedback loop that further stabilizes the signaling complex. Finally, the formation of this complex also appears to be dependent on Mg^{2+} to allow the interaction of Akt and beta-arrestin 2. **(B)** Following treatment with lithium, the formation of this signaling complex can be destabilized by two overlapping mechanisms. First a competition between Li^{+} and Mg^{2+} ions can disrupt the complex directly by preventing the interaction of Akt and beta-arrestin 2. Second direct inhibition of GSK3 by lithium prevents the stabilization of the complex by activated GSK3.

2011). According to this model, a direct inhibition of GSK3 by lithium would thus constitute an additional mechanism that can promote the disassembly of the Akt:βArr2:PP2A in response to lithium.

OTHER REGULATION MODES OF Akt AND GSK3 BY DOPAMINE

While there are several reports that Akt is inhibited following the activation of D2-class receptors, there are also some *in vitro* and indirect *in vivo* observations of Akt activation and subsequent inhibition of GSK3 following dopamine receptor stimulation (Brami-Cherrier et al., 2002; Svenningsson et al., 2003; Mannoury La Cour et al., 2011; Rau et al., 2011). Indeed, activation of both D1R and D2R in primary cultures of embryonic striatal neurons has been shown to increase the phosphorylation/activation of Akt in the nucleus (Brami-Cherrier et al., 2002). Similarly, a recent report has shown that transfection of the long splice variants of human D2R or D3R in Chinese hamster ovary (CHO) cells can activate Akt and inhibit GSK3 by increasing their phosphorylation (Mannoury La Cour et al., 2011). *In vivo*, activation of Akt by cocaine (Brami-Cherrier et al., 2002) and inhibition of GSK3 by amphetamine (Svenningsson et al., 2003) in the mouse striatum, as well as activation of Akt following methamphetamine in a rat model stroke (Rau et al., 2011) have also been reported.

One major difference between these observations and research showing an inactivation of Akt by D2-class receptors resides in the intrinsic temporal dynamics of these signaling mechanisms. In general, increased Akt and/or GSK3 phosphorylation have been reported to occur at short intervals (1–15 min) after receptor activation (Brami-Cherrier et al., 2002; Svenningsson et al., 2003; Mannoury La Cour et al., 2011). In contrast, βArr2-mediated signaling resulting in reduced phosphorylation of Akt and GSK3 occurs at later time periods (30 min or more) following receptor activation (Beaulieu et al., 2004, 2005; Emamian et al., 2004; Li et al., 2009). These variations in response time are important since G protein- and arrestin-mediated signaling (**Figure 3**) are characterized by different temporal dynamics (Ahn et al., 2004; Shenoy et al., 2006; Beaulieu et al., 2007a). Mechanisms that are mediated by G proteins have a fast onset and a short duration as a result of GPCR desensitization in both cultured cells and *in vivo*. In contrast, arrestin-mediated signaling is characterized by much slower and progressive onset and does not have a known desensitization mechanism therefore allowing for prolonged signaling responses lasting from several minutes to hours.

It is thus possible that in addition of the βArr2-mediated signaling that results in the inhibition of Akt and the activation of GSK3, dopamine receptors may also have an opposite effect by activating Akt and inactivating GSK3 via G protein-dependent mechanisms. However, the exact nature of such mechanisms remains mostly unexplained as changes in cAMP production, the hallmark of dopamine receptor G protein-mediated signaling, does not affect the phosphorylation of Akt and GSK3 in the mouse striatum, at least at later time points (Beaulieu et al., 2004). One possibility is that activation of Akt by dopamine under certain conditions may result from alternative G protein signaling mechanisms. Among such potential mechanisms, Gβγ G protein subunits can activate PI3K and therefore result in increased Akt activation, as has been shown following the activation of several different GPCRs (Brock et al., 2003). In addition to this possibility, recent reports also indicate that dopamine receptors may

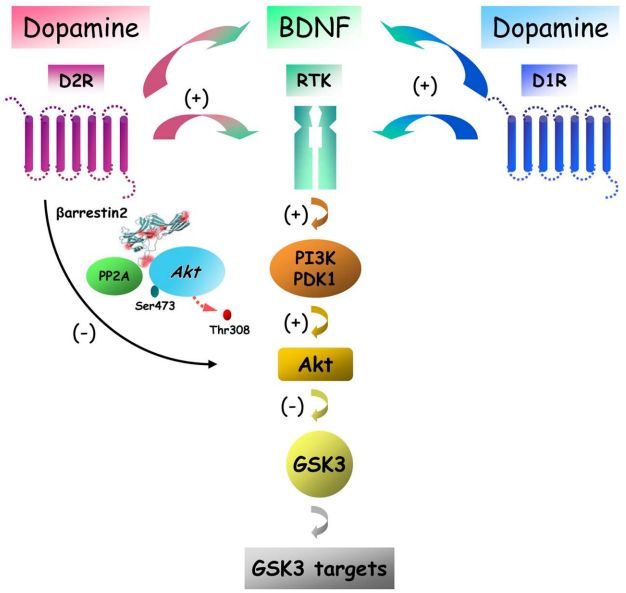

FIGURE 3 | Putative signaling pathways downstream of dopamine receptors with different potential outcomes on Akt/GSK3 signaling. Dopamine receptors can regulate Akt and GSK3 signaling by acting through at least two signaling mechanisms that have an opposite effect on the activity of these two kinases. Activation of D2R leads to a regulated deactivation of Akt by PP2A that is mediated by beta-arrestin 2. In contrast activation of either D1R or D2R can also activate Akt via receptor tyrosine kinase (RTK) transactivation leading to enhancement of Pi3K signaling.

activate Akt by transactivating receptor tyrosine kinase (RTK). For instance, the activation of Akt by D2R and D3R in CHO cells appears to involved the transactivation of the insulin like growth factor 1 receptor (Mannoury La Cour et al., 2011). In a similar way, the development of a new image analysis method to quantify protein dimerization in single cells – spatial intensity distribution analysis (SpIDA) – has shown that stimulation of either transfected D1R or D2R in CHO cells provoques a dose-dependent dimerization and activation of the epidermal growth factor receptor as early as 5 min following dopamine receptor stimulation with apomorphine (Swift et al., 2011). Closer to a naturally occurring situation, SpIDA also revealed that endogenous D1R and D2R can activate endogenous BDNF TrkB receptor within a similar time frame in response to apomorphine in primary neuron cultures obtained from new born mice striatum (Swift et al., 2011). Intriguingly also, a decreased mRNA levels for BDNF and its high affinity receptor TrkB was found in the frontal cortex of hyperdopaminergic DAT-KO mice (Fumagalli et al., 2003). Taken together, these observations indicate that D1R or D2R have the ability to transactivate or interact with at least 3 different types of RTKs and that such a signaling mechanism is compatible with the normal expression of these different receptors in neurons (**Figure 3**). Despite the interesting possibilities raised by this mechanism, further studies will be needed to evaluate the relative contribution of RTK transactivation by GPCRs in the biological functions of dopamine receptors in *in vivo* settings.

INVOLVEMENT OF Akt AND GSK3 IN THE REGULATION OF BEHAVIOR BY DOPAMINE

Importance of Akt/GSK3 signaling for dopamine-related behaviors was evident from the pioneering observations uncovering the role of this pathway in dopamine receptor signaling. Pharmacological and genetic manipulations that increased or reduced dopaminergic activity and caused profound effects on locomotor activity in mice, induced also significant alterations in the phosphorylation of Akt and GSK3 (Beaulieu et al., 2004). At the same time, pharmacological or genetic suppression of GSK3 activity inhibited locomotor hyperactivity related to excessive dopaminergic tone in DAT–KO or amphetamine-treated mice (Beaulieu et al., 2004; Gould et al., 2007). Similarly, several GSK3 inhibitors blocked amphetamine-induced hyperactivity in normal animals (Gould et al., 2007; Kalinichev and Dawson, 2011). In contrast, mice over-expressing GSK3β showed pronounced locomotor hyperactivity (Prickaerts et al., 2006) and transgenic mice that express a GSK3β mutant (lacking an inhibitory phosphorylation site and thus have constitutively active GSK3β), demonstrate increased novelty-driven and amphetamine-induced hyperactivity (Polter et al., 2010).

Mice lacking Akt1 demonstrate enhanced sensitivity to amphetamine as regard to disruption of sensorimotor gating in pre-pulse inhibition (PPI) test, which is classically used to model psychosis in rodents (Emamian et al., 2004). As described above, Akt1 is inhibited following the stimulation of D2R, thus the increased behavioral effect of amphetamine in Akt1–KO mice likely results from the involvement of Akt in dopamine-related behavioral responses.

There are several lines of evidence highlighting the role of βArr2-dependent signaling complex in the actions of dopamine. It has been observed, that βArr2–KO mice display spontaneous locomotor hypoactivity, reduced apomorphine-induced climbing and amphetamine-induced hyperlocomotion (Gainetdinov et al., 2004; Beaulieu et al., 2005). These mutants also have a reduced responsiveness to the dopamine-dependent locomotor effects of morphine (Bohn et al., 2003). In addition, novelty-driven locomotor hyperactivity phenotype typical of hyperdopaminergic DAT–KO mice is less pronounced in mice lacking both βArr2 and DAT (Beaulieu et al., 2005). Finally, the antimanic drug lithium that disrupts βArr2/Akt/PP2A signaling complex and can directly inhibit GSK3, exerts multiple actions on behaviors including suppression of spontaneous locomotor activity in DAT–KO and normal mice, but not in βArr2–KO mice (Beaulieu et al., 2004, 2005).

Interestingly, a recent publication (Miller et al., 2010) also points toward a possible involvement of GSK3 in regulation locomotor responses do D1R stimulation. In this article, the authors report that pharmacological inhibition of GSK3 using SB 216763 leads to reduced behavioral responses to the selective D1R agonist SKF-82958. However, this has to be interpreted with caution since SB216763 is also an inhibitor of cyclin dependent kinases (Meijer et al., 2004), and kinases from this family, namely cyclin dependent kinase 5 (CDK5), are known to contribute to D1R signaling responses (Bibb et al., 1999; Cyr et al., 2003). Furthermore, the expression of a full locomotor response to dopamine agonists is known to depend upon a synergism of D1R and D2R stimulation. Under normal conditions the locomotor effects of a D1R agonist results from an exacerbated stimulation of D1R in a context

where both D1R and D2R are stimulated by dopamine already present in the brain. Since GSK3 is regulated by D2R, it is therefore possible that the results reported by Miller et al., may be explained by an effect of GSK3 inhibitors on locomotion downstream of D2R. In line with this, it is of interest that the full effects of D1R stimulation on locomotion are significantly curtailed in D2R knockout mice where D1R/D2R synergism is abolished (Kelly et al., 1998).

Given the involvement of dopamine in multiple behavioral manifestations beyond locomotor activity, it is imperative to understand the role of AKT/GSK3 signaling cascade in these behaviors. For example, recent publications have suggested that GSK3 is important for psychostimulant-induced sensitization (Chen et al., 2007b; Miller et al., 2009), a process generally related to intermittent dopaminergic stimulation. Certainly, more studies in various behavioral paradigms could be expected in future on this issue.

MOLECULAR TARGETS OF DOPAMINE, REGULATED BY βArr2, Akt, AND GSK3

With growing appreciation of a role for Akt and GSK3 in dopamine receptor signaling, understanding of the nature of the molecular targets of these kinases has become a priority. Akt and GSK3 are known to have multiple substrates, many of them being involved in critical cellular processes such as cytoskeleton organization, trafficking, cell survival, apoptosis, and DNA transcription (Frame and Cohen, 2001; Woodgett, 2001). Here, we overview the major targets of GSK3 that have shown a direct role in dopamine receptor signaling and that could be involved in the pathogenesis of neuropsychiatric disorders. These targets include, but are not limited to, the direct GSK3 targets β-catenin, ionotropic glutamate receptors and clock genes involved in the regulation of circadian rhythms.

β-CATENIN

β-catenin is a common component of the Wnt and Akt/GSK3 signaling pathway. This protein that has multiple roles in the regulation of various aspects of cell cycle (Freyberg et al., 2010). Among other functions, β-catenin can act as a transcription factor and as a scaffolding protein that anchors the actin cytoskeleton to organize adherent junctions. Generally, in the absence of Wnt stimulation, β-catenin predominantly engages in Akt/GSK3 pathway by forming a complex with GSK3 and several other proteins. In this complex, β-catenin is phosphorylated by GSK3, which results in ubiquitination and proteasomal degradation of this protein (Doble and Woodgett, 2003). At the same time, activation of the Wnt receptor Frizzled causes disruption of this complex via involvement of Disheveled (Dvl) protein. Freed β-catenin then translocates to the nucleus and affects gene expression (Fukumoto et al., 2001). Several reports indicate that the action of dopamine and psychotropic drugs on the gene expression and neuronal morphology can involve the Wnt pathway and the Akt/GSK3 signaling cascade-mediated alterations in β-catenin activity. For example, the antipsychotic drugs haloperidol and clozapine may cause an enhancement of Dvl-3 isoform activity in the striatum which in turn may interact with D2R (Sutton et al., 2007) to modulate Akt, GSK3, and β-catenin activities (Sutton and Rushlow, 2011). Furthermore, antipsychotics haloperidol, risperidone, and clozapine can alter GSK3 and β-catenin expression not only in the striatum but also in the prefrontal cortex (Alimohamad et al., 2005b) and ventral midbrain (Alimohamad et al., 2005c). In contrast, direct and indirect dopamine receptor agonists quinpirole and amphetamine, respectively, induce the opposite response (Alimohamad et al., 2005c; Sutton and Rushlow, 2011). Importantly, chronic treatment of mice with the mood stabilizer lithium results in increased β-catenin expression in the amygdala, hypothalamus, and hippocampus (O'Brien et al., 2004; Beaulieu et al., 2008a). Furthermore, mice over-expressing β-catenin recapitulate the behavioral effects of the GSK3 inhibition by lithium in dopamine-dependent tests such as locomotor hyperactivity and in tests used to evaluate antidepressant and anxiolytic actions (Gould et al., 2008). It is likely, that these effects are mediated by β-catenin in the striatum, since the β-catenin forebrain-specific conditional mutant mice demonstrate only minimal behavioral abnormalities (Gould et al., 2008). Certainly, future studies are necessary to fully understand the role of β-catenin in actions of dopamine and psychotropic drugs.

IONOTROPIC GLUTAMATE RECEPTORS

The regulation of Akt/GSK3 signaling cascade by dopamine receptors has important modulatory influences on some specific functions of NMDA ionotropic glutamate receptors and related synaptic plasticity (Chen et al., 2007a). For example, alterations in GSK3 activity modulate the development of both long-term potentiation (LTP) and long-term depression (LTD) in rat hippocampal slices, the synaptic processes known to be regulated by ionotropic glutamate receptors (Peineau et al., 2007, 2008; Zhu et al., 2007). Intriguingly, D2R-mediated Akt/GSK3 signaling seems to be primarily responsible for the regulation of NMDA receptor functions in response to excessive levels of dopamine in the rat frontal cortex (Li et al., 2009). It has been reported that high concentrations of dopamine cause a reduction of NMDA currents and internalization of the NMDA receptor subunit NR2B. These effects of excessive dopaminergic stimulations on NMDA receptor functions are dependent of D2R, GSK3, and PP2A and can not be affected by inhibition of G proteins, thus indirectly suggesting an involvement of G protein-independent βArr2-mediated D2R signaling (Li et al., 2009). However, the postulated contribution of βArr2 to the regulation of NMDA receptor function by dopamine has not been validated yet in direct investigations involving βArr2–KO mice. Furthermore, it would be important to determine if similar type of regulation occurs in the striatum and in other brain areas or if it is restricted only to cortical neurons.

REGULATION OF CLOCK GENE SIGNALING

Several psychiatric disorders such as bipolar disorder and particularly seasonal affective disorder could be related, at least in part, to the dysregulation of circadian rhythms (Benedetti et al., 2004; Mansour et al., 2006). Original observations associating GSK3 activity to the regulation of circadian rhythms performed in *Drosophila melanogaster* (Yuan et al., 2005) revealed that the fly GSK3 ortholog, Shaggy, potently modulates the circadian cycle in response to serotonin. Consequently, several other studies in mammals have demonstrated the regulation of circadian

rhythms and clock genes by GSK3 (Iitaka et al., 2005; Lamont et al., 2007). For example, it has been found that lithium affects the transcription of the clock gene *Bmal1* presumably via GSK3 inhibition (Lamont et al., 2007). Furthermore, regulation of mammalian circadian protein functions by GSK3β was shown *in vitro* (Iitaka et al., 2005). Intriguingly, it has been reported that D2R can influence circadian rhythm-regulated gene expression and related behaviors (Doi et al., 2006; Yujnovsky et al., 2006). Also, it is well known that D2R is expressed at high levels in the retina and thus can play an important role in adaptation to light (Doi et al., 2006). Finally, activation of D2R causes stimulation CLOCK:BMAL1 functions potentially via regulation of circadian gene expression through βArr2/Akt/GSK3 signaling cascade (Sahar et al., 2010). Taken together, these observations indicate a putative mechanism connecting circadian rhythm to D2R functions.

Akt AND GSK3 IN THE ACTION OF ANTIPSYCHOTICS

All clinically effective antipsychotics share the ability to antagonize D2R (Snyder, 1976). Several antipsychotics have been shown to be able to activate Akt and inhibit GSK3 further supporting the role of the βArr2/Akt/GSK3 pathway in D2R signaling (Emamian et al., 2004; Alimohamad et al., 2005b; Li et al., 2007; Beaulieu et al., 2009). For example, the typical antipsychotic haloperidol increases phosphorylation of Akt and GSK3 thereby antagonizing action of dopamine (Beaulieu et al., 2004; Emamian et al., 2004). Furthermore, it has been shown that atypical antipsychotics clozapine, olanzapine, quetiapine, risperidone, and ziprasidone can either activate Akt or mimic its activity by increasing phosphorylation of GSK3α and GSK3β (Alimohamad et al., 2005b; Li et al., 2007). While the role of βArr2 in the actions of antipsychotics has not been validated yet in *in vivo* studies involving mutant mice, *in vitro* studies in cells have indicated that both typical and atypical antipsychotics are highly effective in antagonizing the recruitment of βArr2 to the D2R long isoform in comparison to their action on cAMP signaling (Masri et al., 2008). Particularly, three different patterns of activity of a panel of antipsychotics on cAMP mediated signaling and βArr2 recruitment were observed. Certain antipsychotics, including haloperidol, demonstrated potent antagonism of both the regulation of cAMP and the recruitment of βArr2 to the $D2_LR$ with approximately similar efficacy. Other antipsychotics, such as clozapine showed a stronger antagonist activity on βArr2 recruitment than on the cAMP regulation. Finally, a representative of a third group of antipsychotics, aripiprazole, acted as a partial agonist as regard to cAMP signaling while potently inhibiting βArr2 recruitment to D2R.

In summary, these results indicate that antipsychotics may vary in their ability to antagonize cAMP production but share a common antagonistic action as regard to D2R/βArr2-mediated signaling at least *in vitro*. Future *in vivo* studies are thus necessary to compare the physiological action of each of these drugs with regard to their action on D2R/βArr2/Akt/GSK3 signaling cascade. However, it is noteworthy that several atypical antipsychotics such as clozapine are not only D2R antagonists but are also able to affect brain 5-HT neurotransmission via action on 5-HT2A receptors (Kapur and Remington, 2001). Since drugs acting on 5-HT neurotransmission or decreasing 5-HT synthesis are known to affect GSK3 activity *in vivo* (Li et al., 2007; Beaulieu et al., 2008b), it is likely that atypical antipsychotics can affect GSK3 activity through both dopaminergic and serotonergic mechanisms. Further studies are therefore necessary to delineate the relative contribution of dopamine- and 5-HT-related processes in the modulation of Akt/GSK3 signaling cascade by antipsychotic drugs and thus to define the roles that these mechanisms may play in the treatment of psychotic disorders.

GENETIC ASSOCIATIONS BETWEEN REGULATORS OF Akt/GSK3 SIGNALING AND PSYCHIATRIC DISORDERS

A role for D2R/βArr2/Akt/GSK3 signaling in the action of mood stabilizers and antipsychotics is also supported by clinical studies of genetic risk factors for schizophrenia and bipolar disorders. Psychiatric disorders are multifactorial conditions that involve various combinations of genetic, epigenetic, and environmental factors (Karayiorgou and Gogos, 1997). Several recently published meta-analysis studies have examined the results of ≥1000 genetic association studies attempting to identify candidate genes for schizophrenia (Allen et al., 2008; Jia et al., 2010). Interestingly, in some cases the same allelic polymorphisms have been associated with different neuropsychiatric pathologies such as schizophrenia, bipolar disorder, unipolar depression, and autism. To better understand the mechanisms of such possible common etiology, several genetically altered animal models have been created with the focus on genes known to be altered in these mental illnesses (Gottesman and Gould, 2003). Numerous risk genes for these diseases affect or are being affected by Akt and GSK3. Here, we review genetic associations between psychiatric disorders and the products of three of such genes (*DISC1*, *NRG1*, and *AKT1*) that are known to be involved directly or indirectly in the Akt/GSK3 signaling pathways.

Akt1

Accumulating evidence points to an important role for Akt and related signaling molecules in schizophrenia pathogenesis. In the pioneering study, a reduced levels of Akt1, one of the three Akt isoforms, in the hippocampus and frontal cortex of schizophrenia patients was found (Emamian et al., 2004). A major association of Akt1 haplotype with schizophrenia has been reported following transmission-disequilibrium tests in several other cohorts of patients (Ikeda et al., 2004). Supportive of these associations of AKT1 with schizophrenia, another group has reported a coding variation in AKT1 that affects protein expression in lymphoblasts and which can modulate prefrontal cortical structure and function was associated with risk for schizophrenia (Tan et al., 2008). Importantly, the behavioral outcomes of reduced Akt1 expression both in mice and humans can be affected by alterations in D2R functions. In Akt1–KO mice, amphetamine-induced disruption of sensory–motor gating in the PPI test is enhanced but can be reversed by the D2R antagonist haloperidol (Emamian et al., 2004). In humans, interaction between polymorphisms in the D2R (DRD2 rs1076560) and AKT1 (AKT1 rs1130233) genes was associated with deficient attentional processing in normal individuals and response to olanzapine treatment in schizophrenia patients

(Blasi et al., 2011). Furthermore, it has been reported that Akt1 levels and Akt-dependent phosphorylation of GSK3α and GSK3β isoforms is reduced in the frontal cortex of schizophrenia patients (Emamian et al., 2004). Because Akt activity is affected by D2R and this kinase seems to be important for certain functions of prefrontal cortex such as cognitive performance (working memory; Lai et al., 2006), it is reasonable to consider this kinase as an important mediator in dopamine-related processes involved in etiology and treatment of schizophrenia. Importantly, Akt activity and functions can be also affected by the products of two other genes reported to be associated with schizophrenia: neuregulin 1 (NRG1) and disrupted in schizophrenia 1 (DISC1; Kanakry et al., 2007).

NEUREGULINS

A member of growth factors family, NRG, by acting on ErbB (EGF RTK) signaling pathway influences numerous processes that could be involved in the neuropsychiatric disorders, such as cell migration, myelination, NMDA receptor- dependent neuronal plasticity and even expression of monoamine receptors and transporters (Mei and Xiong, 2008; Pitcher et al., 2011). It has been reported, that in patients with schizophrenia, NRG signaling is increased in the hippocampus causing an up-regulation of Erb receptors and concomitant PI3K activation that results in increased phosphorylation of Akt (Law et al., 2007; Jaaro-Peled et al., 2009). Mice heterozygous for NRG1 or ErbB4 receptor deletion demonstrate some "schizophrenia-related" behavioral abnormalities such as hyperactivity, deficient social behaviors, anxiety, memory deficits, and impaired responses in PPI (Chen et al., 2008; Desbonnet et al., 2009). While the mechanisms involved in these pathologies are still poorly understood, several lines of evidence suggest involvement of NRG1-dependent modulation of the striatal dopaminergic system via Akt (Tosato et al., 2005). In fact, NRG1 mutant mice show a similar phenotype to hyperdopaminergic DAT mutant mice. In addition, Akt is involved in NRG1-associated processes such as myelination (Flores et al., 2000; Li et al., 2001) and Akt1 phosphorylation is stimulated by NRG1 (Keri et al., 2009). Further studies are necessary to explore this hypothesis.

DISC1

DISC1 is a gene locus originally identified as disrupted in Scottish family members suffering from schizophrenia, but also from bipolar disorder and major depression (Chubb et al., 2008). Alterations of DISC1 in cell culture and mice induce abnormal processes that may occur in schizophrenia such as impaired neurite outgrowth, abnormal cortical development and incorrect neuronal migration (Kamiya et al., 2005). Recently, DISC1 has been found to be a direct GSK3β regulator thereby providing further support for a role for this kinase in neuropsychiatric disorders (Mao et al., 2009). Genetic suppression of DISC1 in the dentate gyrus of adult mice increased GSK3 activity and caused abnormal behaviors that might have relevance for depression and schizophrenia (Mao et al., 2009). DISC1 mutant mice have also demonstrated deficits in working memory and these behavioral abnormalities could be corrected by an administration of a direct inhibitor of GSK3 (SB216763; Kvajo et al., 2008). In another study, dominant-negative mutant DISC1 influenced the neurobehavioral and molecular effects of methamphetamine in mice likely via interaction with Akt/GSK3 signaling cascade (Pogorelov et al., 2011). Furthermore, DISC1 can be also involved in modulation of Akt and related signaling in newly generated neurons through its interaction with the potential DISC1-interacting partner KIAA1212, known as a direct activator of Akt (Camargo et al., 2007; Kim et al., 2009). In this model, binding of DISC1 to KIAA1212 prevents its action on Akt function. Importantly, both Akt overexpression and DISC1 suppression result in similar developmental abnormalities of newborn neurons. Taken together, these observations suggest a role of DISC1 interaction with Akt/GSK3 signaling in neurogenesis processes that could be important for both neurotransmission- and neurodevelopment-associated neuropsychiatric abnormalities.

In summary, while behavioral abnormalities observed in mice deficient in NRG1 or DISC1 expression suggest a role of these molecules in modulation of dopaminergic transmission, little evidence still exist supporting this association in clinical genetic studies. Nevertheless, it seems likely that monoamine-related processes involved in the etiology of mental disorders or therapeutic actions of drugs may engage in functional and selective crosstalks with others cellular signaling mechanisms such as those involved in apoptosis, neurogenesis, and plasticity and that are also could be affected by genetic and/or environmental factors in patients with these disorders.

STRIATAL Akt AND GSK3 IN PARKINSON DISEASE

Striatal Akt/GSK3 signaling and related processes may also contribute to mechanisms leading to neurodegeneration or neuroprotection. In fact, recent studies have indicated association of Akt signaling with neurodegenerative disorders such as Parkinson disease. Two neurotoxins, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), induce a massive loss of nigral tyrosine hydroxylase-expressing cells and striatal dopamine depletion. Consequently, these two drugs are routinely used to generate pharmacological animal models of Parkinson's disease (Lane and Dunnett, 2008). Interestingly, both MPTP and 6-OHDA induced neurodegeneration is associated with alterations in striatal Akt/GSK3 signaling cascade (Quesada et al., 2008; Aleyasin et al., 2010). It has been reported also, that chronic treatment with L-DOPA but not DAR agonist pergolide strongly increases the activity of Akt and inhibits GSK3 in the dopamine-depleted striatum of 6-OHDA-lesioned rats suggesting that Akt pathway may be involved in cronic L-DOPA-induced dyskinesia (Bychkov et al., 2007). Indeed, in monkeys, which show dopamine depletion after MPTP administration, chronic L-DOPA treatment with or without two types of antidyskinetic drugs induced a prolonged changes in Akt and GSK3 phosphorylation levels (Morissette et al., 2010). While it is unclear at present if these effects caused by G protein-mediated or βArr2-mediated dopaminergic signaling mechanisms, other neurotransmitter systems like glutamate may also contribute to L-DOPA-induced dyskinesia (Ouattara et al., 2010) by directly regulating Akt/GSK3 or D1 dopamine receptor mediated signaling may be also involved (Santini et al., 2009).

In summary, these results suggest that Akt/GSK3 signaling may contribute to the development and manifestations of neurodegenerative disorders such as Parkinson's disease. Thus, it would seem relevant to investigate alterations in activity and molecular targets of these kinases as possible biomarkers of underlying pathology or treatment complications of this disorder. Understanding these mechanisms may provide better understanding of pathological processes and potentially new therapeutic strategy for the treatment of neurodegenerative disorders.

CONCLUSION AND PERSPECTIVES

Since the first reports published about 7 years ago on the role Akt/GSK3 signaling cascade in the dopamine receptor functions and behavior, this field showed quite remarkable developments. One reason for such noticeable progress is the growing appreciation of the involvement of Akt, GSK3, and related signaling processes in the pathophysiology of various psychiatric and neurological disorders. The role of this pathway in dopamine signaling has also received considerable attention due to multiple lines of evidence for its physiological significance *in vivo*. Numerous studies have convincingly shown significant alterations in dopamine-related functions and behaviors caused by manipulations affecting Akt/GSK3 pathway. Conversely, several genetic or pharmacological manipulation of dopamine receptors or their signaling intermediates have been shown to affect Akt/GSK3 signaling (**Table 1**). Certainly, many outstanding questions still remain unresolved and require further detailed investigations both *in vitro* and particularly at the level of whole organism. Most noticeable among them: understanding mechanisms of formation of arrestin-dependent signaling complexes in response to dopamine, identification of critical downstream targets of Akt and GSK3, clarification of cross-talk mechanisms between this and other signaling pathways, further understanding of the role of this signaling mode in pathological manifestations and effects of therapeutic treatments. It might be predicted that this line of research will continue to develop in very exciting ways and will eventually bring new molecules and pathways under attention as potential novel targets for treatment of neuropsychiatric disorders.

Table 1 | Effect of pharmacological and genetic manipulation of dopamine receptor activation on Akt and GSK3 activity *in vivo*.

Type of intervention		Akt activity	GSK3 activity	Reference
GENE KNOCKOUT				
	D1R	–	–	Beaulieu et al. (2007b)
	D2R	↑	↓	Beaulieu et al. (2007b)
	D3R	↑	↓	Beaulieu et al. (2007b)
	βarrestin2	↑	↓	Beaulieu et al. (2008a)
	DAT	↓	↑	Beaulieu et al. (2004)
PHARMACOLOGICAL				
Miscellaneous	8-Br-cAMP (ICV)	–	–	Beaulieu et al. (2004)
	αMPT (dopamine synthesis inhibitor)	↑	↓	Beaulieu et al. (2004)
	Dopamine (zebrafish)	↓	↑	Souza et al. (2011)
Increase dopamine release	Amphetamine (90 min)	↓	↑	Beaulieu et al. (2004, 2005), Polter et al. (2010), Ghisi et al. (2009)
	Amphetamine (15 min)	N.D.	↓	Svenningsson et al. (2003)
Non-selective DAR agonist	Apomorphine	↑	N.D.	Beaulieu et al. (2005, 2007b)
D1R antagonist	SCH23390 (30 min)	–	–	Beaulieu et al. (2004)
	SCH23390 (subchronic, rat)	–	–	Sutton and Rushlow (2011)
	SCH23390 (zebrafish)	–	–	Souza et al. (2011)
D4R antagonist	L745870	–	–	Beaulieu et al. (2007b)
D3R antagonist	Nafadotride (subchronic, rat)	↑	↓	Sutton and Rushlow (2011)
D2R agonist	Quinpirole (subchronic, rat)	↓	↑	Sutton and Rushlow (2011)
	Quinpirole (zebrafish)	↓	↑	Souza et al. (2011)
D2R antagonist	Eticlopride (DA treated zebrafish)	↑ (compared to DA treated)	↓ (compared to DA treated)	Souza et al. (2011)
	Raclopride (subchronic rat)	↑	↓	Sutton and Rushlow (2011)
	Raclopride (30 min)	↑	↓	Beaulieu et al. (2004)
	Haloperidol (subchronic)	↑	↓	Emamian et al. (2004)

↑ *Increased,* ↓ *decreased,* – *not changed, N.D., no data available. Data obtained in mice except if otherwise indicated.*

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A pivotal role of GSK-3 in synaptic plasticity

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Glycogen synthase kinase-3 (GSK-3) has many cellular functions. Recent evidence suggests that it plays a key role in certain types of synaptic plasticity, in particular a form of long-term depression (LTD) that is induced by the synaptic activation of N-methyl-D-aspartate receptors (NMDARs). In the present article we summarize what is currently known concerning the roles of GSK-3 in synaptic plasticity at both glutamatergic and GABAergic synapses. We summarize its role in cognition and speculate on how alterations in the synaptic functioning of GSK-3 may be a major factor in certain neurodegenerative disorders.

Keywords: GSK-3 (glycogen synthase kinase-3), caspase 3, Akt (PKB, protein kinase B), synaptic plasticity, LTP (long-term potentiation), LTD (long-term depression), AMPAR (AMPA receptor), NMDAR (NMDA receptor)

INTRODUCTION

While long recognized as a critical molecular effector in the cell, and an important target for therapeutic intervention, glycogen synthase kinase-3 (GSK-3) has only recently been discovered to be a key regulator of synaptic plasticity. Here we review the studies that have illuminated our understanding of how GSK-3 is involved in long-term depression (LTD) and long-term potentiation (LTP) in the hippocampus. We describe potential signaling cascades that involve GSK-3 in neurons and how this might affect the expression of AMPA receptors at synapses. In addition, we describe the evidence that suggests that GSK-3 also regulates GABA-A receptor (GABAR) trafficking. Lastly, we relate these emerging mechanisms to cognition and propose a hypothesis of how dysregulation of GSK-3 activity at synapses could explain important aspects of neurodegenerative disease.

ROLE OF GSK-3 IN LTP AND LTD

Information in the brain is stored primarily as changes in the weights of synaptic connections, via the processes of LTP and LTD. LTP, which is a long-lasting increase in the efficiency of synaptic transmission, is usually triggered by the synaptic activation of N-methyl-D-aspartate receptors (NMDARs), though NMDAR-independent forms of LTP also exist in the CNS (Bliss and Collingridge, 1993). LTD, which is the counterpart of LTP, can also be induced by the synaptic activation of NMDA receptors (NMDAR-LTD). Other forms of LTD also exist, such as those triggered by the synaptic activation of metabotropic glutamate receptors (mGluRs) and muscarinic receptors (mAChRs) (Collingridge et al., 2010). Multiple mechanisms exist to express the alteration in synaptic efficiency, including changes in the probability of neurotransmitter release and alterations in the function of postsynaptic receptors. With respect to NMDAR triggered synaptic plasticity, the most extensively investigated mechanisms involve alterations in the number of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs) expressed at synapses, caused via alterations in AMPAR trafficking (Collingridge et al., 2004).

We found that inhibition of GSK-3 prevented the induction of NMDAR-LTD (Peineau et al., 2007). This effect was observed with six structurally distinct inhibitors, at the appropriate concentration range for an action on GSK-3 (Peineau et al., 2007, 2009). In contrast, inhibition of a large number of other serine/threonine (Ser/Thr) protein kinases did not affect NMDAR-LTD (**Figure 1**). This suggests that GSK-3 is likely to be one of a small number of Ser/Thr protein kinases involved in this form of synaptic plasticity. It is not unique, however, since we have more recently also found a role for PI3Kγ, a dual Ser/Thr protein kinase and lipid kinase, in NMDAR-LTD (Kim et al., 2011). Furthermore, a role for tyrosine phosphorylation in NMDAR-LTD has been established (Ahmadian et al., 2004; Hayashi and Huganir, 2004) and recent evidence suggests that the tyrosine kinase Janus kinase 2 (JAK2) is required for the induction of NMDAR-LTD (Nicolas et al., 2012). Therefore, GSK-3 is one component of a complex phosphorylation cascade involved in NMDAR-LTD.

As described elsewhere, the isoform involved in NMDAR-LTD is likely to be GSK-3β, though a complementary role of GSK-3α cannot be discounted. Of the forms of synaptic plasticity that have been examined, GSK-3 is specifically involved in NMDAR-LTD (**Figure 2**). Thus, pharmacological inhibition of GSK-3 has no effect on NMDAR-LTP, depotentiation, or mGluR-LTD at CA3-CA1 synapses (Peineau et al., 2007). Of course, a role in one or more of the several other forms of synaptic

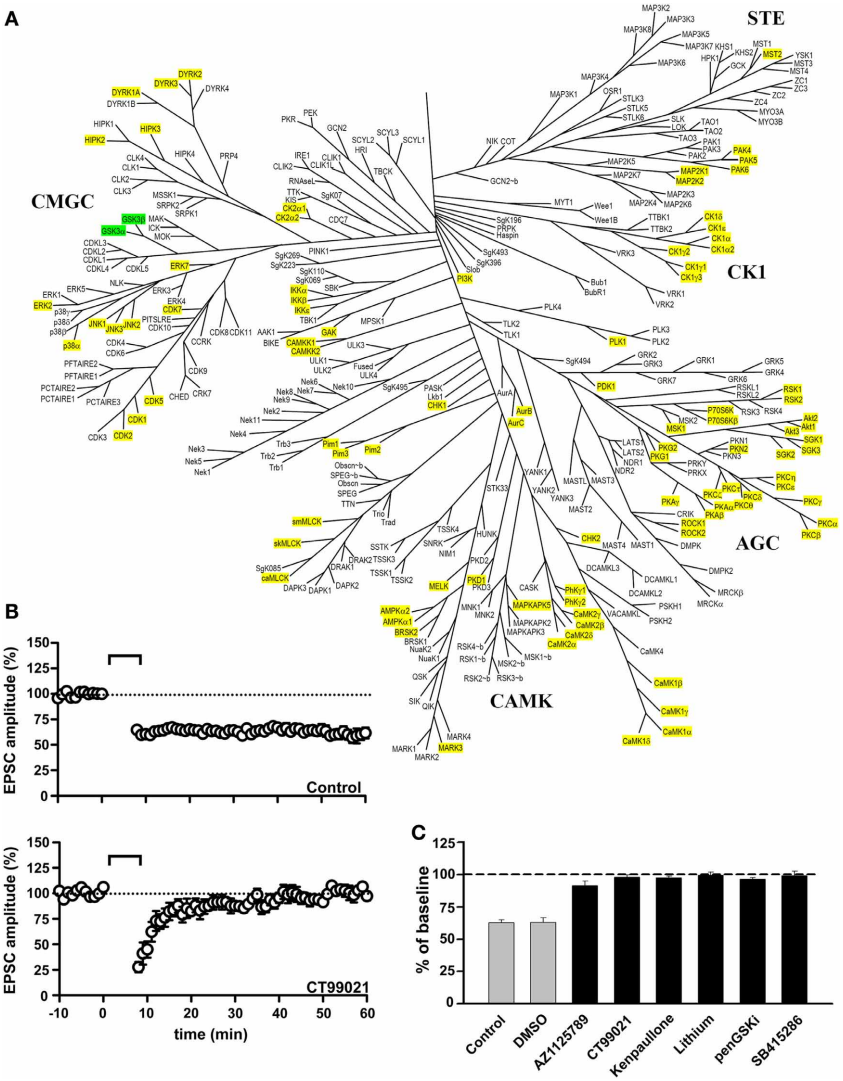

FIGURE 1 | GSK-3 inhibitors block the induction of NMDAR-LTD. **(A)** Family tree of Ser/Thr protein kinases in the human genome (modified from Manning et al., 2002). The two isoforms of GSK-3 (α and β) are highlighted in green. The kinases that were excluded from having a role in the induction of NMDAR-LTD, based on inhibitor studies reported in Peineau et al. (2007, 2009), are highlighted in yellow. **(B)** Control NMDAR-LTD (top) and its block by the GSK-3 inhibitor CT99021 (bottom). **(C)** Block of the induction of NMDAR-LTD by six different GSK-3 inhibitors. See Peineau et al. (2007, 2009) for more details.

plasticity at this or other synaptic pathways in the CNS cannot be discounted.

Although inhibition of GSK-3β has no effect on NMDAR-LTP, over-expression of GSK-3β blocks LTP (Hooper et al., 2007; Zhu et al., 2007). In addition, induction of LTP inhibits the activity of GSK-3β (Hooper et al., 2007; Peineau et al., 2007), suggesting that LTP and LTD may interact via a pathway involving GSK-3β that acts as a molecular switch.

ROLE OF GSK-3 IN METAPLASTICITY

Metaplasticity refers to changes in the ability of synapses to undergo plasticity after a previous synaptic or modulatory event, sometimes called "priming" (see review by Abraham and Bear, 1996). Several different forms of metaplasticity have been described so far. The prototypic form of metaplasticity is depotentiation, which is a form of LTD that can only be induced following the induction of LTP (where it reverses the potentiation to the baseline state; as reviewed by Collingridge et al., 2010). Another form of metaplasticity is where the prior activation of NMDARs can inhibit the subsequent induction of NMDAR-LTP (Coan et al., 1989; Huang et al., 1992). The findings, described above, that NMDAR-LTP can inhibit the activity of GSK-3β and that GSK-3β is required for NMDAR-LTD suggested the existence of another form of metaplasticity whereby LTP regulates LTD.

We explored this prediction experimentally and found that LTP did, indeed, potently inhibit NMDAR-LTD. The priming was induced by a protocol that could induce NMDAR-LTD but the induction of LTP *per se* was not a prerequisite. Indeed, many

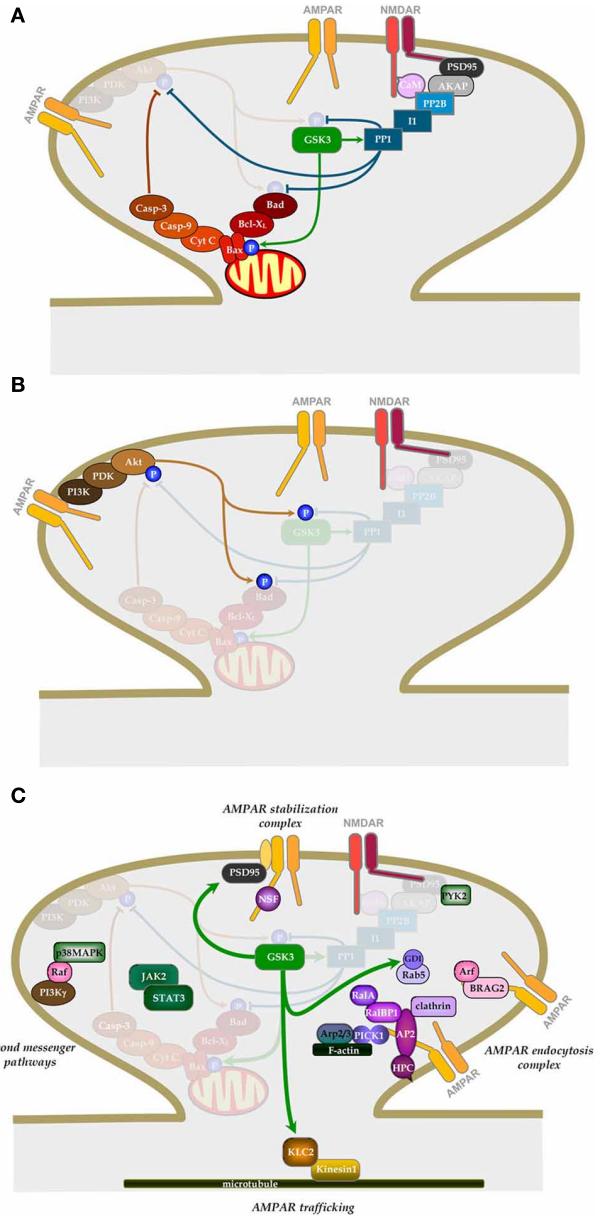

FIGURE 2 | The role of GSK-3 in NMDAR-LTD. (A) Signaling pathways involved in the induction of NMDAR-LTD and the regulation of GSK-3β. **(B)** Signaling pathway by which NMDAR-LTP can inhibit NMDAR-LTD. **(C)** Potential downstream effectors of GSK-3β in NMDAR-LTD. (See text for details).

experiments were performed under conditions in which LTP was deliberately absent, due to the phenomenon of washout (Malinow and Tsien, 1990), so as to eliminate the potentially confounding situation of depotentiation. In other experiments depotentiation was blocked by an mGluR antagonist. We found that the LTP stimulus (priming stimulus) completely prevented the induction of NMDAR-LTD immediately following the priming. Maximal inhibition of NMDAR-LTD lasted for about 20 min, where after there was a slow recovery of the ability to induce NMDAR-LTD such that after approximately 1 h the ability to induce NMDAR-LTD was fully restored. The mechanism of priming involved activation of NMDARs since it was prevented if an NMDAR antagonist was present during the priming. It also involved the canonical pathway for inhibition of GSK-3, namely phosphoinositide 3-kinase (PI3K) and Akt/PKB (protein kinase B; **Figure 2B**; Embi et al., 1980; Peineau et al., 2007).

UPSTREAM REGULATION OF GSK-3 IN SYNAPTIC PLASTICITY

The GSK-3 α and β isoforms are ubiquitous Ser/Thr kinases belonging to the CMGC family of protein kinases that act as key enzymes regulating various cellular signaling pathways. GSK-3 function is modulated through multiple regulatory mechanisms by protein-protein interactions, subcellular localization, priming/substrate specificity, and proteolytic cleavage, which have been recently reviewed by others (Hur and Zhou, 2010; Medina and Wandosell, 2011 and reviewed in this Special Topic by Kaidanovich-Beilin and Woodgett, 2011). Within these levels of functional regulation, phosphorylation and dephosphorylation play prominent roles.

Under certain biochemical conditions such as growth factor deprivation, the mammalian target of rapamycin complex 1(mTORC1)-S6K1 signaling can alternatively regulate and inhibit GSK-3 activity by Ser21/9 phosphorylation (Cohen and Frame, 2001; Zhang et al., 2006). There are also several other kinases such as Erk, ZAK1, MEK1/2, Pyk-2, and Fyn kinases that also have been described to interact with GSK-3 and regulate its function in other cell types (Kim et al., 1999, 2002; Lesort et al., 1999; Hartigan et al., 2001; Ding et al., 2005). However, the extent to which these regulatory processes occur in neurons and, more specifically, are involved in synaptic plasticity is largely unexplored.

We have described an upstream phosphorylation/dephosphorylation-dependent regulation of GSK-3β activity that is involved in NMDAR-LTD (**Figure 2A**). We cannot discount an additional role of GSK-3α, but focus on the β isoform in this review. Thus far, three sites of phosphorylation have been identified on GSK-3β: Ser9 (Sutherland et al., 1993), Tyr216 (Hughes et al., 1993), and Ser389 (Thr 390 in humans; Thornton et al., 2008) and have also been shown to be important regulatory elements in neurons. It should be noted that while regulation at Thr43 has been demonstrated in other cell types (Ding et al., 2005; Thornton et al., 2008), it remains to be explored in neurons.

The basal activity of GSK-3β is dependent on phosphorylation on Tyr216 (Hughes et al., 1991, 1993). The mechanism by which this Tyr residue becomes phosphorylated is still under debate. It is unclear whether there is an autophosphorylation mechanism (Cole et al., 2004; Lochhead et al., 2006) and/or a regulation by tyrosine kinases such as Fyn (Lesort et al., 1999) or Pyk2 (Hartigan et al., 2001). In resting neurons, Tyr216 is phosphorylated, and thus, GSK-3β is constitutively active (Hur and Zhou, 2010).

The main way neurons regulate GSK-3β activity is by controlling the phosphorylation level of Ser9 (for review see Doble and Woodgett, 2003). The phosphorylation of this site inhibits enzymatic activity. At least eight distinct signaling pathways have

been identified as a regulator of the Ser9 phosphorylation state (**Figure 3**). Seven of them are inhibitory and mediated by kinases. These are the Akt pathway (Hong and Lee, 1997), which we have shown to be important in mediating the phosphorylation of GSK-3β during LTP (Peineau et al., 2007; **Figure 2B**). In addition, CaMKII has been shown to phosphorylate and inhibit GSK-3 in neurons, where it functions in a pro-survival manner (Song et al., 2010). Phosphorylation of GSK-3β (and not GSK-3α) by classical protein kinase C (PKC) isotypes (α, β1, β2, and γ) results in its inactivation (Espada et al., 2009; Ortega et al., 2010) and may protect neurons from Aβ toxicity (Garrido et al., 2002). In addition, protein kinase A (PKA; Li et al., 2000; O'Driscoll et al., 2007; Shelly et al., 2010, 2011), PrkG1 (Zhao et al., 2009), p90 ribosomal protein S6 kinase (RSK; Valerio et al., 2006) and Integrin-linked kinase (ILK; Naska et al., 2006) also regulate GSK-3 activity in neurons. Interestingly, ILK directly interacts and phosphorylates GSK-3β and also phosphorylates Akt to inhibit GSK-3β indirectly (Delcommenne et al., 1998).

A third site to regulate GSK-3β activity has been recently identified. The highly enriched neuronal enzyme p38 mitogen-activated protein kinase (p38MAPK) was found to phosphorylate GSK-3β at Ser389 (Thr390 in humans) causing inhibition of its activity similar to that of inhibition via Ser9 (Thornton et al., 2008). Inhibition of GSK-3β via p38MAPK stimulates accumulation of β-catenin.

Only one pathway has been shown to stimulate GSK-3β activity in neurons and is mediated by protein phosphatase 1 (PP1) removing the phosphate group on Ser9 (Bennecib et al., 2000; Morfini et al., 2004). Noteworthy, a positive loop between PP1 and GSK-3β has been identified (Szatmari et al., 2005). We have observed a marked activation of GSK-3β during NMDAR-LTD via dephosphorylation of Ser9 mediated by PP1 (Peineau et al., 2007). However, the involvement of additional pathways that activate GSK-3β activity during NMDAR-LTD may also exist. Indeed, in other systems there are a variety of phosphatases, which dephosphorylate and disinhibit GSK-3 activity (Wera and Hemmings, 1995). In terms of the regulation by PP1, it is likely that during NMDAR-LTD this involves Ca^{2+} entry through NMDARs, binding of Ca^{2+} to calmodulin, activation of PP2B (calcineurin) leading to dephosphorylation of Inhibitor 1 (I-1) which in turn leads to activation of PP1 (**Figure 2A**). This pathway has been shown to be important for NMDAR-LTD (Mulkey et al., 1994).

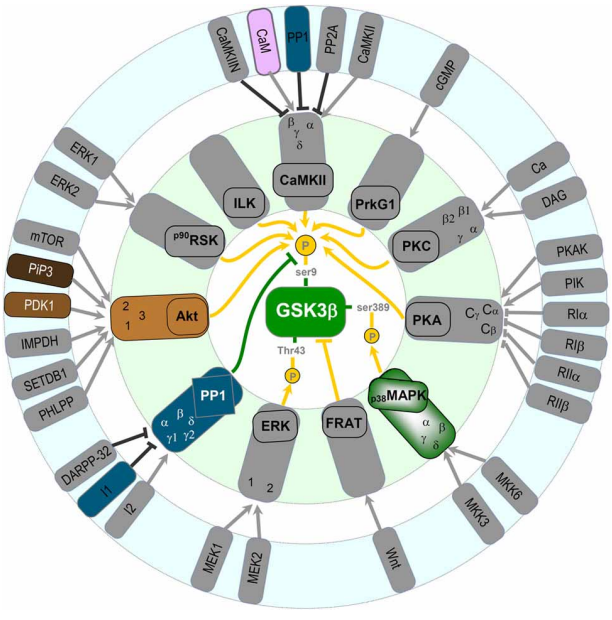

FIGURE 3 | Potential regulators of GSK-3 β in neurons (see text for details).

DOWNSTREAM EFFECTORS OF GSK-3 IN SYNAPTIC PLASTICITY

How GSK-3β activation contributes to the expression of NMDAR-LTD remains unknown. Given that the activation of NMDARs and endocytosis of AMPARs are, respectively, involved in the induction and expression of LTD, it appears quite reasonable to investigate if GSK-3β can exert its actions by regulating either or both of the processes. Indeed, one study has reported a GSK-3 activity dependent rapid internalization of NMDARs (Chen et al., 2007). Thus, LTP might inhibit LTD by regulating the levels of NMDARs that are required for LTD induction. However, in this case, one would expect to see a rapid depression of NMDAR-mediated synaptic transmission as a result of a rapid internalization of the receptors following LTP induction. However, most studies of LTP that have monitored NMDAR-mediated synaptic transmission have reported LTP [e.g., (Bashir et al., 1991)] rather than a transient depression. Thus, further work is needed to establish the extent to which the regulation of NMDAR trafficking by GSK-3β accounts for its involvement in synaptic plasticity. Any inhibition of NMDAR activity by GSK-3 antagonists is highly unlikely to have accounted for the inhibition of NMDAR-LTD in our experiments (Peineau et al., 2007). First, the effects observed on NMDARs were typically around 20% inhibition and this may be too small to prevent NMDAR-LTD induction. Indeed, whilst we observed a complete block of NMDAR-LTD there was sufficient NMDAR activation for NMDAR-LTP to be induced (Peineau et al., 2007). GSK-3β affects not only the induction, but also the expression of LTD as lithium was able to fully block NMDAR-LTD even when applied after the induction protocol (Peineau et al., 2007). Thus, even if the inhibition of NMDAR function may contribute to the effects under certain circumstance it cannot be the sole mechanism.

Wei and colleague (2010) recently showed that constitutive GSK-3β activity enhances basal AMPAR endocytosis. They found that either pharmacological inhibition or knockdown of the protein resulted in a decrease in mEPSC amplitude in cultured hippocampal neurons (Wei et al., 2010). They went on to demonstrate that internalization of AMPARs is via enhanced activity of Rab5 and is dependent on phosphorylation of guanine nucleotide dissociation inhibitor (GDI) at Ser45. They also showed that the effects of inhibition of GSK-3 and insulin occluded, suggesting that insulin-induced endocytosis of AMPARs (Man et al., 2000) is due to inhibition of GSK-3 (**Figure 2C**). At first glance these observations seem at variance with the reports that inhibition

of GSK-3β blocks NMDAR-LTD (Peineau et al., 2007, 2009) and Aβ-mediated inhibition of LTP (Jo et al., 2011) without affecting baseline AMPAR-mediated transmission. Although the effects on mEPSC amplitude where small (15–18%) they should have been detected as a small reduction in baseline transmission, but this was never observed. A potential explanation for these differences is the levels of activity of GSK-3β in the two preparations. As described above, GSK-3β is tightly regulated by inhibition on Ser9 by a variety of signaling pathways and is actively suppressed by LTP (via the PI3K/Akt pathway). Loss of any of these regulatory systems might result in a higher constitutive activity of GSK-3 in cultured dissociated neurons. It, therefore, remains to be determined whether endocytosis of AMPARs during LTD involves GSK-3β-dependent phosphorylation of GDI and activation of Rab5.

We have observed a complex formation between GSK-3β and AMPARs and found that the activity of GSK-3β within this complex is regulated by LTP (Peineau et al., 2007). These results strongly suggest that GSK-3β may regulate AMPAR function or trafficking, thereby affecting the expression of LTD. Consistent with this conjecture, a recent study (Du et al., 2010) has reported that GSK-3β inhibition prevented the NMDA-induced AMPAR endocytosis in a model of chemical LTD (Collingridge et al., 2010). How GSK-3β is involved in AMPAR endocytosis during LTD expression remains to be established. Given that a serine phosphorylation of the GluA2 subunit has been reported to be critical for receptor endocytosis during LTD (Kim et al., 2001a; Chung et al., 2003), GSK-3β may play an important role in mediating the expression of LTD by triggering the GluA2 phosphorylation dependent process. However, *in vitro* phosphorylation assays have failed to demonstrate an ability of GSK-3β to phosphorylate the carboxyl tail of GluA2 (authors' unpublished observation), suggesting that GSK-3β may phosphorylate one or more other proteins that are required for the regulated AMPAR endocytosis.

It has been proposed that one such potential protein is kinesin light chain 2 (KLC2; Du et al., 2010), a key molecule of the kinesin cargo delivery system, which may be involved in the transport of AMPAR-containing membrane-bound vesicles in neurons (Setou et al., 2002) and has previously been demonstrated as a target of neuronal GSK-3 (Morfini et al., 2002). In this study, Du et al. (2010) have shown that GSK-3β phosphorylation of KLC2 promotes its dissociation from the AMPAR/KLC2 protein complex (**Figure 2C**). This suggests that GSK-3 phosphorylation of KLC2 may lead to the dissociation of AMPAR-containing vesicles from the kinesin cargo system, thereby affecting the trafficking of AMPARs in neurons. To directly demonstrate that phosphorylation of KLC2 is one of the critical steps in GSK-3β promoted LTD, the authors developed a membrane permeable

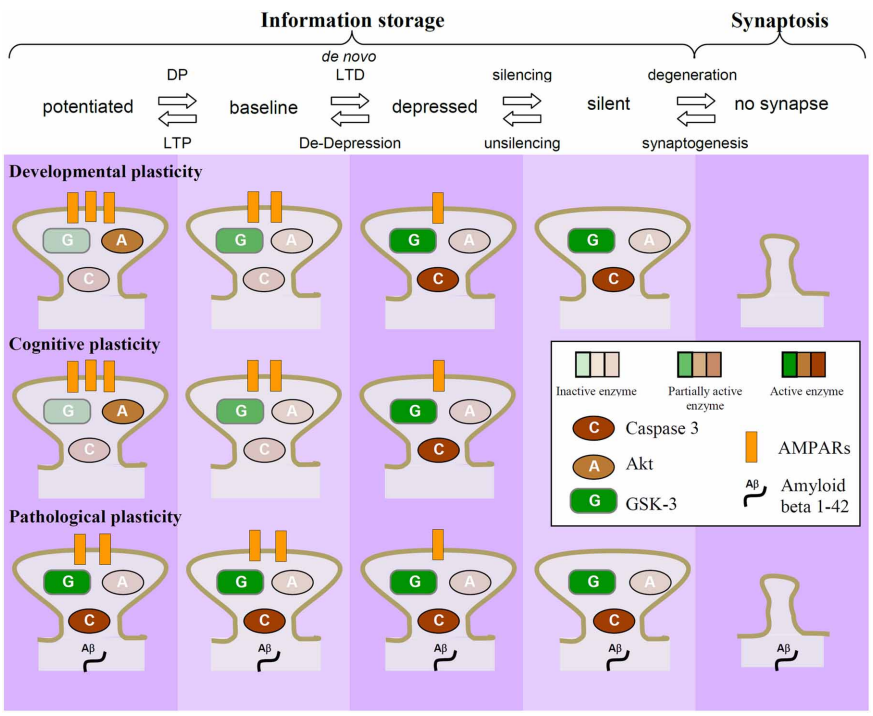

FIGURE 4 | Dysregulation of GSK-3 as a potential causal factor in neurodegeneration. NMDAR-LTD results in a reduction in the number of AMPARs at synapses. It can lead to the removal of all AMPARs (termed "silencing") and synapse elimination, via the process of synaptosis (localized apoptosis). This process is likely to be prominent early in development, where it may be important for the pruning of unwanted synaptic connections. During adulthood it may be more tightly regulated so that LTD involves mainly reductions in synaptic efficacy rather than the elimination of synapses. In particular, synapses that experience regular LTP will have the activity of GSK-3 dampened, by virtue of the PI3K-Akt pathway. In response to stressors (genetic, epigenetic, or environmental) the same mechanisms may be rekindled, resulting in unwanted synaptic elimination. In this example, the dysregulation of GSK-3β activity is due to activation of caspases leading to cleavage of Akt.

peptide TAT-KLCpCDK, a specific inhibitor for KLC2 phosphorylation by GSK-3β, and showed that by preventing GSK-3β phosphorylation of KLC2, it inhibited both AMPAR endocytosis and the expression of hippocampal CA1 LTD. Thus, this study has provided strong evidence that KLC2 may be a cellular target of GSK-3β capable of regulating AMPA receptor trafficking and hence synaptic plasticity. Of course, GSK-3β may also affect other components of the AMPAR endocytosis pathway. In addition to AMPAR endocytosis, GSK-3β may influence LTD expression by regulating other processes, some of which are illustrated in **Figure 2C**. Other potential targets include tau and presenilin-1, as well as transcription factors that may be involved in the protein synthesis dependent phase of NMDAR-LTD (Manahan-Vaughan et al., 2000).

ROLE IN PATHOLOGICAL SYNAPTIC PLASTICITY

A large body of evidence suggests that changes in synaptic plasticity may be responsible for the cognitive deficits that are observed in various brain disorders (Bowers et al., 2010; Goto et al., 2010; Kasai et al., 2010; Russo et al., 2010; van Spronsen and Hoogenraad, 2010; Proctor et al., 2011; Quartarone and Pisani, 2011; Zhuo et al., 2011). Increasing evidence suggests, additionally, that alterations in various molecular components of synaptic plasticity pathways may actually be the cause of many of these disorders (see review by Collingridge et al., 2010). We term this process pathological plasticity, to distinguish it from the forms of plasticity that are utilized by the CNS during the formation and stabilization of synaptic connections (developmental plasticity) and for learning and memory (cognitive plasticity; **Figure 4**). This basic premise is that NMDAR-LTD is a mechanism that is used by the CNS to remove AMPARs from synapses and thereby weaken the efficiency of synaptic transmission. This enables the storage of information, and indeed there is growing evidence that NMDAR-LTD is involved in forms of learning and memory (see review by Collingridge et al., 2010). NMDAR-LTD can involve the removal of all AMPARs from individual synapses (Luthi et al., 1999), via a process known as silencing. These silent synapses can then either (1) have new AMPARs inserted via the process of unsilencing (Isaac et al., 1995; Liao et al., 1995) or (2) are eliminated (Bastrikova et al., 2008; Kano et al., 2008; Egashira et al., 2010). Since NMDAR-LTD is particularly prevalent early in development (Dudek and Bear, 1993) when there is considerable pruning of superfluous synaptic connections (Rabacchi et al., 1992; Bhatt et al., 2009), it is likely that NMDAR-LTD is a physiological mechanism used to eliminate synapses during development.

With respect to a molecular mechanism, we previously showed that NMDAR triggered apoptosis, and the associated increase in caspase-3 activity, requires AMPAR endocytosis (Wang et al., 2004). More recently we found that NMDAR-LTD also involves activation of a caspase cascade (Li et al., 2010). These studies suggest that the internalization of AMPARs associated with LTD triggers a local apoptosis. We will refer to this process as *synaptosis*, a term originally coined for the compartmentalized retraction of synapses at the neuromuscular junction (Gillingwater and Ribchester, 2001). After the period of synaptic pruning during development is over and the organization of the brain is largely complete then it makes sense that the mechanisms of synaptosis are down-regulated. We, therefore, assume that for most of the normal life-span NMDAR-LTD is used more sparingly and for mainly adjusting synaptic weights rather than for eliminating synapses because it would be an energetically more efficient way to store information. However, if these latent synaptosis mechanisms were once again awakened then synapses could be eliminated. Our hypothesis is that neurodegeneration is often triggered by just this reactivation of synaptosis, which if left unchecked leads to apoptosis of vulnerable neuronal populations. The idea that the weakening of connections between nerve cells may explain dementia has been around for over 100 years. However, the observation that GSK-3β regulates the balance between LTP and LTD provides the basis of a molecular mechanism that gives credence to this idea.

In terms of a molecular mechanism, we suggest that GSK-3β is activated to enable NMDAR-LTD and that this mechanism is especially prominent early in development for synaptic pruning. In adulthood, whilst this mechanism may still occur it is utilized less often so as to preserve synaptic connectivity. The ability of NMDAR-LTP to inhibit NMDAR-LTD via GSK-3β activity provides a potential mechanism to enable this regulation. Thus, as long as synapses are engaging LTP processes then they are protected from NMDAR-LTD by the PI3K/Akt regulatory pathway. In other words, it is a classic case of "use it or lose it" where this translates into "use LTP or lose the synapse" (because it is cognitively unimportant).

Pathological plasticity could then result from any one of a number of influences, genetic, epigenetic, or environmental, that affect the LTD process directly or its regulation by LTP so that the LTD mechanism becomes more actively re-engaged. As a formal test of this hypothesis, we used the acute application of $A\beta_{1-42}$. This treatment is well known to cause a synaptic toxicity, which is characterized by an inhibition of NMDAR-LTP and facilitation of NMDAR-LTD (Cullen et al., 1997; Chen et al., 2000; Kim et al., 2001b; Li et al., 2009; Shankar et al., 2008; Witton et al., 2010). We studied the former, and tested the ability of a GSK-3β inhibitor to prevent the effect (Jo et al., 2011). What we found was that this inhibitor protected synapses from the deleterious effects of Aβ, such that LTP was no longer affected. Similar observations were made in parallel by another group using a different GSK-3 inhibitor (Shipton et al., 2011). Thus, it seems that over-activation of GSK-3β is indeed responsible for this pathological effect on plasticity.

Remarkably, in our study, the GSK-3β inhibitor was still fully effective if applied after the Aβ peptide had exerted its effects to inhibit LTP. This experiment is illustrated in **Figure 5**. The implications of this finding are that the Aβ-mediated inhibition of LTP is due, at least for a while, to the increased activity of GSK-3β. It is possible that in AD a chronic activation of GSK-3β (Hooper et al., 2008) is responsible for the synaptosis and consequently that GSK-3 inhibitors will reverse the cognitive decline. Of course, GSK-3 inhibitors will have many side-effects relating to the ubiquitous actions of this kinase throughout the body (see other chapters in this volume). But understanding the full cascades involved in the pathological activation of GSK-3β in synaptosis offers the possibility of finding more specific targets.

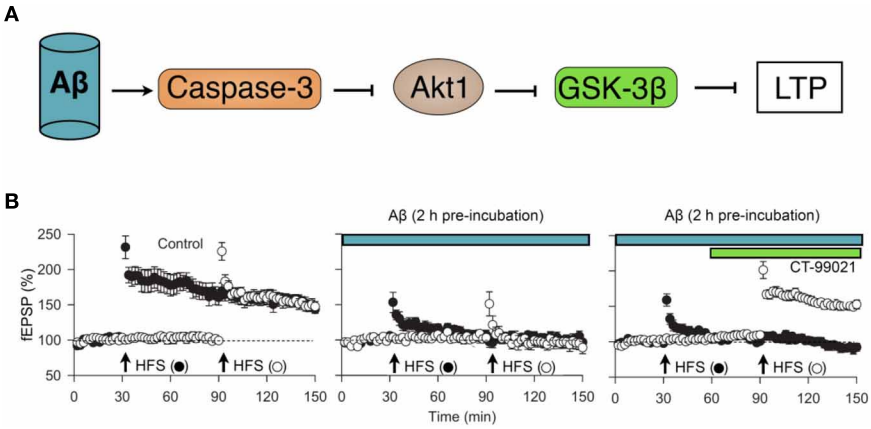

FIGURE 5 | The CAG cascade. (A) potential mechanism by which various neuronal insults (exemplified here by Aβ1–42) may inhibit NMDAR-LTD via the activation of caspase-3 (C) which, by resulting in cleavage of Akt (A), results in persistent activation of GSK-3β (G). **(B)** Experimental data in support of the "CAG" cascade hypothesis. Pooled data (from left to right) showing LTP in two inputs, in response to high frequency stimulation (HFS), blockade of LTP in both inputs by pre-incubation with Aβ1–42 (500 nM, 2 h) and recovery of LTP in input two following treatment with the GSK-3 inhibitor CT-99021 (1 μM). Data re-plotted from Jo et al. (2011).

In terms of a molecular mechanism, we found that inhibition of caspase-3 and the prevention of the caspase-mediated cleavage of Akt was also able to completely prevent the Aβ-mediated inhibition of LTP. Thus, it seems that Aβ activates caspase-3, which cleaves Akt to persistently activate GSK-3β and thereby trigger synaptosis; we have termed this the CAG cascade (**Figure 5**). What is not known is how Aβ activates casapse-3 to initiate the process, though an action via mitochondria seems likely. Also the downstream effectors of GSK-3β pathological plasticity are not established. A role for tau is likely given that the Aβ inhibition of LTP is prevented in the tau knockout (Shipton et al., 2011) and since, more generally, tau is a likely effector of GSK-3β regulated toxicity in AD (Morris et al., 2011).

ROLE IN SYNAPTIC PLASTICITY AT GABAergic SYNAPSES

In addition to playing an important role in synaptic plasticity at glutamatergic synapses, recent evidence also suggests a role for GSK-3β in plasticity at GABAergic synapses in the CNS.

GABAergic plasticity has been demonstrated in many brain regions (for reviews see Gaiarsa et al., 2002; Mendez and Bacci, 2011). Some forms of GABAergic plasticity are dependent on Ca^{2+} entry via stimulation of NMDARs (Wang et al., 2003a; Marsden et al., 2007, 2010; Muir et al., 2010) and alterations in the post-synaptic GABAR distribution (for review see Luscher et al., 2011). Indeed, activation of calcineurin, which forms a complex with the intracellular loop of the γ2 subunit of GABARs, may be a critical step in the induction of GABAergic LTD (Wang et al., 2003a). Recent work has since demonstrated that activity causes rapid dispersion of GABAR clusters (Bannai et al., 2009) and is dependent on Ca^{2+} entry and Ser327 of the γ2 subunit (Muir et al., 2010). We have also previously shown that insulin, via the PI3 kinase-Akt pathway, causes a rapid increase of GABAergic synaptic transmission and concomitant LTD at glutamatergic synapses (Wan et al., 1997; Man et al., 2000; Wang et al., 2003b). Though GSK-3β stimulation via the insulin pathway has not yet been demonstrated in neurons, it would not be surprising to see that GSK-3 may regulate GABAergic transmission under these conditions as well.

Indeed, modulation of GABAergic function, via alteration of the scaffolding molecule gephyrin, has recently been demonstrated under conditions of chronic treatment with GSK-3β inhibitors (Tyagarajan et al., 2011). In this study the authors identified Ser270 on the inhibitory scaffolding protein gephyrin as a unique phosphorylation site targeted by GSK-3β. Mutation of this site to an alanine residue increased both the number of post-synaptic gephyrin clusters and the frequency of mIPSCs. The pharmacological inhibition of GSK-3β similarly induced greater post-synaptic clustering of gephyrin. Mechanistically, it appears that GSK-3β phosphorylation of Ser270 may cause gephyrin to be targeted for proteolytic degradation by the Ca^{2+}-dependent protease calpain-1, and thus reduce gephyrin clusters and decrease GABAergic transmission.

However, in this study it was not clear what the implications are to GABARs on the surface of the cell as the authors did not extend their findings to look at localization of GABARs. Thus far, α1, α2, and α3 subunits of the GABARs have been demonstrated to interact with gephyrin (Mukherjee et al., 2011; Tretter et al., 2008, 2011 respectively). Gephyrin appears to have different affinities for all these receptors (Maric et al., 2011), and strongly interacts with glycine receptors (Pfeiffer et al., 1982). Thus, each receptor type may be affected differentially by GSK-3β.

Wei et al. (2010) present biochemical data that β2/3 containing GABARs were increased on the cell surface within 10 min of GSK-3β inhibition, suggesting that GABAergic transmission may be rapidly increased by the actions of this kinase. Because the previous study used long treatments with GSK-3β inhibitors more work is required to establish how rapid down-regulation or up-regulation of GSK-3β affects gephyrin clustering and GABAergic transmission.

GSK-3β, LTD, AND THE IMPLICATIONS FOR LEARNING AND MEMORY

Since dysregulation of GSK-3 activity has been strongly implicated in a number of mood-related disorders (Lovestone et al., 2007; Du et al., 2010 and reviewed in this Special Topic by Jope, 2011) as well as AD (Giese, 2009 and reviewed in this Special Topic by Kremer et al., 2011), understanding the effects of GSK-3β's involvement in normal learning and memory, and the importance of the LTD and LTP interaction in this context, is of great interest. Spatial memory impairments on the Morris water maze task have been demonstrated in transgenic mice with a conditional over-expression of GSK-3β (Hernandez et al., 2002). In another study by Engel et al. (2006), these same mice showed significant deficits in the object recognition task, which were completely reversed when GSK-3β over-expression was restored to normal levels (Engel et al., 2006).

More recently, Kimura and colleagues sought to elucidate the role of GSK-3 by examining hypogene function on memory formation and maintenance (Kimura et al., 2008). In adult mice heterozygous for GSK-3β expression (GSK-$3\beta^{+/-}$), the animals learned the Morris water maze task at a similar rate as wild type mice, but retrograde amnesia began to affect learning of the task in days 3–9 suggesting that long-term memory formations were adversely affected. Further testing of these heterozygous GSK-3β knock-out mice, using the contextual fear conditioning (CFC) paradigm, suggested that hypo-expression of GSK-3β affected reconsolidation of memory specifically. Inhibition of GSK-3β with the inhibitor AR-A014418 before consolidation or before reconsolidation in CFC showed severely disrupted reconsolidation. They further demonstrated with western blotting that memory reconsolidation is associated with activation of synaptosomal GSK-3β in wild type hippocampal neurons and that intermediate levels of GSK-3β activity were observed upon learning or in the consolidation state relative to naive animals. Though direct evidence is still required, because of the role of GSK-3β activity in LTD mechanisms, these experiments may indicate a greater importance of an LTD dependent cascade in reconsolidation memory.

Periods of sleep may also enhance consolidation of certain types of learning and memory (for reviews see Born et al., 2006; Ribeiro, 2011). Interestingly, in a study on sleep and awake states of plasticity, alterations in molecular, and electrophysiological LTD correlates have also been found. The sleep state (relative to awake state) was associated with low Ser9 phosphorylation of GSK-3β, and hence greater activity and low GluA1 levels as well as a long lasting depression of responses to evoked stimulation (Vyazovskiy et al., 2008). This suggests that during sleep there is an increase in LTD mechanisms relative to LTP mechanisms and that this could potentially underlie the enhancement of memory formation through improved consolidation.

CONCLUSIONS

The last few years have seen GSK-3 firmly established as a key player in synaptic plasticity. In particular, it has been shown to be involved in NMDAR-LTD and to be regulated during NMDAR-LTP. We have developed the hypothesis that the ability of LTP to inhibit LTD, via the PI3K/Akt/GSK-3β pathway, serves to stabilize synapses, which would otherwise be lost through the process of synaptosis. We argue that synapses live in a precarious state whereby if they are not protected by LTP then they die at the hands of LTD. This process may serve key physiological functions particularly during development, where synaptic pruning of unwanted connections is of paramount importance, but also during adulthood. However, we postulate that pathological alterations in this pathway, leading to excessive GSK-3β activity, may actually be a causal factor in some forms of neurodegeneration. The understanding of how GSK-3 is involved in synaptic plasticity is in its infancy. Clearly, it will be important to establish a more complete picture of how GSK-3β contributes to synaptic plasticity in both health and disease.

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GSK-3β and memory formation

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In Alzheimer's disease (AD), tau hyperphosphorylation and neurofibrillary tangle (NFT) formation are strongly associated with dementia, a characteristic and early feature of this disease. Glycogen synthase kinase 3β (GSK-3β) is a pivotal kinase in both the normal and pathological phosphorylation of tau. In the diseased state, hyperphosphorylated tau is deposited in NFTs, the formation of which, drive the disease process. GSK-3β which is also involved in long-term depression induction, interacts with tau to inhibit synaptic long-term potentiation. Strong lines of evidence suggest that the activation of GSK-3β is responsible for the memory deficits seen in both advanced age and AD. In this review, we will focus on the role of GSK-3β in brain function, particularly in memory maintenance. We will examine human and mouse studies which suggest a role for GSK-3β in memory maintenance and the eventual development of memory deficits.

Keywords: Alzheimer's disease, aging, memory formation, memory impairment, tau

ALZHEIMER'S DISEASE AND MEMORY

Memory impairment in old age is a hallmark of the initial stage of Alzheimer's disease (AD), with dementia developing in the final stages (Poissonnet et al., 2012). AD is characterized by the extensive deposition of amyloid β (Aβ), outside of neurons, and the formation of neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau, as intraneuronal inclusions (Selkoe, 1986). The relationship between the clinical course of AD and the observed pathological changes is not yet fully understood. Genetic studies of familial AD identified three causative genes, *APP*, *PSEN 1*, and *PSEN 2* (Tandon et al., 2000). Since these genes form part of a cascade that results in Aβ generation, the Aβ hypothesis emerged as a mechanism for AD pathophysiology (Hardy and Selkoe, 2002). This theory states that Aβ deposition directly affects neurons, inducing NFTs and neuronal death, leading to dementia. Inheritance of the APP mutation leads to AD with 100% penetrance (Goate and Hardy, 2011). Mice engineered to overexpress mutant human APP, show memory impairment along with Aβ deposition (Gotz and Ittner, 2008), supporting the Aβ hypothesis. Electrophysiological analyses indicate an inverse correlation between Aβ levels and the amplitude of hippocampal long-term potentiation (LTP; Walsh et al., 2002; Westerman et al., 2002), an underlying mechanism of memory. A recent study found that reducing tau alleviated Aβ-induced memory impairment in APP transgenic (Tg) mice (Roberson et al., 2007), suggesting that tau contributes to memory impairment in APP Tg mice. However, contrary to these results, recent clinical trials show that reducing Aβ generation, or removing Aβ deposits fail to halt the progression of dementia (Holmes et al., 2008).

NFT FORMATION PROMOTES MEMORY IMPAIRMENT AND DEMENTIA

The number of NFTs, unlike the extent of Aβ deposition, correlates strongly with the degree of dementia (Gomez-Isla et al., 1997). In diseased brains, synaptic and neuronal loss are prominent in regions with detectable NFTs, implicating NFT formation in AD associated memory impairment and dementia (Masliah et al., 1992). Based on the observations of Braak and Braak (1990), as AD progresses, NFTs are observed first in the entorhinal cortex, a region integral to memory formation and maintenance, later spreading into the limbic cortex and neocortex, regions associated with emotions, and higher functioning such as thought, respectively. Considering the role of these regions in normal brain function, this sequential formation of NFTs could go some way to explaining the clinical progression of AD. Before NFT formation, tau is hyperphosphorylated by glycogen synthase kinase 3β (GSK-3β) activation and forms granular tau oligomers. This hyperphosphorylated tau is associated with synapse loss (Kimura et al., 2007), while granular tau oligomers are involved in neuronal death. These data imply that the neuronal dysfunction resulting from synaptic and neuronal loss (Kimura et al., 2010), occurs when NFTs are formed.

NFT FORMATION PROMOTES NEURONAL DYSFUNCTION

Mice that overexpress P301L, a mutant form of tau, display age-related NFTs, neuronal death, and memory deficits (Ramsden et al., 2005; Santacruz et al., 2005). Although inhibiting mutant tau overexpression in these mice blocks neuronal death and improves memory, NFTs continue to form (Ramsden et al., 2005; Spires et al., 2006). This suggests that NFTs in themselves are not toxic, but instead, the processes of NFT formation, neuronal death and neuronal dysfunction underly the pathogenic mechanism.

The formation of tau fibrils follows three sequential steps (Maeda et al., 2007; Kimura et al., 2008; Takashima, 2008), and has been studied using atomic force microscopy (AFM). AFM allows direct observation of tau aggregation in experimental solutions, with no special pretreatments, in contrast to scanning electron microscopy which requires several pretreatment steps.

First, hyperphosphorylated monomeric tau binds together to form soluble oligomers. The structure of these oligomers however, is not discernible under AFM. Second, the soluble tau oligomers take on a β-sheet structure, forming insoluble tau aggregates. These aggregates become granular-shaped oligomers consisting of approximately 40 tau molecules, which are detectable under AFM. Third and finally, the increased concentration of granular tau causes these oligomers to fuse, forming tau fibrils (Maeda et al., 2007).

As a major tau kinase, GSK-3β induces tau hyperphosphorylation, as one of the earliest events in NFT formation (Ishiguro et al., 1988, 1993). Hyperphosphorylated tau or soluble tau oligomers are associated with loss of synapses in wild type tau Tg mice (Kimura et al., 2007), while granular tau oligomers are associated with loss of neurons in P301L tau Tg mice (Kimura et al., 2010). Thus, the intermediary, soluble and granular tau oligomers can promote synaptic and neuronal loss before NFT formation. This suggests that rather than being the cause of cell death, NFTs represent a biological tombstone, marking the sites of neuron death. Therefore, memory impairment probably occurs when NFTs are seen in the entorhinal cortex and hippocampus, since synaptic and neuronal loss occur before the formation of NFTs in these regions.

TAU PHOSPHORYLATION BY GSK-3β

Tau protein kinase I (TPKI; Ishiguro et al., 1988), is encoded by a nucleotide sequence identical to that of GSK-3β (Ishiguro et al., 1993), but not GSK-3α. This kinase is activated by aggregated Aβ and induces tau hyperphosphorylation as seen in NFTs and neuron death, in hippocampal cultures (Takashima et al., 1993, 1996). Phosphorylation of the tau Ser422 residue, a site not phosphorylated by GSK-3β, is specifically seen in NFTs (Morishima-Kawashima et al., 1995) indicating the involvement of additional kinases in this process. While the Ser422 residue can be phosphorylated by c-Jun amino-terminal kinase (JNK), this is not enough to promote tau aggregation. In cultured cells at least, both JNK and GSK-3β activation are needed to generate tau aggregation (Sato et al., 2002). These results point to GSK-3β activation as a requirement for AD pathogenesis.

Mice overexpressing GSK-3β show an accumulation of hyperphosphorylated tau, neuronal death in the hippocampus, and memory impairment in object recognition tests (Lucas et al., 2001; Hernandez et al., 2002). These mice also exhibit reduced hippocampal LTP (Hooper et al., 2007), and this memory deficit is reversed when tau expression stops (de Barreda et al., 2010). Reducing tau levels (Roberson et al., 2007) and inhibiting GSK-3 (Sereno et al., 2009) can each rescue memory impairment in APP Tg mice. Aβ activates GSK-3β, inducing tau hyperphosphorylation in hippocampal neurons, and it is this GSK-3β activation that leads to reduced LTP and eventual memory impairment in APP Tg mice. Again, evidence shows that activation of GSK-3β is a key factor in AD associated memory impairment, promoting the idea that inhibitors of GSK-3β, may be potential therapeutic agents for this disease.

GSK-3 INHIBITORS

Peineau et al. (2007) showed that GSK-3β localizes to postsynaptic regions and that GSK-3 inhibitors block NMDA-dependent long-term depression (LTD) induction. Our own data (unpublished) shows a blockade of LTD induction in GSK-3β heterozygote knockout mice. Although several companies have developed GSK-3 inhibitors, there are currently no successful candidates in Phase III trials. Lithium, a longstanding therapeutic drug used in bipolar disorder (Gould et al., 2006), is a specific inhibitor for GSK-3 (Klein and Melton, 1996). Lithium inhibits GSK-3 directly by competing with magnesium binding sites. It also acts indirectly, by enhancing serine phosphorylation of GSK-3, as well as through β-arrestin complex formation (reviewed in this Research Topic series: Eldar-Finkelman and Martinez, 2011; Freland and Beaulieu, 2012). Lithium treatment inhibits tau hyperphosphorylation, and NFT formation (Engel et al., 2006; Leroy et al., 2010), alleviating memory deficits not only in mice overexpressing tau, but also mice expressing both APP and PS1 (Zhang et al., 2011). Therefore hypothetically, lithium inhibition of GSK-3β should halt the clinical progression of AD in humans. While short-term lithium treatment failed to improve cognitive function, a biomarker for AD (Hampel et al., 2009), long-term treatment significantly reduced phosphorylated tau levels in cerebrospinal fluid, a potential biomarker for AD, and improved cognitive function (Forlenza et al., 2011). Interestingly, a retrospective study of bipolar and unipolar-depression patients with a history of lithium treatment, found that these patients had a higher risk of developing dementia (Dunn et al., 2005). It therefore appears that GSK-3 performs a dual role. In patients without dementia, GSK-3 activity maintains cognitive function, whereas patients with dementia show excessive activation of GSK-3.

THE ROLE OF GSK-3β IN SYNAPTIC PLASTICITY

GSK-3 exists as two isoforms, α and β, which share high sequence identity and are encoded by genes on chromosomes 19 and 3 respectively, in humans (Woodgett, 1990). GSK-3β and GSK-3α localize to different compartments. GSK-3β, but not GSK-3α localizes to the mitochondria and synaptosomes (Hoshi et al., 1995). Therefore it is likely that GSK-3β may be directly involved in synaptic plasticity, while GSK-3α may act indirectly, via the regulation of gene expression (more details in this Research Topics series, as reviewed by Beaulieu et al., 2011; Polter and Li, 2011). These isoforms share common substrates including tau, but they also have distinct functions. While knockout of GSK α in mice induces increased insulin sensitivity, knockout of GSK-3β in mice is embryonically lethal (Hoeflich et al., 2000; MacAulay et al., 2007).

GSK-3β is pivotal in the cascade leading to NFT formation, which in turn drives dementia in AD. GSK-3β could be seen as a time-delayed ignition switch in the brain, which in old age triggers the process of dementia. As mentioned previously, patients with a long history of lithium therapy, and consequently suppressed levels of GSK-3, show a higher risk for developing dementia compared with lithium naïve patients (Dunn et al., 2005). These observations imply that controlled levels of GSK-3 activity are required for maintaining normal brain function, and as we already know, excessive activation of GSK-3β, drives NFT formation, leading to disease. Unraveling the dual role of GSK-3β requires an understanding of the physiological function of this protein in healthy adult brains and how this changes with aging. As we know, GSK-3β

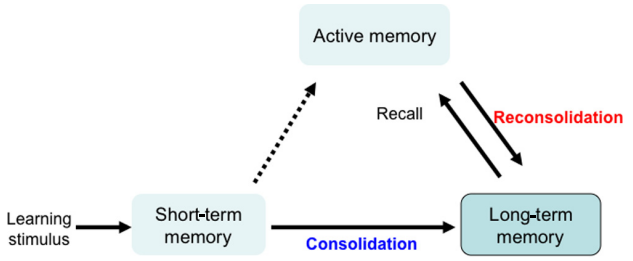

FIGURE 1 | Mechanism for memory formation and maintenance. Learning stimulation leads to short-term memory, which lasts a few hours and is converted to long-term memory through a process of consolidation. Active memory is formed by recalling and updating long-term memory. This updated memory becomes long-term memory through a process of memory reconsolidation. Reconsolidation is a protein synthesis-dependent process that is required for updating the reactivated memory and for maintaining long-term memory.

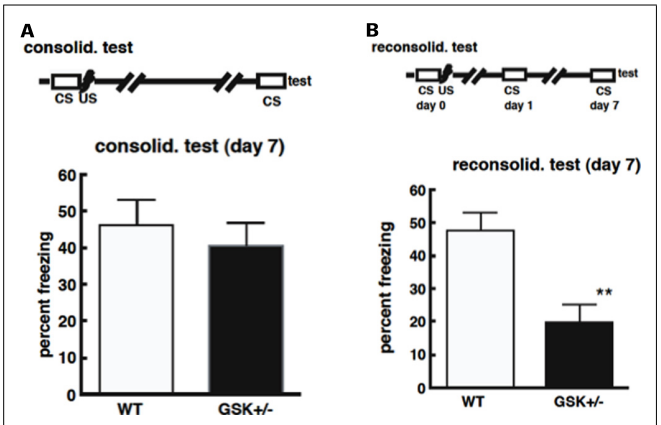

FIGURE 2 | (A) Deficiencies in GSK-3β do not affect the process of memory consolidation. Mice were placed in a novel environment for 5 min (CS) and then subjected to three sequential foot-shocks (US). On day 7 after conditioning, animals were placed in the original environment, without shocks, for 5 min. The freezing time was recorded for these animals. **(B)** Deficiencies in GSK-3β impair memory reconsolidation. Mice were placed in the environmental for 5 min (CS), and then subjected to three sequential foot-shocks (US). One day after conditioning, animals were exposed to the same environment for 5 min. Six days after their first exposure to conditioning, animals were re-exposed again and the freezing time was recorded.

is required for NMDA-dependent LTD induction (Peineau et al., 2007). It is this requirement for GSK-3β in synaptic plasticity that fuels the analysis of GSK-3β in memory formation.

GSK-3β ACTIVATION IS REQUIRED FOR MEMORY RECONSOLIDATION

Learning stimuli first lead to short-term memory formation, which lasts a few hours and is then converted to long-term memory, through a process of memory consolidation. Active memory is formed by recalling and updating long-term memory. This updated memory becomes long-term memory through a process of memory reconsolidation. Reconsolidation is required for updating reactivated memory, and maintaining long-term memory (**Figure 1**). Although memory consolidation and reconsolidation are thought to have distinct molecular pathways, both are protein synthesis-dependent (Nader et al., 2000; Riccio et al., 2002; Eisenberg et al., 2003; Biedenkapp and Rudy, 2004; Dudai and Eisenberg, 2004; Lee et al., 2006; Morris et al., 2006). We used GSK-3β heterozygous knockout mice ($^{+/-}$) to understand how GSK-3β fits into these pathways. While the homozygous GSK-3β mutation is embryonically lethal, heterozygous mice express GSK-3β at approximately 50%, and a relative activity was about 70% of wild type mice (Kimura et al., 2008). For GSK-3α, the paralog of GSK-3β, the total amount and relative activity of GSK-3α did not differ between GSK-3β$^{+/-}$ and wild type mice. As previously reported (Hoeflich et al., 2000), GSK-3β$^{+/-}$ mice are healthy and fertile, with normal circadian rhythms, life span, and locomotor activity, compared to wild type mice.

In the contextual fear conditioning paradigm, GSK-3β$^{+/-}$ mice showed similar freezing times in response to unconditioned stimuli as wild type mice, and there was no difference in the freeze times between GSK-3β$^{+/-}$ and WT mice in the consolidation test (**Figure 2A**; Kimura et al., 2008). This suggests no impairment in the ability of GSK-3β$^{+/-}$ mice to form and consolidate memories, and that these memories can be maintained for at least 7 days, the time period examined in this study (Kimura et al., 2008). In reconsolidation however, GSK-3β$^{+/-}$ mice, showed significantly less freeze time compared with wild type mice, at day 7 (**Figure 2B**; Kimura et al., 2008). These results indicate that GSK-3β heterozygotes are capable of learning and stabilizing long-term memory for 7 days, if memory is not reactivated. However, GSK-3β$^{+/-}$ mice failed to achieve reconsolidation when memory was reactivated once before testing (Kimura et al., 2008). The retrograde amnesia exhibited by these mice in the reconsolidation test of contextual fear conditioning, points to possible impaired memory reconsolidation, in keeping with the theory that GSK-3β activation is required for memory reconsolidation or maintenance.

PROSPECTIVE ROLE OF GSK-3β IN BRAIN AGING

GSK-3β is involved in NMDA-dependent LTD induction and memory reconsolidation (Peineau et al., 2007; also reviewed in this Research Topic series by Bradley et al., 2012). However, the relationship between LTD induction and memory reconsolidation is unclear, although there are reports that LTD is important for memory formation or new object recognition. Focusing on synaptic plasticity, particularly LTP, genetic ablation of the NMDA receptor impaired place learning in a LTD dependent manner (Tsien et al., 1996). Further analysis using CaMKIV knockout mice indicates that late LTP is involved in the consolidation process of memory formation (Kang et al., 2001). Thus, both LTP and LTD contribute to memory formation, in which the memory consolidation processes may preferentially depend on LTP, and memory reconsolidation processes require LTD. In memory reconsolidation, LTD maintains a prior potentiated circuit by competitive synaptic maintenance (Diamond et al., 2005) and protects stable memory traces. This may explain why activation of GSK-3β is required in reconsolidation but not in consolidation processes in normal brain function. GSK-3β activation in the entorhinal cortex and hippocampus is required for spatial recognition, in aged but not young brains (unpublished result). While this process is required for maintaining normal brain function in old age, the

frequent activation of GSK-3β induces NFTs in the entorhinal cortex (Braak and Braak, 1996) and hippocampus. It would therefore appear that GSK-3β activation is an early event in normal brain aging as well as AD.

We put forward that, generally, as we age, we learn and accumulate many memories. When we are confronted with a new idea or task, we draw on our experiences, that is, we recall related memories to help us understand new information. The frequent need to recall and reconsolidate memories relies on increased activation of GSK-3β and consequently, tau phosphorylation. Over time, NFTs accumulate in the entorhinal cortex, which is a very early pathological change in sporadic AD.

CONCLUSION

Tau hyperphosphorylation and NFT formation are early features of dementia associated with AD. This major change in the phosphorylation state of tau leads to deposition of pathological tau in NFTs, and these tangles are formed in a specific spatial and temporal pattern within the brain. It is the formation rather than the presence of these NFTs that induces neuronal dysfunction and death, leading to tauopathies.

GSK-3β is a major kinase for tau phosphorylation associated with both physiological brain function and AD pathophysiology. GSK-3β is also required for synaptic plasticity. Reduced GSK-3 expression in GSK-3β$^{+/-}$ mice results in impaired memory reconsolidation emphasizing the importance of GSK-3 in promoting memory maintenance via reconsolidation. A greater understanding of how synaptic plasticity changes with aging, through the analysis of AD-related molecules such as GSK-3β and tau, would provide a solid platform of knowledge, from which new therapeutic targets and innovative agents could be developed for AD.

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Inhibition of GSK3 by lithium, from single molecules to signaling networks

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For more than 60 years, the mood stabilizer lithium has been used alone or in combination for the treatment of bipolar disorder, schizophrenia, depression, and other mental illnesses. Despite this long history, the molecular mechanisms trough which lithium regulates behavior are still poorly understood. Among several targets, lithium has been shown to directly inhibit glycogen synthase kinase 3 alpha and beta (GSK3α and GSK3β). However *in vivo*, lithium also inhibits GSK3 by regulating other mechanisms like the formation of a signaling complex comprised of beta-arrestin 2 (βArr2) and Akt. Here, we provide an overview of *in vivo* evidence supporting a role for inhibition of GSK3 in some behavioral effects of lithium. We also explore how regulation of GSK3 by lithium within a signaling network involving several molecular targets and cell surface receptors [e.g., G protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs)] may provide cues to its relative pharmacological selectivity and its effects on disease mechanisms. A better understanding of these intricate actions of lithium at a systems level may allow the rational development of better mood stabilizer drugs with enhanced selectivity, efficacy, and lesser side effects.

Keywords: lithium, glycogen synthase kinase 3, arrestin, Akt, mood stabilizer, bipolar disorder, pharmacology, protein-protein interactions

INTRODUCTION

Lithium is an alkali metal that is used medically under the form of a cationic salt Li^+ in association with carbonate (COO^-) or citrate. Lithium salts have been used medically since the late nineteenth century for instance as part of popular tonics such as the "Bib-Label Lithiated Lemon-Lime Soda" better known under the name of 7^{UP} (Williams and Harwood, 2005). The first publication on the use of lithium to treat acute mania was published by John Cade more than 60 years ago (Cade, 1949). However, it took 22 years for the U.S. food and Drug administration to approve lithium for the treatment of bipolar disorder. Over time, lithium has become a gold standard for the management of bipolar mood disorders and cyclothymia. Each of these mental illnesses affects 1–2% of the population and is characterized by alternating episodes of manic and depressive symptoms (Cade, 1949; Schou et al., 1954; Blanco et al., 2002). Lithium has been reported to reduce suicide rates and prevent manic episodes in individuals with bipolar disorder, major depression, or schizoaffective disorders (Bowden, 2000; Cipriani et al., 2005; Muller-Oerlinghausen et al., 2005; Tondo and Baldessarini, 2009). Lithium is also used as part of combination therapies for augmenting the effects of other psychoactive drugs such as antidepressants and antipsychotics affecting monoaminergic neurotransmission (De Montigny et al., 1981; Valenstein et al., 2006).

Despite important clinical applications, the molecular mechanisms by which lithium exerts its therapeutic effects in mental disorders are still not well understood (Phiel and Klein, 2001; Beaulieu and Caron, 2008). Over the years, lithium has been shown to inhibit various enzymes directly *in vitro* (**Figure 1**). These include inositol monophosphatases (IMPAs) (Berridge et al., 1989), bisphosphate 3′-nucleotidase (BPNT1) (Spiegelberg et al., 2005), cyclooxygenase (COX) (Rapoport and Bosetti, 2002) and isoforms of glycogen synthase kinase 3 (GSK3) (Klein and Melton, 1996; Stambolic et al., 1996; Phiel and Klein, 2001). Studies conducted using different cellular models or organisms (e.g., *Mus. musculus*, *Caenorhabditis elegans* or *Dictyostelium discoideum*) have documented the importance of these effects of lithium on different physiological or pathological processes including development and epilepsy (Klein and Melton, 1996; Williams et al., 2002; Shaldubina et al., 2006; Cryns et al., 2007, 2008). However, the relative involvement of these direct molecular targets of lithium in behavioral effects that would be clinically relevant to depression or mania has often not been demonstrated. Furthermore, it is also unclear how the action of lithium on ubiquitously expressed molecules that are mostly involved in the regulation of cell signaling and not to specific neurotransmitter systems can explain its relative selectivity as a therapeutic agent in psychiatry.

Here we provide an overview of several independent line of evidence suggesting that lithium can exert "anti-manic" and "antidepressant" like effects by affecting the activity of GSK3α and β both as a direct inhibitor and as a modulator of cell signaling pathways regulating the activity of these kinases. We also speculate on the possible importance of such intricate regulation mechanisms for lithium efficacy in several types of combination therapies and for its relative clinical selectivity.

THE REGULATION OF GSK3 SIGNALING BY AKT AND CELL SURFACE RECEPTORS

The GSK3 family of serine threonine kinases is composed of two isoenzymes/paralogous proteins GSK3α and GSK3β, which

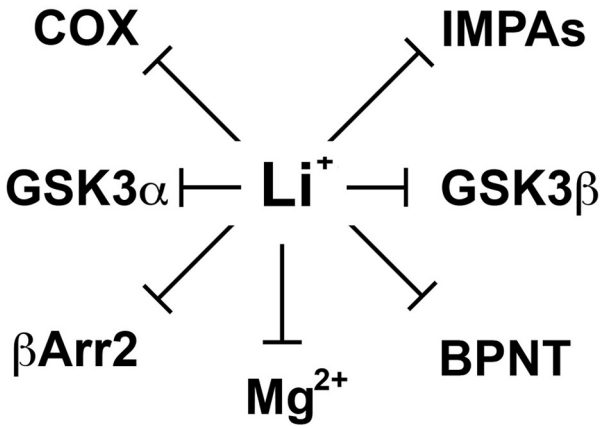

FIGURE 1 | Schematic representation of different direct molecular targets of lithium. These include inositol monophosphatases (IMPAs), Bisphosphate 3′-nucleotidase (BPNT), cyclooxygenase (COX), beta-arrestin 2 (βArr2), and members of the glycogen synthase kinase 3 alpha and beta (GSK3α and β). Since the mechanism by which lithium would inhibit several of these molecules is by competing with magnesium ions (Mg^{2+}) acting as a co-factor we also included the possibility that other molecular targets may be affected through such competition.

were originally identified for their role in insulin receptors signaling (Embi et al., 1980; Parker et al., 1983; Woodgett, 1990; Cross et al., 1995). Since then, the biological roles of these kinases have been demonstrated to be much broader, including implications in the regulation of development, immunity/inflammation, cancer, neurotransmission, and several other biological processes (Cohen and Frame, 2001; Woodgett, 2001; Kaidanovich-Beilin and Woodgett, 2011). GSK3 is constitutively active in cells under resting/unstimulated conditions (Sutherland et al., 1993; Stambolic and Woodgett, 1994) and is primarily regulated through inhibition of its activity either by phosphorylation or the regulation of its incorporation into protein complexes. For instance activation of phosphoinositide 3-kinase (PI3K) signaling regulates GSK3 by increasing its phosphorylation on negative regulatory serine residues (Sutherland et al., 1993; Stambolic and Woodgett, 1994; Sutherland and Cohen, 1994; Cross et al., 1995). In contrasts, activation of canonical Wnt signaling prevents the action of GSK3 on βcatenin by preventing the formation of a protein complex involving adenomatous polyposis coli (APC) and axin that targets active GSK3 to βcatenin (Behrens et al., 1998; Kishida et al., 1998).

The activity of GSK3α/β is positively regulated by phosphorylation on tyrosine residues (Thy 279 for GSK3α and Thy 216 for GSK3β) (Hughes et al., 1993; Lochhead et al., 2006) and negatively regulated by serine phosphorylation (Ser 21 for GSK3α and Ser 9 for GSK3β) that are situated in the N-terminal domains of these kinases (Sutherland et al., 1993; Stambolic and Woodgett, 1994; Sutherland and Cohen, 1994; Cross et al., 1995). While several serine/threonine kinases can phosphorylate these domains, the most prevalent negative regulators of GSK3 are members of a small family of kinases termed Akt. In mammals, this family is composed of three isoforms (Akt1, 2, and 3) that are themselves regulated by PI3K signaling that leads to an activatory phosphorylation of two residues (Alessi and Cohen, 1998). The Thr 308 residue of Akt1 is phosphorylated by phosphatidyl-dependant kinases, PDK1 and the Ser 473 residue is phosphorylated by the PDK2/rictor-mTOR, in response to PI3K signaling (Scheid and Woodgett, 2001; Jacinto et al., 2006). Of these two phosphorylation events, phosphorylation of Akt on Thr 308 has been shown to be essential and sufficient for the regulation of GSK3 by Akt (Jacinto et al., 2006). Over the years, myriads of cell surface receptors including several G protein coupled receptors (GPCRs) and most receptor tyrosine kinases (RTKs) have been shown to be involved in the activation of Akt isoforms via PI3K mediated signaling (Lemmon and Schlessinger, 2010; Liao and Hung, 2010; Beaulieu, 2011; Swift et al., 2011).

In addition to these kinase-mediated mechanisms, phosphatases are also playing a role in the regulation of Akt and GSK3 activity. For instance, the serine/threonine protein phosphatase 1 (PP1) is known to dephosphorylate the N-terminal serine residues of GSK3, therefore leading to its activation (Zhang et al., 2003). The protein phosphatase 2A (PP2A) has also been shown to participate in the inhibition of Akt (Beaulieu et al., 2005). Importantly, there is evidence for a regulation of Akt activity by PP2A in response to GPCR stimulation. Activation of the dopamine receptor 2 (D2R) has been shown to stimulate the inactivation of Akt by PP2A (Beaulieu et al., 2005; Beaulieu and Gainetdinov, 2011), therefore providing a mechanism through which GPCR activation can inhibit Akt in response to extracellular signals.

As a general mechanism, activation of GPCR leads to the activation of their cognate G proteins, which is rapidly followed by homologous receptor desensitization. This latter process involves the phosphorylation of the GPCR by GPCR kinases and the recruitment of the adaptor proteins β-arrestin 1 and β-arrestin 2 (βArr2) (Lohse et al., 1990; Ferguson et al., 1996). The recruitment of β-arrestins to GPCR leads to an uncoupling of the G protein and to clathrin mediated receptor internalization. However, β-arrestins can also participate to GPCR signaling by acting as molecular scaffolds for signaling molecules such as kinases and phosphatases (Luttrell et al., 1999; Luttrell and Gesty-Palmer, 2010). In the specific case of D2R, activation of the receptor by dopamine promotes the formation of a signaling complex composed of Akt, βArr2, and PP2A, which favor the dephosphorylation of Akt on Thr 308 and Akt inactivation. This in turn results in an activation of GSK3 by dopamine (Beaulieu et al., 2004, 2005). This mechanism of D2R signaling appears to play important roles in the regulation of locomotor behavior and sensory motor gating by dopamine (Beaulieu et al., 2004; Emamian et al., 2004). It could also contribute to the therapeutic and/or adverse effects of psychoactive drugs like amphetamines and antpsychotics that act on dopamine neurotransmission (Emamian et al., 2004; Masri et al., 2008; Beaulieu et al., 2009).

DIRECT AND INDIRECT REGULATION OF GSK3 BY LITHIUM

In 1996, two independent studies (Klein and Melton, 1996; Stambolic et al., 1996) of the effects of lithium on cell signaling and development have identified a direct effect of lithium on the activity of GSK3 both *in vitro* and in cells. However, the therapeutic relevance of this finding has been unclear mostly

because the high K_i values of lithium for GSK3α (∼3.5 mM) and GSK3β (∼2.0 mM) are superior to therapeutic lithium serum concentrations in humans (0.5–1.2 mM) (Phiel and Klein, 2001; Gould et al., 2004; Beaulieu and Caron, 2008; Beaulieu et al., 2008). However, these values can be affected by experimental conditions including the availability of Mg^{2+} ions in the assay system. Indeed, one hypothesis explaining the inhibition of GSK3 by lithium is that Li^{+} ions act as uncompetitive inhibitors for the binding of the co-factor magnesium to GSK3 (Ryves and Harwood, 2001). Magnesium and lithium share similar ionic radii (0.072 and 0.076 nm, respectively) and it has been suggested long ago (Birch, 1974) that this may explain several of the biochemical effects of lithium since Mg^{2+} is a cofactor for multiple enzymes (**Figure 1**). The proposed competitive mechanism of inhibition by lithium predicts that the *in vivo* inhibition of GSK3 may be greater than inhibition levels observed *in vitro* at optimal concentrations of magnesium ions (Ryves and Harwood, 2001).

In addition to direct inhibition, lithium can also affect both isoforms of GSK3 indirectly by activating Akt (**Figure 2**). This results in an increased phosphorylation/inactivation of these kinases by Akt in cultured neurons (Chalecka-Franaszek and Chuang, 1999). A similar activation of Akt by lithium has also been found in the striatum, frontal cortex, and hippocampus of rodents following either acute or chronic treatment with lithium under conditions resulting in lithium brain concentrations that are compatible with therapeutic serum concentrations in humans (De Sarno et al., 2002; Beaulieu et al., 2004).

The activation of Akt by lithium is explained in part by an effect of lithium on the regulation of Akt by βArr2 and the D2R. Co-immunoprecipitation studies conducted both *in vitro* and *in vivo*, have shown that the Akt:βArr2:PP2A signaling complex can be dissociated in response to therapeutically relevant lithium concentrations, thus providing a mechanism for the activation of Akt by lithium (Beaulieu et al., 2008). In support to this, both chronic and acute lithium treatment failed to activate Akt and increase GSK3 phosphorylation in mice lacking βArr2, in which the Akt:βArr2:PP2A signaling complex cannot be formed (Beaulieu et al., 2008). Furthermore, lithium also failed to regulate GSK3 phosphorylation in mice with reduced Akt1 activity (Pan et al., 2011). The exact detailed mechanism through which lithium interferes with the formation of the Akt;βArr2;PP2A signaling complex is only partially understood. *In vitro* experiments with recombinant purified Akt1 and βArr2 suggest that the interactions of these two proteins and thus complex formation, requires magnesium (Beaulieu et al., 2008). Therefore, competition between lithium and magnesium could underlie the instability of the Akt;βArr2;PP2A signaling complex in the presence of lithium (Birch, 1974; Beaulieu et al., 2008).

The direct inhibition of GSK3 by lithium also appears to contribute to the activation of Akt (**Figure 2**). Early experiments conducted in cells have suggested that GSK3 may promote its own activation by preventing the activation of Akt (Zhang et al., 2003). GSK3β has also been shown to interact with βArr2 (Beaulieu et al., 2005). Experiments conducted using transgenic mice overexpressing frog GSK3β in neurons, suggest the existence of a feed forward mechanism by which GSK3 promotes its own activation by stabilizing the Akt;βArr2;PP2A signaling complex (O'Brien et al., 2011). According to this model, direct inhibition of GSK3 would constitute an additional mechanism that can promote the disassembly of the Akt;βArr2;PP2A signaling complex in response to lithium. Interestingly, such feedback effects resulting from GSK3 inhibition may also contribute to strengthen the effects of lithium under other conditions. For instance there is evidence that GSK3 can regulate its own activation by phosphorylation of the Thy 279/Thy 216 residues (Lochhead et al., 2006). In addition, GSK3 can dampen PI3K signaling by destabilizing the insulin receptor substrate 1 (IRS1) protein that contributes to PI3K and Akt activation downstream of the insulin receptor (Eldar-Finkelman and Krebs, 1997; Liberman et al., 2008; Waraich et al., 2008). Therefore, it would be interesting to examine how such effects of GSK3 inhibition at the level of signaling networks can contribute to the amplification and maintenance of the effects of lithium in different cell types or organs.

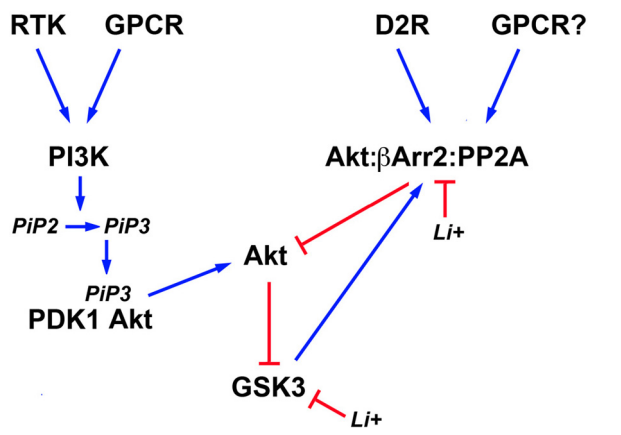

FIGURE 2 | Schematic representation of signaling pathways regulating the activity of brain GSK3 and its regulation by lithium. Activation of different cell surface receptors activates Phosphatidylinositol 3-kinases (PI3K) that in turn phosphorylates Phosphatidylinositol 4,5-bisphosphate (PIP2) into Phosphatidylinositol (3,4,5)-triphosphate (PIP3). Availability of PIP3 causes the co-recruitment of the 3-phosphoinositide dependent protein kinase-1 (PDK1) and Akt to the cell membrane and the activation of Akt by PDK1. Phosphorylation of N-terminal serine residues of glycogen synthase kinase 3 (GSK3) isoforms by activated Akt results in GSK3 inactivation. Conversely, activation of the D2 dopamine receptor (D2R) and potentially of other G protein coupled receptors (GPCR) triggers the formation of a signaling complex composed of Akt, beta-arrestin 2 (βArr2), and protein phosphatase 2A (PP2A) that results in an inactivation of Akt and concomitant relived GSK3 inhibition. Lithium can affect the equilibrium of this signaling network by inhibiting GSK3 directly and by disrupting the assembly of the Akt;βArr2;PP2A signaling complex. In addition, activated GSK3 would contribute to its own regulation by Akt by stabilizing the formation of this same protein complex.

EVIDENCE FOR A ROLE OF AKT AND GSK3 SIGNALING IN THE BEHAVIORAL EFFECTS OF LITHIUM

To our knowledge, there is no human study implicating GSK3 or any of the other potential molecular targets of lithium in its therapeutic effects. Furthermore, research has been hindered by a lack of a good animal model of bipolar disorder with construct validity. However, behavioral tests with predictive validity for antimanic or antidepressant drug effects have been used

extensively. Interestingly, inhibition of GSK3 activity seems to replicate both the "antidepressant" and "antimanic" like effects of these drugs in rat and mice, at least in some genetic backgrounds (Beaulieu et al., 2004, 2008; Kaidanovich-Beilin et al., 2004; O'Brien et al., 2004; Mines et al., 2010).

While reduction of GSK3 activity does replicate several behavioral effects of lithium it remains improbable that direct inhibition of GSK3 by lithium solely explains its effects on behavior. One major reason for this is that lithium concentrations in excess of therapeutic concentrations are often necessary to inhibit GSK3 in cell based assays (Kremer et al., 2011). Furthermore, molecules acting upstream of GSK3 also appear to be essential for the behavioral effects of lithium. Indeed, βArr2-KO mice, in which lithium does not affect the phosphorylation of Akt (Thr 308) and GSK3β (Ser 9), have been shown not to respond behaviorally to either chronic or acute lithium treatment (Beaulieu et al., 2008). Similarly, a recent study conducted on different strains of inbred mice has shown that lithium fails to affect the phosphorylation of Thr 308 Akt in DBA/2J mice (Pan et al., 2011). This lack of an effect of lithium on Akt phosphorylation was accompanied by a lack of antimanic and antidepressant like behavioral responses to lithium in these mice. Importantly, increased expression of Akt1 using a *herpes simplex* (HSV) viral vectors in the brain of DBA/2J mice restored lithium sensitivity (Pan et al., 2011). Therefore, not only βArr2 but also Akt1 would be necessary for the regulation of GSK3β Ser 9 phosphorylation and behavior by lithium. However, the effects of lithium have not been tested in these behavioral paradigms in mice lacking a functional *Akt1* gene and it remains possible that the effects observed in DBA/2J mice might be associated to other characteristics of this genetic background.

The overall picture emerging from several studies of the role of GSK3 in the effects of lithium suggests a contribution of this kinase to at least some of its actions on behavior. However, this is based mostly on studies conducted with rodents using tests that were developed to predict the "antimanic" or "antidepressant" like effects of drugs. In the absence of a better understanding of the etiology of bipolar disorder, it may be more difficult to establish the exact contribution of GSK3 inhibition to the treatment of this psychiatric condition. That being said, a convergence of pharmacological and genetic evidences also supports the role of GSK3 in mood regulation as well as in the action of several drugs used for the management of psychiatric illnesses (Beaulieu et al., 2011; Jope, 2011). In this context, a contribution of GSK3 inhibition to the clinical effects of lithium provides a working model that may explain its relative clinical selectivity as well as its efficacy as an augmentation agent for other psychoactive drugs.

CONVERGENCE OF ACTIONS, MULTIPLE HITS, AND TARGET SELECTIVITY

The Akt1/GSK3 pathway sits at a convergence of several signaling mechanisms that have been associated to mental disorders (**Figure 1**). Among those, partial loss of function mutations in the genes encoding cognate ligands for the RTKs tropomyosin-receptor-kinaseB (TrkB) and epidermal growth factor receptor family 4 (ErbB-4) have been associated with enhanced risk for bipolar disorder, depression and schizophrenia (Neves-Pereira et al., 2002; Law et al., 2007; Jaaro-Peled et al., 2009; Schmidt and Duman, 2010). By reducing the activation of PI3K signaling, such genetic variation should result in a reduction in the activity of Akt and increased GSK3 activation. Similarly, genetic variation affecting the well-established negative regulator of GSK3—Akt1— and of potential negative GSK3 regulator—disrupted in schizophrenia 1 (Disc1)—have also been associated with bipolar disorder and schizophrenia (Emamian et al., 2004; Chubb et al., 2008; Kvajo et al., 2008; Mao et al., 2009). This suggests that several genetic risk factors for mental illnesses could lead to an increase in brain GSK3 activity. In contrast, lithium, antidepressant, and several antipsychotics have been shown to increase the inhibitory N-terminal phosphorylation of brain GSK3 in rodents (Beaulieu et al., 2004, 2009; Emamian et al., 2004; Li et al., 2004). Taken together, these observations suggest that inhibition of GSK3 is a plausible model for the action of lithium that may allow to reconcile its therapeutic effects with disease etiology, at least in some patient populations. Furthermore, this model predicts that lithium and several other psychoactive drugs may have an additive effect on GSK3 by inhibiting its activity trough different mechanisms that would complement and/or facilitate each other. Such a "multiple hit" inhibition of GSK3 by several drugs acting through different mechanisms may be one of the reasons underlining their mutual reinforcing effects in the clinic.

Interestingly, lithium also appears to inhibit GSK3 *in vivo* through a combination of mechanisms including its direct and indirect inhibition following Akt activation (**Figure 2**). This second type of inactivation mechanism would involve the disruption of a protein complex that is held together by βArr2 and stabilized by GSK3 (Beaulieu et al., 2008; O'Brien et al., 2011). While the direct inhibition of GSK3 by lithium is a relatively simple general mode of action that can affect all cells, its inhibition by an Akt/βArr2 dependent mechanism points toward the possibility that lithium may inhibit GSK3 with varying levels of efficacy in different cellular/neuronal populations. Indeed, the formation of βArr2 signaling complexes is dependent upon the activation of GPCRs (Luttrell et al., 1999) and such complex would only exist in cells that express the specific receptors (e.g., the D2R) that are responsible for their formation. By disrupting a βArr2 signaling complex that is responsible for the inhibition of Akt, lithium may, therefore, have a stronger effect on GSK3 activity in cells in which this complex is formed. In this case, targeting this complex may confer some level of selectivity, which may explain why lithium can be useful in the treatment of bipolar disorders without significantly disrupting GSK3 mediated physiological functions in the whole organism.

CONCLUSIONS AND FUTURE PERSPECTIVES

Lithium's mechanism of therapeutic action remains a mystery, more than 60 years after its first use for the treatment of mania in a clinical context. This situation has seriously prevented the development of improved pharmacotherapies for bipolar disorder. Some of the data reported in this review indicate that inhibition of GSK3 either directly or—more possibly—indirectly is a credible mechanism that may be responsible for some of lithium's actions.

However, this remains not solidly established and several additional studies will be needed to clarify the exact contribution of GSK3 inhibition in lithium therapy.

A first important question is the role played by GSK3 inhibition in the effects of lithium in humans. Studies conducted over the past decade have relied mostly on studies conducted in vertebrates. While this approach is powerful and allows for a comparison between molecular and behavioral effects of lithium, it nevertheless remains analogical and correlative. Most of the behavioral tests used in animals have good predictive validity for a first evaluation of antimanic or antidepressant like drug effects (Crawley, 2008). However, these tests certainly do not reflect the complex and variable symptoms of bipolar disorder in humans. A logical next step of investigation could be to combine studies of drug responsiveness in human subjects with different genetic risk factors that can affect GSK3 activity. In this regard, recent studies have shown a possible association between a GSK3β promoter polymorphism and responsiveness to lithium (Benedetti et al., 2004; Adli et al., 2007; Serretti et al., 2008). However to be truly convincing such studies should be replicated across independent laboratories and expended to several risk factors.

The study of GSK3's involvement in humans would also be facilitated by the development of surrogate biomarkers for central nervous system GSK3 activity. The development of such markers would allow monitoring the activity of GSK3 in patients in correlation with disease states and responsiveness to treatment. In line with this, a study has shown that GSK3β phosphorylation in peripheral blood cells can be used as an indicator of responsiveness to treatment (Li et al., 2007). However, this approach has several limitations as it remains biochemical and not functional. Future investigations may add to this by establishing correlates between brain GSK3 activity and, for instance, physiological/electrophysiological response in peripheral tissue or changes in brain activity/physiology that can be monitored by magnetic resonance imaging (Blasi et al., 2011).

In addition to human studies, investigations in mammals can be expanded to address several questions in rodents and eventually non-human primates. Among questions that can be addressed in this way is the identification of molecular targets that are responsible for the impact of GSK3 activity on behavior. Furthermore, it would be important to establish neuroanatomical correlates of GSK3 activity and behavioral regulation. Finally, animal models probably remain the most powerful tool at our disposal to understand how lithium acts not on a single molecular target but on several interconnected cellular signaling networks to produce its therapeutic actions. For instance, the action of lithium on both GSK3 and a βArr2 signaling complex that regulates and is regulated by GSK3 suggests that lithium may affect GSK3 activity differently in cells that express this signaling complex. To our knowledge, only the D2R has been shown to trigger the formation of this complex. This is interesting as this receptor is also a major pharmacological target of antipsychotics (Snyder, 1976; Roth et al., 2004). The relevance of this to the therapeutic selectivity of lithium and its combined action with antipsychotics can be further explored by identifying the complement of GPCRs that are responsible for the formation of this complex and the impact of these receptors on lithium actions *in vivo*. Similarly, lithium does not only affect GSK3 activity but has several other molecular targets that are also involved in cell signaling (Gould and Manji, 2002; Beaulieu and Caron, 2008). It would thus be of interest to use animal models to explore the possible involvement of modulations of cell signaling landscape involving several of these targets in the overall behavioral effects of lithium. Such an approach may reveal unexpected and important new information that would not be obtainable using a research program aimed at identifying a single molecular target while excluding all others.

Overall, recent studies of the involvement of GSK3 in the action of lithium have established a strong correlation between GSK3 activity and the regulation of behavior. They have also indicated that lithium is regulating GSK3 not only directly but also through more complex network effects affecting more than one molecular target at a time. In the near future, a continuation of these studies in animal models and the establishment of strong associations with human data may allow furthering this knowledge. This could lead to the development of an understanding of the role of GSK3 as well as that of other molecular targets of lithium in its relatively specific effects on several mental illnesses. Such knowledge may be the key to the development of new, safer mood stabilizers having better efficacy and fewer side effects.

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GSK-3 and Wnt signaling in neurogenesis and bipolar disorder

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The canonical Wnt signaling pathway is critical for development of the mammalian central nervous system and regulates diverse processes throughout adulthood, including adult neurogenesis. Glycogen synthase kinase-3 (GSK-3) antagonizes the canonical Wnt pathway and therefore also plays a central role in neural development and adult neurogenesis. Lithium, the first line of therapy for bipolar disorder, inhibits GSK-3, activates Wnt signaling and stimulates adult neurogenesis, which may be important for its therapeutic effects. GSK-3 also regulates other critical signaling pathways which may contribute to the therapeutic effects of lithium, including growth factor/neurotrophin signaling downstream of Akt. Here we will review the roles of GSK-3 in CNS development and adult neurogenesis, with a focus on the canonical Wnt pathway. We will also discuss the validation of GSK-3 as the relevant target of lithium and the mechanisms downstream of GSK-3 that influence mammalian behavior.

Keywords: Wnt, GSK-3, neurogenesis, lithium, bipolar disorder, behavior, development, psychiatric

INTRODUCTION

Wnt signaling is essential for patterning, cell fate specification, and stem cell regulation during the development of many tissues and organs, including the mammalian brain (reviewed in Ciani and Salinas, 2005; Petersen and Reddien, 2009; Kim and Snider, 2011). In adults, Wnt signals continue to influence the maintenance and regeneration of many tissues by regulating stem cell homeostasis and cell proliferation (reviewed in Gu et al., 2010; Wend et al., 2010; Yeung et al., 2011). In the adult brain, Wnt signaling also regulates critical processes such as neurite outgrowth, axon remodeling, synapse formation and plasticity, and neurogenesis (reviewed in Ciani and Salinas, 2005; Budnik and Salinas, 2011). In the canonical Wnt pathway, Wnts inhibit glycogen synthase kinase-3 (GSK-3) to activate downstream signaling. Lithium, which has been used to treat bipolar disorder (BPD) for decades, inhibits GSK-3 both directly and indirectly, raising the possibility that the therapeutic effects of lithium may involve activation of downstream Wnt signaling (reviewed in MacDonald et al., 2009; O'Brien and Klein, 2009). However, GSK-3 also regulates other pathways – distinct from the canonical Wnt pathway – that are involved in neuronal development and function, and these may also play important roles in the response to lithium. As these pathways are discussed in detail elsewhere in this volume (reviewed in Kaidanovich-Beilin and Woodgett, 2011; Kim and Snider, 2011), this review will focus on GSK-3 and canonical Wnt signaling in CNS development, adult neurogenesis, and BPD.

GSK-3 IS A CRITICAL REGULATOR OF DIVERSE SIGNALING PATHWAYS

Glycogen synthase kinase-3 constitutively antagonizes the canonical Wnt signaling pathway and must be inhibited for the pathway to function (**Figure 1**). In the absence of Wnt ligands, GSK-3, the transcriptional co-activator β-catenin, and the tumor suppressor adenomatous polyposis coli (APC) bind directly to the scaffolding protein Axin in a complex that facilitates phosphorylation of β-catenin by GSK-3, which targets β-catenin for proteasome-dependent degradation. Wnt ligands bind to the receptor Frizzled, inducing phosphorylation of the essential co-receptors low density lipoprotein receptor-related protein 5 (LRP5) and LRP6 which results in GSK-3 inhibition and β-catenin stabilization. Stabilized β-catenin enters the nucleus and interacts with the lymphocyte enhancer factor/T-cell factor (LEF/TCF) family of transcription factors to activate transcription (reviewed in Clevers, 2006; MacDonald et al., 2009; Wu and Pan, 2010).

In addition to its critical role in regulating Wnt signaling, GSK-3 phosphorylates over 100 substrates and regulates multiple signaling pathways, such as Sonic Hedgehog, Notch, and growth factor signaling through Akt which influences cell survival decisions in the brain (reviewed in Kockeritz et al., 2006; Kaidanovich-Beilin and Woodgett, 2011; Kim and Snider, 2011). Insulin, neurotrophins, and other growth factors activate phosphatidylinositol-3-kinase (PI3K) and Akt which phosphorylates GSK-3 at an N-terminal serine residue (Ser21 on the GSK-3α isoform, Ser9 on GSK-3β; Cross et al., 1995; McManus et al., 2005). This creates a pseudosubstrate motif that inhibits GSK-3 allowing activation of downstream effectors such as Glycogen Synthase and the mammalian target of rapamycin (mTOR; Dajani et al., 2001; reviewed in Proud, 2006; Kim et al., 2011). Importantly, GSK-3 in the Wnt-responsive Axin complex is not regulated by N-terminal serine phosphorylation, as neither insulin/Akt nor Wnt ligands induce Ser9/21 phosphorylation of Axin-associated GSK-3 (Ding et al., 2000; Ng et al., 2009). Additionally, Wnt signaling

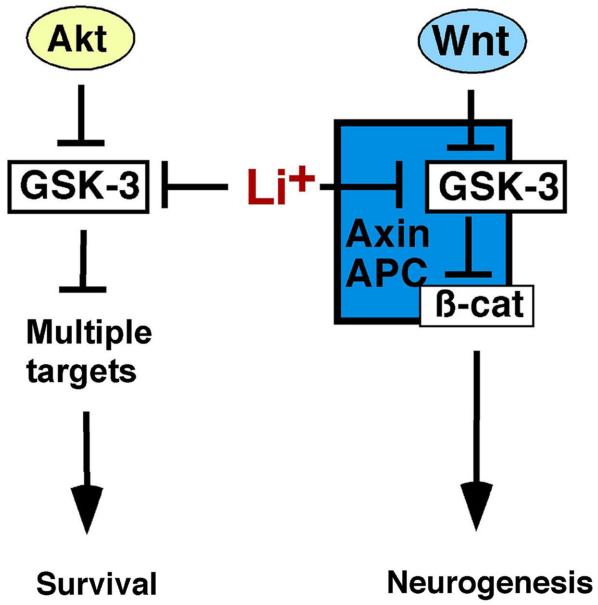

FIGURE 1 | Glycogen synthase kinase-3 regulates Wnt and Akt-dependent signaling. Growth factors, including neurotrophins, activate Akt, which phosphorylates, and inhibits GSK-3 allowing activation of downstream effectors to promote cell survival. Both Akt and GSK-3 have multiple targets not shown here for the sake of clarity. Wnts inhibit GSK-3 within the Axin complex stabilizing β-catenin, which activates Wnt target genes, and promotes neurogenesis. Axin-associated GSK-3 is not regulated by N-terminal phosphorylation and is thus distinct from Akt-regulated GSK-3, and both pools are inhibited by lithium. Thus lithium may stimulate neurogenesis by activating Wnt/β-catenin signaling and enhancing cell survival.

is normal in double knockin mice in which the *Gsk3a*^{ser21} and *Gsk3b*^{ser9} phosphorylation sites have been mutated to alanine, further demonstrating that Wnt ligands inhibit GSK-3 by a mechanism other than Ser21/9 phosphorylation (Ding et al., 2000; McManus et al., 2005). Therefore Wnt-responsive GSK-3 and growth factor/Akt responsive GSK-3 represent distinct subcellular pools regulated by distinct mechanisms.

CANONICAL Wnt SIGNALING DURING CENTRAL NERVOUS SYSTEM DEVELOPMENT

Wnt signaling regulates numerous critical processes throughout the development of the vertebrate central nervous system. These include patterning and cell fate specification, proliferation, and neuronal morphology (Chenn and Walsh, 2002; Woodhead et al., 2006; reviewed in Ciani and Salinas, 2005; Budnik and Salinas, 2011).

PATTERNING

In the early vertebrate embryo, Wnt signaling promotes posterior development and suppresses anterior development of the neural tube. Thus inhibition of Wnt signaling reduces posterior development and expands anterior regions, whereas aberrant Wnt pathway activation enhances posterior and reduces anterior development (reviewed in Ciani and Salinas, 2005). Consistent with this framework, anterior localization of Wnt antagonists such as DKK1 is required for anterior neural tube development (reviewed in Glinka et al., 1998; Mukhopadhyay et al., 2001; Mudher et al., 2004; Ciani and Salinas, 2005). At later stages, Wnt signaling further patterns the neural tube by establishing signaling centers such as the midbrain–hindbrain boundary, and restricting rhombomere boundaries in the developing hindbrain (reviewed in McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Kim et al., 2000; Lekven et al., 2001; Kapsimali et al., 2004; Ciani and Salinas, 2005).

Wnt signaling is also essential for dorsal/ventral patterning of the neural tube. Several Wnts including Wnt1 and Wnt3a are expressed in the dorsal neural tube and combined deletion of Wnt1 and Wnt3a results in expansion of ventral cell fates at the expense of dorsal fates (Megason and McMahon, 2002; Muroyama et al., 2002). Overexpression of Wnt1 or Wnt3a causes expansion of dorsal cell fates (Dickinson et al., 1994; Muroyama et al., 2002). Wnts also promote dorsal and suppress ventral cell fates in the telencephalon and are essential for the specification of neural crest (reviewed in Saint-Jeannet et al., 1997; Wu et al., 2003; Ciani and Salinas, 2005).

PROLIFERATION

Wnt signaling also regulates proliferation of neural precursor cells throughout CNS development. In the developing chick neural tube, overexpression of Wnt1, Wnt3a, or stabilized β-catenin increases neural precursor proliferation while expression of dominant negative TCF4 (dnTCF4) reduces cell proliferation (Megason and McMahon, 2002). In mice, Wnt1 overexpression increases proliferation and neuronal cell size in the caudal midbrain leading to significant midbrain overgrowth (Panhuysen et al., 2004). Furthermore, β-catenin loss of function in the diencephalon, mesencephalon, and hindbrain diminishes progenitor cell domains and decreases midbrain size, while β-catenin gain of function expands progenitor cell domains and increases midbrain size (Zechner et al., 2003). Wnts also regulate proliferation in the developing hippocampus. Wnt3a loss of function reduces hippocampal neural progenitor proliferation and disrupts hippocampal development (Lee et al., 2000). Similar defects are observed when β-catenin is deleted from the dorsal telencephalon (Machon et al., 2003). These data suggest Wnt/β-catenin signaling promotes progenitor proliferation in the developing neural tube as well as the midbrain and hippocampus.

Deletion of both *Gsk3a* and *Gsk3b* from mouse neural progenitors activates Wnt signaling and causes dramatic hyperproliferation of Sox2-positive early neural progenitors (known as radial progenitors) and increases proliferation as measured by the number of phospho-Histone H3, BrdU, and Ki67 positive cells, without affecting apoptosis. *Gsk3* deletion also reduces differentiation into intermediate neural progenitors and postmitotic neurons. Deletion of *Gsk3a* or *Gsk3b* alone does not significantly affect brain development demonstrating mostly redundant functions for the two isoforms. Expression of a dominant negative TCF to block downstream Wnt signaling in neural precursors cultured from *Gsk3a/b* double mutant mice partially reduces cell proliferation, suggesting that *Gsk3* loss of function increases neural precursor proliferation in part by activating Wnt signaling (Kim et al., 2009). In support of this conclusion, expression of stabilized β-catenin

in neural precursors also increases precursor proliferation (Chenn and Walsh, 2002), and *in utero* electroporation of dnTCF reduces cell proliferation (Woodhead et al., 2006). Taken together, these data suggest a role for canonical Wnt signaling in promoting neural precursor proliferation in the embryo.

NEURAL MORPHOLOGY

In addition to regulating patterning and cell proliferation, Wnts promote neurite outgrowth and influence axon size and branching, as well as growth cone size, complexity, and remodeling (Purro et al., 2008). Wnt7a increases neurite, axon, and growth cone size in cultured cerebellar granular cells and the Wnt antagonist secreted frizzled related protein (sFRP)-1 reduces growth cone size and prevents axon remodeling (Lucas and Salinas, 1997; Hall et al., 2000). GSK-3 inhibitors also increase neurite size, promote neurite outgrowth and axon formation, and increase axon size and branching in many cell types, including cerebellar granular cells, dorsal root ganglion neurons, and hippocampal neurons (Lucas and Salinas, 1997; Hall et al., 2000; Gartner et al., 2006; Dill et al., 2008). Wnts can also act as attractants to regulate commissural axon guidance after crossing the midline in the developing spinal cord; however, this activity is dependent on PI3K and atypical protein kinase C (aPKC) but not on LRP6, suggesting it is not through the canonical Wnt/β-catenin pathway (Lyuksyutova et al., 2003; Wolf et al., 2008). Wnts and β-catenin also promote dendrite growth and branching and promote synapse formation and plasticity (reviewed in Yu and Malenka, 2003; Rosso et al., 2005; Budnik and Salinas, 2011). Wnt7a increases the number of excitatory synapses in the hippocampus and promotes synapse formation in the cerebellum (Hall et al., 2000; Ciani et al., 2011).

Neurodevelopmental phenotypes in mice containing Wnt pathway mutations are summarized in **Table 1**.

Table 1 | Wnt pathway components are essential for neural development.

Mutated genes	Phenotype	Reference
Wnt1	Absence of midbrain, cerebellum	McMahon and Bradley (1990), Thomas and Capecchi (1990)
Wnt3	Reduced hippocampal neurogenesis	Lie et al. (2005)
Wnt3a	Absence of hippocampus	Lee et al. (2000)
Wnt1/Wnt3a double mutant	Loss of dorsal interneurons in the neural tube and expansion of ventral interneuron fates	Muroyama et al. (2002)
Wnt7a	Reduced hippocampal neurogenesis, delayed cerebellar synapse maturation	Hall et al. (2000)
Fzd3/Fzd6 double mutant	Midbrain morphogenesis defects	Stuebner et al. (2010)
Fzd4	Progressive loss of cerebellar granule and Purkinje cells	Wang et al. (2001)
Fzd5	Decreased number of neurons in the parafascicular nucleus	Liu et al. (2008)
Axin	Axis duplication	Zeng et al. (1997)
Apc	Embryonic lethality prior to gastrulation	Moser et al. (1995)
**Gsk3a/b* double mutant	Hyperproliferation of neural progenitors	Kim et al. (2009)
Lrp6	Midbrain and cerebellar malformations	Pinson et al. (2000)
Dkk1	Absence of anterior head structures	Mukhopadhyay et al. (2001)
b-catenin	Defects in anterior/posterior axis formation	Huelsken et al. (2000)
Lef1	Loss of granule cells of dentate gyrus	Galceran et al. (2000)

*Neurodevelopmental phenotypes are summarized in mice carrying loss of function mutations in the indicated genes. *Gsk3a/b indicates conditional deletion from neural progenitor cells. For more information please see Kim and Snider (2011) in this volume and also visit The Wnt Homepage at http://www.stanford.edu/group/nusselab/cgi-bin/wnt/*

CANONICAL Wnt SIGNALING IN ADULT NEUROGENESIS

Neurogenesis, the generation of new neurons, is a dynamic process that involves proliferation and differentiation of neural progenitors to produce new neurons which can then migrate, mature, and integrate into neuronal circuitry. Neurogenesis was once thought to occur during development only, but has since been observed throughout the lifespan of adult mammals including humans (Eriksson et al., 1998; reviewed in Suh et al., 2009; Ming and Song, 2011). In adult mammals, neurogenesis occurs in the subgranular zone (SGZ) of the dentate gyrus within the hippocampus and in the subventricular (SVG) zone adjacent to the lateral ventricles in the forebrain. Neurons generated in the SGZ migrate into the granular layer of the dentate gyrus where they mature and integrate into the existing neuronal circuitry and eventually behave similar to their older neighbors. Neurons arising in the SVZ migrate anteriorly through the rostral migratory stream and differentiate into interneurons in the olfactory bulb (reviewed in Gage, 2000; Lledo et al., 2006; Ming and Song, 2011; Mongiat and Schinder, 2011).

Although definitive evidence of a functional role for adult neurogenesis is limited, work from many groups suggests adult neurogenesis may be important for learning, memory, and a subset of behavioral responses to antidepressant medications in adult mammals. Spatial and object recognition memory, stress response, and contextual fear conditioning are among the behaviors that neurogenesis has been proposed to influence. Hippocampal irradiation inhibits neurogenesis and impairs spatial learning, contextual fear conditioning, and behavioral and hormonal responses to stress (Snyder et al., 2005, 2011). Importantly, similar results have been observed using alternative methods to block hippocampal neurogenesis (Saxe et al., 2006; Jessberger et al., 2009). Adult neurogenesis in both the SGZ and SVZ has also been proposed to play roles in pattern separation and/or memory resolution (Clelland et al., 2009; reviewed in Aimone et al., 2011; Sahay et al., 2011). SVG neurogenesis may also contribute to olfaction (reviewed in Lazarini and Lledo, 2011). Interestingly new neurons in adult

mammals exhibit unique properties at various stages of their maturation, including hyperexcitability, enhanced synaptic plasticity, and unique response to GABA, suggesting they may be able to carry out unique functions in the adult brain (reviewed in Ge et al., 2008; Deng et al., 2010). Thus, adult neurogenesis appears to be an important regulator of learning, memory, and other behaviors, although behavioral data from some groups challenges these conclusions, perhaps due to differing experimental conditions (reviewed in Deng et al., 2010). A better understanding of the regulation of neural stem and progenitor cell homeostasis will also likely contribute to the treatment of neurodegenerative disorders. Thus, considerable recent attention has focused on the signaling pathways and local factors that control adult neurogenesis.

Much of this attention has focused on the critical role for Wnt signaling in adult neurogenesis. Several Wnt ligands including Wnt3 and Wnt7a are expressed in adult hippocampal progenitors (AHPs) and astrocytes near the SGZ (Lie et al., 2005; Wexler et al., 2009). Adult mice lacking Wnt7a show reduced BrdU labeling in the dentate gyrus and expression of dominant negative Wnt in the dentate gyrus also decreases BrdU incorporation and decreases the number of cells expressing Doublecortin (DCX), which marks neuroblasts and newborn neurons (Lie et al., 2005; Qu et al., 2010). Similarly, deletion and shRNA mediated knockdown of β-catenin reduces the number of cells expressing neuronal markers in the adult dentate gyrus (Kuwabara et al., 2009). Conversely, overexpression of Wnt3 in the dentate gyrus increases the number of BrdU, DCX double positive cells (Lie et al., 2005). These data suggest Wnt signaling promotes proliferation of neural progenitor cells and hippocampal neurogenesis *in vivo*.

Additional support for the role of the Wnt pathway in neurogenesis comes from experiments with cultured AHPs. The Wnt pathway is active under basal conditions in cultured AHPs and is further activated by co-culture with adult hippocampal astrocytes (Lie et al., 2005; Wexler et al., 2009). Treatment with Wnt antagonists, including the Frizzled extracellular domain and sFRPs, reduces AHP proliferation and the number of cells expressing neuronal markers. Similarly, overexpression of Axin, GSK-3, dominant negative TCF (dnTCF), or a truncated N-cadherin that binds and sequesters β-catenin blocks Wnt signaling and results in long term depletion of multipotent AHPs and reduction of neuronal marker expression (Lie et al., 2005; Wexler et al., 2008, 2009). Furthermore, activating Wnt signaling in cultured AHPs by expressing Wnt3, stabilized β-catenin, a kinase dead GSK-3 mutant, or by adding the GSK-3 inhibitor lithium, increases AHP proliferation, BrdU incorporation, and neuronal marker expression (Lie et al., 2005; Wexler et al., 2008). Lithium-induced BrdU uptake is blocked by β-catenin knockdown, further suggesting lithium induces AHP proliferation by activating Wnt signaling (Wexler et al., 2008). Taken together, these data suggest Wnt/β-catenin signaling promotes progenitor proliferation and neurogenesis both in culture and *in vivo*.

The disrupted in schizophrenia 1 (DISC1) gene is mutated in affected family members in a Scottish pedigree with a high incidence of schizophrenia and depression (Millar et al., 2000). Although this familial syndrome and corresponding mutation are extremely rare, investigation of DISC1 function has been highly informative, as DISC1 protein was recently shown to bind and inhibit GSK-3 (Mao et al., 2009). DISC1 knockdown increases phosphorylation of β-catenin at the GSK-3 phosphorylation sites, reduces total β-catenin levels, and reduces Wnt reporter activation and Wnt target gene expression, consistent with the proposed role for DISC1 as an endogenous GSK-3 inhibitor. DISC1 knockdown also reduces proliferation of AHPs in culture and *in vivo*, and this effect is rescued by treatment with a GSK-3 inhibitor (Mao et al., 2009). These data suggest DISC1 promotes proliferation in the adult hippocampus by inhibiting GSK-3.

Several studies suggest Wnt signaling promotes progenitor proliferation and neurogenesis in the adult SVZ as well as in hippocampus. Expression of the Wnt antagonist DKK1 in the adult mouse SVZ reduces neural progenitor cell proliferation, and inhibition of GSK-3 or expression of stabilized β-catenin increases progenitor cell proliferation. Inhibition of GSK-3 also increases the number of new neurons in the olfactory bulb (Adachi et al., 2007). Adult mice lacking Wnt7a show reduced BrdU labeling in the subventricular zone, and overexpression of Axin in the SVZ of wild type mice also reduces BrdU incorporation (Qu et al., 2010). In cultured neural progenitor cells isolated from the SVZ, exposure to Wnt3a, Wnt7a, or expression of stabilized β-catenin promotes proliferation and neurogenesis (Yu et al., 2006; Qu et al., 2010).

Evidence that Wnt signaling is essential for adult neurogenesis has led to investigation of upstream factors that may promote neurogenesis by activating Wnt signaling and downstream effectors that may mediate the effects of Wnt signaling on neurogenesis. Recent evidence suggests the orphan nuclear receptor TLX and hypoxia inducible factor-1 (HIF-1) regulate neurogenesis upstream of Wnt/β-catenin signaling. TLX is expressed in adult neural stem cells in the dentate gyrus and the SVZ (Shi et al., 2004). TLX binds to the Wnt7a promoter in adult neural stem cells and promotes Wnt7a transcription, β-catenin stabilization, and activation of Wnt target genes. Furthermore, TLX knockdown reduces NSC proliferation, and this effect is blocked by Wnt7a treatment or expression of stabilized β-catenin. *In vivo,* TLX null mice show reduced BrdU labeling in the SVZ, and this is rescued by expression of stabilized β-catenin (Qu et al., 2010). These data suggest TLX promotes adult neurogenesis by activating Wnt/β-catenin signaling.

Similarly, HIF-1 promotes Wnt signaling and neurogenesis (Mazumdar et al., 2010). HIF-1 is heterodimer of HIF-1α and HIF-1β/ARNT that regulates the response to hypoxia. Knockout of *Hif1a,* which encodes the HIF-1α subunit, reduces Wnt target gene expression in the adult hippocampus. *Hif1a* knockout also reduces BrdU incorporation and the number of newborn DCX positive neurons in the hippocampus. These effects are rescued by inhibition of GSK-3 and by expression of stabilized β-catenin, suggesting that HIF-1 functions upstream of the Wnt pathway to promote neurogenesis (Mazumdar et al., 2010). Interestingly, AHPs occupy a hypoxic niche *in vivo,* and hypoxia dramatically increases TLX protein levels in cultured AHPs (Mazumdar et al., 2010; Chavali et al., 2011). Furthermore, TLX knockdown under hypoxic conditions reduces AHP proliferation, suggesting TLX promotes neural stem or progenitor cell proliferation under hypoxic conditions (Chavali et al., 2011). These data suggest a potential link between HIF-1 and TLX functions upstream of Wnt signaling.

Downstream of Wnt signaling, NeuroD1 and the prospero-related homeodomain transcription factor Prox1 represent two

intriguing candidates for Wnt target genes that may mediate the effects of Wnt signaling on neurogenesis. The NeuroD1 promoter contains several LEF/TCF binding sites and activating Wnt signaling by addition of either Wnt3a or the GSK-3 inhibitor TDZD8 increases NeuroD1 mRNA expression in cultured adult rat hippocampal NSCs, while blocking Wnt signaling with DKK1, dominant negative Wnt or β-catenin shRNA reduces NeuroD1 mRNA. Wnt3a treatment increases the number of cells expressing neuronal markers in cultured adult hippocampal NSCs, and in neurospheres cultured from adult mice, and this effect is blocked by NeuroD1 loss of function (Kuwabara et al., 2009). Overexpression of NeuroD in cultured adult hippocampal NSCs increases neuronal marker expression, mimicking the effect of Wnt3a (Hsieh et al., 2004). In the adult SGZ, NeuroD1 is expressed in neural progenitors and neuroblasts, including Ki67, and BrdU positive cells (Gao et al., 2009; Kuwabara et al., 2009). NeuroD1 deletion mimics Wnt loss of function by reducing the number of newborn neurons in the adult dentate gyrus as well as in the olfactory bulb, while NeuroD1 overexpression in the forebrain induces neurogenesis in the SVZ and rostral migratory stream (Gao et al., 2009; Boutin et al., 2010). These data suggest Wnt signaling may promote neurogenesis by inducing NeuroD1 expression.

Prox1 may also mediate the effects of Wnts on neurogenesis. β-Catenin associates with LEF/TCF binding sites in the Prox1 enhancer and promotes Prox1 expression in adult hippocampal NSCs. Prox1 is expressed in neural progenitors and both immature and mature neurons in the adult dentate gyrus. Expression of dominant negative LEF (dnLEF) in the dentate gyrus reduces Prox1 expression and expression of stabilized β-catenin induces Prox1 expression. Prox1 knockdown in the dentate gyrus reduces the number of newborn neurons and Prox1 overexpression increases the number of newborn neurons (Karalay et al., 2011). These data suggest Prox1 is a direct Wnt target that promotes neurogenesis. Importantly, further investigation is necessary to determine *in vivo* whether NeuroD1 or Prox1 expression can rescue neurogenesis defects resulting from Wnt pathway inhibition or whether Wnt-induced neurogenesis can be blocked by NeuroD1 or Prox1 loss of function.

LITHIUM ACTION IN BIPOLAR DISORDER

Bipolar disorder is a common neuropsychiatric disorder characterized by episodes of mania and depression, affecting an estimated 50–100 million people world-wide, and is associated with significant morbidity and mortality (reviewed in Belmaker, 2004; Goodwin and Jamison, 2007; Chen et al., 2010). Lithium has been widely used to treat BPD for over half a century, but its mechanism of action is not fully understood. Lithium also has a narrow therapeutic index and therapy is associated with multiple side effects, including thyroid dysfunction, nephrogenic diabetes insipidus, weight gain, arrhythmias, leukocytosis, tremor, and a variety of CNS and neuromuscular side effects (reviewed in Gilman, 1996). Thus, defining the molecular and cellular mechanisms of lithium action may provide insights into the pathogenesis of BPD and also lead to the development of better treatments for this common and devastating disorder.

An animal model of BPD would be extremely valuable in testing pathogenic and therapeutic mechanisms, but accurately assessing affect or affective disorders, especially with the added complexity of cycling of mood, in a model organism amendable to experimental manipulation is inherently challenging. Thus we and others have turned to animal behaviors that are influenced by mood stabilizers, including lithium, and can be measured in an objective and reproducible manner. Until recently, surprisingly few behaviors had been described that are sensitive to chronic lithium, most notably the amphetamine-induced hyperactivity behavior (reviewed in Murphy, 1977; Crawley, 2000). We have found that the forced swim test, classically used to assess antidepressant effects in rodents, is robustly sensitive to chronic lithium, with marked decrease in immobility in lithium treated mice (O'Brien et al., 2004). In addition, chronic lithium affects behavior in the elevated zero maze, a test often used to assess anxiolytic efficacy, and exploratory behavior (O'Brien et al., 2004; reviewed in O'Brien and Klein, 2009). Beaulieu et al. (2008) have shown that lithium also affects the tail suspension test and light–dark emergence, and Thakker-Varia et al. (2010) showed that lithium affects the novelty suppressed feeding paradigm.

LITHIUM REGULATES MAMMALIAN BEHAVIORS BY INHIBITING GSK-3

Numerous hypotheses have been put forth to explain the mechanisms of lithium action in mammalian behavior and in BPD (reviewed in Gurvich and Klein, 2002), but the number of direct lithium targets is quite limited. These include GSK-3 (Klein and Melton, 1996), inositol monophosphatase (IMPase) and structurally related enzymes (reviewed in Berridge et al., 1989; York et al., 2001; Gurvich and Klein, 2002), phosphoglucomutase (reviewed in Gurvich and Klein, 2002), and a β-arrestin-2 complex that regulates response to dopamine in the striatum (Beaulieu et al., 2008). Given the multiple plausible targets of lithium, we have suggested that validation of a proposed target of lithium should include evidence that (1) lithium inhibits the target at therapeutically relevant concentrations *in vitro* and *in vivo*, (2) structurally distinct inhibitors of the target should mimic lithium, (3) genetic loss of function of the target should also mimic lithium, and (4) the effects of lithium should be reversed by restoring function of the target (reviewed in Phiel and Klein, 2001; O'Brien and Klein, 2009).

Evidence from invertebrates suggests lithium can regulate behavior by inhibiting inositol phosphatases. In the nematode *Caenorhabditis elegans,* mutation of the inositol monophosphatase TTX-7 disrupts thermotaxis, and this behavioral phenotype is mimicked by lithium treatment; lithium also causes synaptic defects that phenocopy the *ttx-7* mutant and both behavior and synaptic defects are prevented by inositol treatment or by TTX-7 overexpression (Tanizawa et al., 2006). These findings strongly support a role for inositol depletion in the effect of lithium on thermotaxis in *C. elegans*. Mutation of inositol polyphosphate 1-phosphatase (*IPP*) in *Drosophila* causes defects in synaptic vesicle release that are phenocopied by lithium (Acharya et al., 1998), although it is not known whether lithium or loss of *IPP* affects myo-inositol levels in this setting. However, inositol depletion in mammals does not mimic lithium effects on behavior; heterozygous deletion of the sodium dependent myo-inositol cotransporter-1 (SMIT1) in mice reduces brain myo-inositol levels to a similar or greater degree than lithium, but has no effect

on behavioral phenotypes that are robustly affected by lithium (Shaldubina et al., 2006). These data suggest that global inositol depletion is not responsible for the behavioral effects of lithium in mammals.

We have proposed that the behavioral effects of lithium in mammals are mediated through inhibition of GSK-3 (O'Brien et al., 2004). GSK-3 fulfills each of the validation criteria described above: Lithium inhibits GSK-3 both *in vitro* and *in vivo* at therapeutically relevant concentrations (Klein and Melton, 1996; Stambolic et al., 1996; Hedgepeth et al., 1997; Hong et al., 1997; Munoz-Montano et al., 1997; Mudher et al., 2004; O'Brien et al., 2004), and structurally distinct GSK-3 inhibitors mimic the effects of lithium on mouse behaviors such as the forced swim test, exploratory behavior (Kaidanovich-Beilin et al., 2004; Pan et al., 2011; reviewed in O'Brien and Klein, 2009), light/dark emergence, and amphetamine-induced hyperactivity (Gould et al., 2004; Beaulieu et al., 2008). Furthermore, heterozygous loss of *Gsk3b* mimics lithium action in multiple behaviors (Beaulieu et al., 2004; O'Brien et al., 2004) and homozygous deletion of *Gsk3a* was also recently shown to mimic the behavioral effects of lithium and other GSK-3 inhibitors (**Table 2**; Kaidanovich-Beilin et al., 2009). Importantly, *Gsk3b* overexpression blocks the behavioral effects of LiCl (O'Brien et al., 2011). Taken together, these data strongly support GSK-3 as the relevant target of lithium in mammalian behavior.

In an elegant series of experiments, Beaulieu, Caron, and colleagues showed that, in the striatum, β-arrestin-2 forms a scaffold that binds Akt and protein phosphatase-2 (PP2A). Within this scaffold, PP2A dephosphorylates and deactivates Akt, preventing Akt-mediated phosphorylation and inhibition of GSK-3. They also showed that lithium disrupts this complex and proposed that this leads to Akt-mediated inhibition of GSK-3 (Beaulieu et al., 2008). Their findings therefore suggested that lithium inhibits GSK-3 indirectly.

However, these data are also compatible with direct inhibition of GSK-3 by lithium. We recently showed that GSK-3, which also binds to β-arrestin-2, maintains the stability of the β-arrestin-2 complex and lithium disrupts the complex by directly inhibiting GSK-3 (**Figure 2**; Beaulieu et al., 2005; O'Brien et al., 2011). In support of this mechanism, structurally distinct GSK-3 inhibitors as well as heterozygous loss of *Gsk3b* disrupt the complex and overexpression of *Gsk3b* restores basal levels of complex in the presence of lithium (O'Brien et al., 2011). These data suggest that GSK-3 stabilizes the β-arrestin-2 complex and that lithium disrupts the complex by inhibiting GSK-3.

Thus, the hypothesis that lithium modulates behaviors through direct inhibition of GSK-3 is strongly supported by each of the validation criteria described above, and while the data do not rule out contributions from other proposed targets, they provide a compelling rationale for pursuing GSK-3 substrates and downstream signaling pathways that mediate the behavioral and therapeutic effects of lithium.

POSITIVE FEEDBACK REGULATION OF GSK-3

These observations demonstrate both direct and indirect mechanisms of GSK-3 inhibition by lithium and imply a positive feedback system in which GSK-3 maintains its own activity by stabilizing the β-arrestin-2 complex, leading to dephosphorylation and inhibition of Akt (**Figure 2**). As Akt inhibits GSK-3, stabilization of the β-arrestin-2 complex by GSK-3 antagonizes this inhibition. In this model, a direct GSK-3 inhibitor is predicted to lead to indirect inhibition through enhanced N-terminal phosphorylation of GSK-3, as has been observed in many *in vivo* and cell culture contexts. This model can also explain how lithium activates Akt, as observed previously (Chalecka-Franaszek and Chuang, 1999). Through a parallel positive feedback circuit, GSK-3 regulates protein phosphatase 1 (PP1) and the PP1 inhibitor I-2 (Zhang et al., 2003). PP1 dephosphorylates and activates GSK-3; I-2 inhibits this reaction but I-2 in turn is deactivated by GSK-3; hence GSK-3 enhances its own activity by activating PP1. Taken together, these positive feedback systems provide discrete mechanisms to explain tissue-specific enhancement of GSK-3 inhibition by lithium (**Figure 3**), and may also help to explain why lithium is effective, both clinically and in mouse behaviors, at 1 mM, close to its *in vitro* IC_{50} (O'Brien et al., 2004).

POTENTIAL MECHANISMS MEDIATING BEHAVIORAL EFFECTS OF GSK-3 INHIBITION

Since GSK-3 has emerged as the likely target of lithium in mammalian behavior, much attention has focused on mechanisms

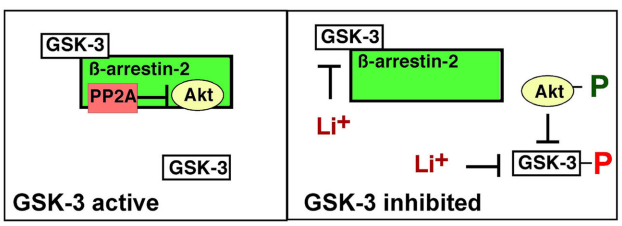

FIGURE 2 | Glycogen synthase kinase-3 regulation of the β-arrestin-2 scaffold. GSK-3 binds to and stabilizes the β-arrestin-2/PP2A/Akt complex, promoting PP2A-mediated dephosphorylation, and inactivation of Akt, which in turn maintains GSK-3 in an active state. Direct inhibition of GSK-3 disrupts the β-arrestin-2/PP2A/Akt interaction allowing activation of Akt, which then phosphorylates, and inhibits GSK-3.

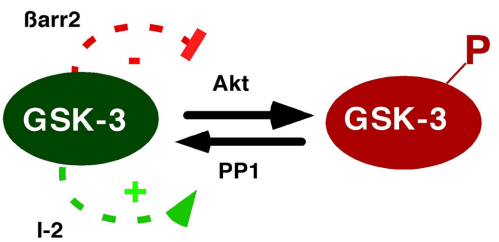

FIGURE 3 | Positive feedback regulation of GSK-3 through β-arrestin-2 and PP1. GSK-3 regulates its own activity by modulating its N-terminal phosphorylation state. As shown in **Figure 2**, GSK-3 enhances stability of the β-arrestin-2 scaffold, leading to inactivation of Akt, and thereby reducing GSK-3 phosphorylation. In addition, PP1 dephosphorylates and activates GSK-3, but is inhibited by the PP1-specific inhibitor-2 (I-2). GSK-3 inactivates I-2, preventing PP1 inhibition, and thereby maintaining dephosphorylation of GSK-3. Thus GSK-3 activity is regulated by positive feedback loops involving β-arrestin-2 and PP1.

downstream of GSK-3 that may contribute to lithium effects. GSK-3 regulates dozens of substrates directly, regulates multiple signaling pathways including Wnt and Akt-dependent signaling, and controls cellular processes such as neurogenesis and survival (Doble et al., 2007). Lithium promotes adult hippocampal neurogenesis and several groups have proposed that this is an essential function of mood stabilizers (Chen et al., 1999; Son et al., 2003; Silva et al., 2008; Fiorentini et al., 2010; Hanson et al., 2011; reviewed in Samuels and Hen, 2011). In this regard, there are interesting parallels between the effects of mood stabilizing drugs and antidepressants in rodents: Structurally diverse antidepressants also stimulate neurogenesis (Malberg et al., 2000; Santarelli et al., 2003), have similar effects as chronic lithium in multiple behaviors (**Table 2**), and stimulate N-terminal inhibitory phosphorylation of GSK-3 (Li et al., 2004; reviewed in Polter and Li, 2010), suggesting that they may act through similar mechanisms. Hippocampal irradiation blocks the effects of antidepressants in the chronic unpredictable stress and novelty suppressed feeding paradigms suggesting hippocampal neurogenesis is required for the behavioral effects of antidepressants (Santarelli et al., 2003). By analogy, enhancing neurogenesis may be critical for the mood stabilizing effects of lithium. Although the requirement for neurogenesis in the behavioral response to lithium has not been tested, it would be intriguing and somewhat surprising to find that antimanic agents and antidepressants act through similar mechanisms.

Lithium inhibits GSK-3 to activate canonical Wnt signaling which promotes adult neurogenesis; thus chronic lithium may influence behavior by stimulating Wnt-dependent neurogenesis. In support of this proposed mechanism, chronic lithium activates Wnt signaling in the dentate gyrus in mice and expression of an activated form of β-catenin reduces immobility in the forced swim test (Gould et al., 2007). However, whether Wnt signaling is *required* for behavioral responses to lithium has not been explicitly tested. In addition, the requirement for Wnt-induced neurogenesis in behavior has not been studied. Lithium also activates Wnt signaling in the amygdala and hypothalamus, areas not known to support adult neurogenesis (O'Brien et al., 2004). Thus, Wnt target genes could function independently of neurogenesis to regulate lithium-sensitive behaviors. Importantly, lithium effects on non-neuronal cell types may also be important for its effects on behavior.

Glycogen synthase kinase-3 negatively regulates cell survival pathways suggesting additional mechanisms by which lithium could promote neurogenesis. Growth factors such as insulin, EGF, and neurotrophins activate Akt, which phosphorylates and inhibits GSK-3 to promote cell survival and proliferation (reviewed in Wada, 2009). In addition to inhibiting GSK-3 directly, lithium increases brain-derived neurotrophic factor (BDNF) levels (Fukumoto et al., 2001), and stimulates Akt and Akt-mediated inhibitory phosphorylation of GSK-3 (at Ser9/21) in the striatum (Beaulieu et al., 2008; Pan et al., 2011). Inhibitory GSK-3 phosphorylation may be important for the behavioral effects of lithium as Akt inhibition blocks the effects of lithium on amphetamine-induced hyperlocomotion (Pan et al., 2011), and Ser9/21 to alanine mutations have the opposite effects as lithium in some behaviors including increased exploratory behavior and increased amphetamine-induced hyperactivity (Ackermann et al., 2010; Polter et al., 2010). Ser9/21 to alanine mutations also reduce BrdU incorporation in the adult dentate gyrus and block stimulation of neurogenesis by co-treatment with lithium and fluoxetine (Eom and Jope, 2009a), suggesting GSK-3 phosphorylation promotes adult hippocampal neurogenesis and may be required for the effects of lithium and antidepressants on neurogenesis and behavior.

Interestingly, Akt may also enhance canonical Wnt pathway activation by lithium. As discussed above, lithium can activate Akt indirectly. Furthermore, lithium-induced Wnt reporter activation is attenuated by inhibition of Akt or PI3K, as well as by overexpression of the Akt inhibitor phosphatase and tensin homolog (PTEN; Pan et al., 2011). As N-terminal phosphorylation of GSK-3 does not promote Wnt signaling (Ding et al., 2000; McManus et al.,

Table 2 | Parallels between GSK-3 inhibitors and antidepressants in adult neurogenesis and behavior.

Phenotype	Lithium	Alternative GSK-3 inhibitors	GSK-3 deletion	Antidepressants
Adult neurogenesis	↑	↑ (SB216763)		↑ (Fluoxetine, tranylcypromine, reboxetine)
Forced swim test (immobility)	↓	↓ (CHIR99021, AR-A014418, L803mts)	↓ (*Gsk3b*$^{+/-}$ or *Gsk3a*$^{-/-}$)	↓ (Fluoxetine, desipramine)
Exploratory behavior (hole pokes)	↓		↓ (*Gsk3b*$^{+/-}$)	
Elevated zero/plus maze (time in open area)	↑		↑ (*Gsk3b*$^{+/-}$)	↑ (Citalopram)
Tail suspension test (immobility)	↓		↓ (*Gsk3a*$^{-/-}$)	↓ (Citalopram)
Novelty suppressed feeding	↓			↓ (Fluoxetine, imipramine, desipramine)
Novelty induced locomotor activity	↓	↓ (TDZD-8)	↓ (*Gsk3a*$^{-/-}$)	
Amphetamine induced hyperlocomotion	↓	↓ (CHIR99021, TDZD-8, 6-BIO, AR-A014418, SB216763, alsterpaullone)	↓ (*Gsk3b*$^{+/-}$)	

Lithium stimulates adult neurogenesis and affects mammalian behaviors. Structurally diverse GSK-3 inhibitors as well as genetic loss of GSK-3 function mimic the effects of lithium, strongly implicating GSK-3 as the relevant target mediating lithium effects on behavior. Interestingly, distinct classes of antidepressants have similar effects as lithium on neurogenesis and many behaviors.

2005), Akt may either phosphorylate GSK-3 at other sites or regulate other components of the Wnt pathway. So far, alternative Akt phosphorylation sites in GSK-3 have not been described. However, Akt can phosphorylate β-catenin directly at a C-terminal site (Ser552), distinct from the GSK-3 phosphorylation sites, promoting β-catenin cytoplasmic and nuclear localization while reducing membrane localization (Fang et al., 2007). While speculative, these data raise the interesting possibility that Akt could contribute to β-catenin signaling in the response to lithium or other GSK-3 inhibitors.

Lithium may also affect behavior by inhibiting GSK-3 in the β-arrestin-2 complex in the striatum. β-Arrestin-2 prevents Akt-mediated inhibition of GSK-3, as β-arrestin-2 knockout activates Akt and increases inhibitory phosphorylation of GSK-3. Furthermore, β-arrestin-2 is required for effects of lithium on light/dark emergence and time immobile in the tail suspension test, and mimics the effect of lithium on novelty induced locomotor activity (Beaulieu et al., 2008).

Akt-mediated GSK-3 phosphorylation promotes cell growth, proliferation, and survival by activating downstream effectors such as mTOR, a key signaling complex that promotes protein translation and cell growth, regulates cell metabolism, and also regulates stem cell fate decisions (Huang et al., 2009; Duvel et al., 2010; Sato et al., 2010). GSK-3 negatively regulates mTOR by phosphorylating tuberous sclerosis complex-2 (TSC2), a core component of TSC (Inoki et al., 2006). The TSC is a GTPase activating factor that antagonizes the function of the small GTPase rheB, which is required to activate mTOR in response to nutrients (Inoki et al., 2003). Thus, inhibition of GSK-3 enhances rheB function and leads to activation of mTOR. Activation of mTOR by lithium or Gsk3 knockdown was demonstrated in cell culture and confirmed *in vivo* (Inoki et al., 2006; Huang et al., 2009). Interestingly, mTOR is also regulated by the Axin–GSK-3 complex, and canonical Wnt ligands activate mTOR in a β-catenin independent manner (Inoki et al., 2006). These data suggest mTOR may contribute to effects of Wnts, Akt, and lithium (**Figure 4**).

Hypoxia inducible factor-1 is another compelling candidate to mediate the effects of lithium as it promotes hippocampal neurogenesis and is functionally integrated with Insulin/PI3K signaling, Wnt signaling, GSK-3, and mTOR (**Figure 4**). GSK-3 phosphorylates HIF-1α resulting in proteasome-dependent HIF-1α degradation, and inhibition of GSK-3 by lithium, *Gsk3* knockdown, or exposure to insulin increases HIF-1α protein levels (Flugel et al., 2007). HIF-1α stabilization in response to hypoxia can also be blocked by PI3K inhibition, which prevents inhibitory phosphorylation of GSK-3 (Mottet et al., 2003; Schnitzer et al., 2005).

Alternatively, GSK-3 may regulate HIF-1α abundance through regulation of mTOR (**Figure 4**). As discussed above, inhibition of GSK-3 activates mTOR, and mTOR activation increases HIF-1α translation and activates HIF-1α target genes including vascular endothelial growth factor (VEGF; Duvel et al., 2010). Fluoxetine and desipramine increase VEGF levels in the hippocampus, and inhibition of the VEGF receptor blocks the increase in neurogenesis and the behavioral effects caused by these two antidepressants (Warner-Schmidt and Duman, 2007; Greene et al., 2009). Increasing VEGF levels in the hippocampus is sufficient

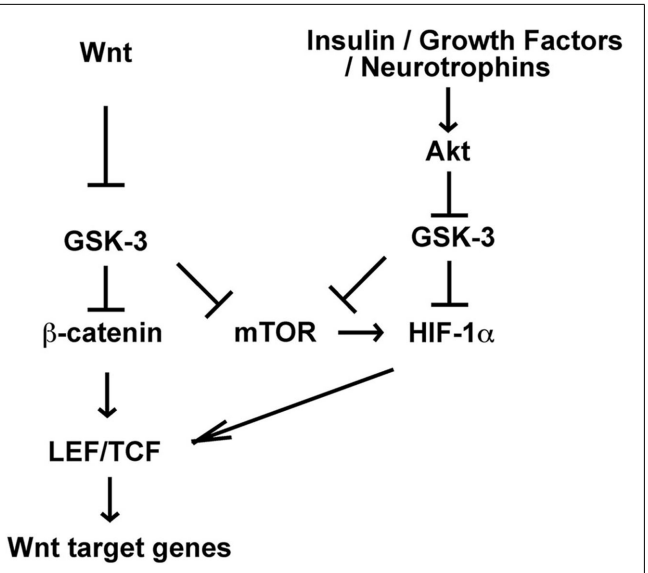

FIGURE 4 | Potential for crosstalk between Wnt and Akt-dependent signaling pathways through mTOR and HIF-1α. Wnt and Akt-dependent signaling pathways activate mTOR and mTOR promotes HIF-1α translation. Thus Wnt-induced mTOR activation may enhance HIF-1α function downstream of Akt. Furthermore HIF-1α promotes Wnt target gene expression by enhancing LEF/TCF transcription in embryonic and neural stem cells, suggesting that HIF-1α activation downstream of Akt may promote Wnt signaling. Interestingly, GSK-3 has also been shown to contribute to HIF-1α degradation, and therefore inhibition of GSK-3 by either small molecules or Akt-dependent pathways may also stabilize HIF-1α and enhance HIF-1 signaling (Mottet et al., 2003; Schnitzer et al., 2005; Flugel et al., 2007).

to stimulate neurogenesis, and has similar effects as lithium and antidepressants on behavior (Warner-Schmidt and Duman, 2007; Segi-Nishida et al., 2008; Udo et al., 2008). These data suggest HIF-1 and its target VEGF may be important for the effects of lithium on neurogenesis and behavior. Interestingly, HIF-1α also promotes canonical Wnt signaling (Mazumdar et al., 2010). HIF-1α promotes LEF-1 and TCF-1 transcription and antagonizes expression of APC, a negative regulator of Wnt signaling (Mazumdar et al., 2010; Newton et al., 2010). Thus HIF-1 may regulate neurogenesis and behavior through multiple downstream effectors.

Much evidence suggests GSK-3 also promotes apoptosis in many cell types, including neurons. Lithium blocks Bax and caspase-3 activation in response to trophic factor withdrawal and genotoxic stress in cultured neural precursor cells and protects against apoptosis resulting from glutamate induced excitotoxicity in multiple cell types (Nonaka et al., 1998; Eom et al., 2007). Lithium also increases levels of anti-apoptotic B-cell lymphoma protein-2 (bcl-2) in the hippocampus (Chen and Chuang, 1999; Chen et al., 1999). Neuronal apoptosis induced by trophic factor withdrawal or PI3K inhibition can be blocked by structurally diverse GSK-3 inhibitors, by an inhibitory GSK-3 binding protein (GBP), or by dominant negative GSK-3 (Hetman et al., 2000; Cross et al., 2001). Lithium and other GSK-3 inhibitors also block neuronal apoptosis induced by constitutively active c-Jun (Hongisto et al., 2003). In addition, GSK-3 enhances p53 function through

direct interaction with p53 and through phosphorylation and activation of Tip60, an acetyltransferase required for p53-dependent apoptosis (Watcharasit et al., 2003; Eom and Jope, 2009b; Charvet et al., 2011). Thus, lithium and other GSK-3 inhibitors promote survival in part by attenuating p53 levels and/or activity. *Gsk3b* overexpression also induces apoptosis in cultured cortical neurons (Pap and Cooper, 1998; Hetman et al., 2000). Taken together, these data suggest GSK-3 promotes apoptosis and inhibiting GSK-3 blocks apoptosis in response to diverse challenges. Thus reduced apoptosis resulting from GSK-3 inhibition by lithium may contribute to increased neurogenesis and effects on behavior.

CONCLUSION

Wnt signaling regulates many aspects of mammalian CNS development and continues to play critical roles in the adult CNS, including adult neurogenesis. Canonical Wnt signaling may also play a key role in the therapeutic response to lithium in BPD, as lithium activates the pathway by inhibiting GSK-3. In animal models, lithium regulates multiple mammalian behaviors by inhibiting GSK-3. However, GSK-3 regulates multiple pathways in addition to Wnt signaling, most notably Akt-dependent signaling, and each of these pathways may contribute to neurogenesis, cell proliferation, cell survival, and neural morphology. Thus, how distinct GSK-3 regulated pathways interact to mediate the effects of lithium on neurogenesis and behavior has not yet been delineated. Furthermore, the evidence that GSK-3 inhibition mediates the therapeutic effects of lithium in patients with BPD is still limited. Exciting recent work has shown that inhibitory GSK-3 phosphorylation is reduced in peripheral blood mononuclear cells (PBMCs) of patients with BPD compared to healthy controls suggesting GSK-3 is aberrantly active in BPD patients (Polter et al., 2010). In addition, lithium and other mood stabilizers increase GSK-3 phosphorylation (reviewed in Li and Jope, 2010) in PBMCs of BPD patients. Thus, while the number of patients studied is still small, the therapeutic effects of lithium in BPD may involve both direct and indirect inhibition of GSK-3. Finally, an outstanding question has been whether lithium is a sufficiently potent GSK-3 inhibitor to explain its therapeutic action. Recent findings support several positive feedback circuits that confer GSK-3 autoregulation and these may enhance the sensitivity to lithium in a tissue-specific manner. Better understanding of these intriguing complexities will aid in the development of new therapeutics for patients with BPD.

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Glycogen synthase kinase-3 in the etiology and treatment of mood disorders

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The mood disorders major depressive disorder and bipolar disorder are prevalent, are inadequately treated, and little is known about their etiologies. A better understanding of the causes of mood disorders would benefit from improved animal models of mood disorders, which now rely on behavioral measurements. This review considers the limitations in relating measures of rodent behaviors to mood disorders, and the evidence from behavioral assessments indicating that glycogen synthase kinase-3 (GSK3) dysregulation promotes mood disorders and is a potential target for treating mood disorders. The classical mood stabilizer lithium was identified by studying animal behaviors and later was discovered to be an inhibitor of GSK3. Several mood-relevant behavioral effects of lithium in rodents have been identified, and most have now been shown to be due to its inhibition of GSK3. An extensive variety of pharmacological and molecular approaches for manipulating GSK3 are discussed, the results of which strongly support the proposal that inhibition of GSK3 reduces both depression-like and manic-like behaviors. Studies in human postmortem brain and peripheral cells also have identified correlations between alterations in GSK3 and mood disorders. Evidence is reviewed that depression may be associated with impaired inhibitory control of GSK3, and mania by hyper-stimulation of GSK3. Taken together, these studies provide substantial support for the hypothesis that inhibition of GSK3 activity is therapeutic for mood disorders. Future research should identify the causes of dysregulated GSK3 in mood disorders and the actions of GSK3 that contribute to these diseases.

Keywords: glycogen synthase kinase-3, lithium, mood stabilizers, depression, antidepressants

INTRODUCTION

Major depressive disorder and bipolar disorder, in which patients experience manic episodes typically interspersed with depressive episodes, are commonly referred to as mood disorders. These are debilitating and prevalent illnesses, with a lifetime incidence of approximately 20% in the United States, and they are life-threatening due to suicide as well as other causes (Wong and Licinio, 2001; Nestler et al., 2002; Berns and Nemeroff, 2003; Belmaker, 2004). The pathophysiological underpinnings of mood disorders are unknown. Research into the causative mechanisms has been greatly hampered by the lack of adequate animal models of these diseases. However, studies of behaviors in rodents, including investigations of the mechanisms of action of therapeutic agents, have provided substantial evidence of a number of connections between glycogen synthase kinase-3 (GSK3) and mood disorders. Altogether, these findings suggest that dysregulation of GSK3 that causes its increased activity in specific cellular locations, pathways, and circuits, promotes susceptibility to mood disorders, and that inhibition of GSK3 is an important component of the therapeutic actions of interventions used to treat mood disorders.

ASSESSING CHARACTERISTICS OF MOOD DISORDERS IN RODENTS

Progress in understanding and treating mood disorders faces a difficult paradox. On one hand, there is no doubt that progress would be enhanced by mood disorder models in animals, preferably rodents. On the other hand, it is unlikely that rodents are capable of experiencing mood states that are equivalent to mania or major depression. Thus, unlike most other diseases, it may not be possible to precisely model the defining characteristics of mood disorders in animals. This has led to measurements of behaviors, instead of mood, in rodents, although it remains unclear to what extent assessments of rodent behaviors can be useful for clarifying the causes of mood disorders and for developing new therapeutics (Chen et al., 2010; Nestler and Hyman, 2010). Nonetheless, much effort has been exerted in the pursuit of measuring behavioral characteristics in rodents because of the high value of such models in developing better treatments for mood disorders (Matthews et al., 2005; Gould and Einat, 2007). In this regard, it is important to remember that the classical mood stabilizer lithium was initially conceived as a possible therapeutic for psychiatric diseases based on its behavioral effects in guinea pigs (Cade, 1949).

The rationale for using rodent behaviors to estimate mood is strengthened when it is possible to obtain predictable actions of drugs, such as by administration of antidepressants or mood stabilizers. However, care must be taken that administration of these drugs reasonably models therapeutic usage in human patients. For example, antidepressants have differential effects when administered acutely or chronically, and the latter have traditionally been favored as modeling actions in patients because of their delayed

therapeutic effects in major depression. Regarding lithium, many studies originally used injections that produce large fluctuations in serum levels of lithium. More recently, relatively stable therapeutic levels have been achieved by administering lithium in the food of rodents in a manner that produces serum lithium concentrations that are within the therapeutic range in humans. Generally, the range of 0.4–1.2 mM lithium is achieved in rodent serum using food pellets that contain 0.2–0.4% lithium, which can be maintained for months as long as the rodents are provided with a source of extra sodium to prevent hyponatremia (Thomsen and Olesen, 1974).

Quite a few models have been developed for studying antidepressant actions and depression-like behavior in rodents. Hereafter this will be referred to as depression for simplicity with the understanding that there are immense limitations in this categorization since behavior, rather than mood, is measured. Extensive reviews of these developments and their limitations have been published previously (Nestler et al., 2002; Cryan and Mombereau, 2004; O'Donnell and Gould, 2007; Chen et al., 2010). Therefore, only limited examples will be discussed here with a focus on those that have been employed in studies of the role of GSK3 in depression. One widely used model is the learned helplessness model of depressive-like behavior (Chourbaji et al., 2005). In this test, exposure to uncontrollable and inescapable shock will afterward cause depressed rodents to exhibit a deficit in escape when it is available, and this failure to escape can be ameliorated by chronic administration of antidepressants. The forced swim test (FST) and tail suspension test (TST) also have been used to identify in rodents drugs with antidepressant effects (Porsolt et al., 1977). These are only a few examples of a growing number of tests that have been applied to measure depressive-like behavior in rodents and antidepressant actions (Nestler et al., 2002; Cryan and Mombereau, 2004; O'Donnell and Gould, 2007).

In contrast to depression, progress has been more limited in developing rodent models of bipolar disorder. Furthermore, these studies have primarily focused on the manic-like component of behavior. This will be referred to as mania for simplicity, again with the understanding that there is currently not a true rodent model of the condition that humans experience as mania. Mania is a complex group of symptoms and no single measure can identify a manic rodent. Therefore, investigators have attempted to model characteristics of mania in rodents while understanding the limitations of these models. These models are most useful when disturbances in several rodent behaviors are observed that fall within the definition of mania, although not unique to mania, and that are ameliorated by a mood stabilizer (Machado-Vieira et al., 2004; Gould and Gottesman, 2006; Einat, 2007; Kovacsics et al., 2009). The two most common assessments of mania in rodents are basal locomotor activity, either in the home cage or in a novel environment, and amphetamine-induced hyperactivity. The latter has been used in part because amphetamine can worsen symptoms or induce mania relapse in patients, and mood stabilizers can alleviate these responses (reviewed in Einat et al., 2003; O'Donnell and Gould, 2007). Further behavioral measurements have been employed to attempt to study additional components of manic-like behavior in rodents. Recent evidence was reported that increased preference for sweet solutions provides a model for increased reward seeking, a central component of manic behavior that can be reduced by administration of mood stabilizers (Flaisher-Grinberg et al., 2009). Enhanced sucrose preference was displayed by CLOCK mutant mice that were characterized as exhibiting mania-like behavior (Roybal et al., 2007) and by heterozygote bcl-2 deficient mice (Lien et al., 2008). Furthermore, CLOCK mutant mice exhibiting characteristics of manic-like behavior were resistant to learned helplessness-induced depression-like behavior (Roybal et al., 2007). Increased acoustic startle response has also been observed in mice characterized as manic-like or bipolar-like, including mice expressing neuron-specific mutant mitochondrial DNA polymerase (Kasahara et al., 2006) and mice postnatally overexpressing constitutively active S9A–GSK3β in neurons (Prickaerts et al., 2006).

Thus, several approaches have been developed for measuring behaviors in rodents that have provided a wealth of novel information and that may be relevant for studying depression and mania. However, still needed are more behavioral approaches and methods to validate the applicability of these behavioral measurements to mood disorders. Particularly lacking are animal models that display the episodic and progressive natures of mood disorders.

ACTIONS OF LITHIUM ON RODENT BEHAVIORS

Since lithium is an effective mood stabilizer for bipolar disorder, its effects on locomotor activity of rodents has been the focus of much research. The activity of normal rodents in a familiar environment has generally been found to be unaffected by lithium, although this depends on the administration protocol (Smith, 1980; Gould et al., 2007b; O'Donnell and Gould, 2007). Additionally, strain-selective actions of lithium on basal and drug-induced behaviors is an important variable (Can et al., 2011; Pan et al., 2011). The absence of lithium effects in wild-type rodent locomotor activity heightens interest in the reduced locomotor activity induced by lithium in transgenic mice that exhibit increased locomotor activity and in mice given stimulant drugs that increase locomotor activity. For example, rodents that spontaneously exhibit increased locomotor activity that is reduced by lithium treatment include sleep-deprived rats (Gessa et al., 1995), rats administered ouabain (Jornada et al., 2010; Gao et al., 2011), mouse knockouts of the kainate receptor subunit GluR6 (Shaltiel et al., 2008), mouse knockouts of the *fmr1* gene that model Fragile X Syndrome (Yuskaitis et al., 2010), diacylglycerol kinase β knockout mice (Kakefuda et al., 2010), muscarinic M1 receptor knockout mice (Creson et al., 2011), and mouse knockouts of the AMPA receptor GluA1 subunit (Fitzgerald et al., 2010). This differential effect of lithium on normal compared with abnormal states of locomotor activity provides some support for the rationale that studies of rodent locomotor behaviors may be useful for examining therapeutic interventions for bipolar disorder. These studies also exemplify the well-known fact that multiple single alterations (e.g., various genetic manipulations) can cause equivalent behavioral outcomes in mice (locomotor hyperactivity), which is undoubtedly also the case for mood disorders. Altogether, it is remarkable that lithium is capable of normalizing behavioral locomotor hyperactivity in such a variety of mutant mice.

Studies of drug-induced increased locomotor activity that is reduced by lithium have predominantly used amphetamine, but other drugs also have been examined. Long ago, lithium was found to reduce hyperactivity and/or stereotypic behavior induced by amphetamine (Cox et al., 1971; Berggren et al., 1978; Borison et al., 1978) or by cocaine (Flemenbaum, 1977; Antelman et al., 1998). There has recently been a resurgence in studies examining lithium's control of amphetamine-induced hyperactivity (reviewed in Einat et al., 2003; O'Donnell and Gould, 2007). For example, lithium antagonized locomotor behaviors induced by amphetamine and dopamine D2 receptor stimulation (Beaulieu et al., 2004, 2005, 2008a). Lithium administration also reversed heightened amphetamine-induced hyperactivity or sensitization to amphetamine displayed by ERK1 knockout mice that exhibit increased behavioral excitement (Engel et al., 2009), heterozygote bcl-2 deficient mice (Lien et al., 2008), and omega-3 fatty acid deficient mice (McNamara et al., 2008). In addition to locomotor hyperactivity, lithium treatment also has been reported to reduce heightened sweet solution preference in CLOCK mutant mice that were characterized as exhibiting mania-like behavior (Roybal et al., 2007) and in heterozygote bcl-2 deficient mice (Lien et al., 2008).

STRATEGIES TO STUDY THE ACTIONS OF GSK3 AS A THERAPEUTIC TARGET OF LITHIUM

The discovery that lithium directly inhibits GSK3 raised the possibility that this action contributes to the mood stabilizing action of lithium in bipolar disorder (Klein and Melton, 1996; Stambolic et al., 1996). In addition to directly inhibiting the activity of GSK3, *in vivo* treatment with a therapeutically relevant level of lithium also increases the inhibitory serine-phosphorylation of GSK3, which was suggested to amplify the direct inhibitory action of lithium on GSK3 (De Sarno et al., 2002). This amplification mechanism has received support from a variety of studies (Eom and Jope, 2009; Polter et al., 2010; Pan et al., 2011). There is increasing evidence that many of the behavioral actions of lithium in rodents results from inhibiting GSK3 (Jope, 1999; Manji et al., 2000; Phiel and Klein, 2001; Harwood and Agam, 2003; Jope and Johnson, 2004), and that the diverse effects of lithium may largely be due to the numerous substrates of GSK3 and its consequential influences on many cellular functions. Evidence has been reported that GSK3 phosphorylates more than 100 substrates, and projections suggest that there may be many more proteins that are phosphorylated by GSK3 (Pilot-Storck et al., 2010; Taelman et al., 2010). Thus, it is inevitable that an inhibitor of GSK3, such as lithium, would have many effects on cellular functions. However, GSK3 is clearly not the only target of lithium (Chiu and Chuang, 2010), which also directly inhibits phosphoglucomutase (Ray et al., 1978), bisphosphate 3′-nucleotidase 1 (Spiegelberg et al., 1999), inositol monophosphatase (Hallcher and Sherman, 1980), and other inositol polyphosphatases (Inhorn and Majerus, 1987). Another proposed target of lithium, destabilization of the Akt-β-arrestin–protein phosphatase 2A protein complex (Beaulieu et al., 2004), was recently attributed to inhibition of GSK3 (O'Brien et al., 2011). Thus, multiple targets of lithium must be considered as potential contributors to its behavioral effects, although many of the major advances in recent years have identified the outcomes of GSK3 inhibition.

The identification of behavioral, or mood-altering, effects of lithium raised the possibility that these may be mediated by inhibition of GSK3, a potential causal relationship that requires verification. The two major strategies for doing so utilize pharmacological and molecular approaches. The pharmacological approach has been strengthened in recent years because many academic and pharmaceutical laboratories have engaged in intensive efforts to develop new small molecule selective inhibitors of GSK3 (Meijer et al., 2004; Martinez et al., 2006). This was particularly driven by the discovery that lithium inhibits GSK3 and the abundant evidence that GSK3 contributes to prevalent diseases, such as diabetes and Alzheimer's disease, as well as mood disorders. This has resulted in the availability of several selective agents for testing if other GSK3 inhibitors cause effects similar, or not, to lithium, and several of these new inhibitors have been widely used. Frequently used inhibitors include indirubin derivatives (Leclerc et al., 2001), L803-mts (Plotkin et al., 2003), SB216763, with care taken concerning its solubility as originally described (Coghlan et al., 2000), TDZD derivatives (Martinez et al., 2002), paullone derivatives (Leost et al., 2000), and AR-A014418 (Bhat et al., 2003), although the reports of behavioral effects of AR-A014418 are mitigated by other studies indicating that it does not significantly enter the CNS (Vasdev et al., 2005; Selenica et al., 2007; Hicks et al., 2010). Particularly valuable are studies of the kinase specificities of several GSK3 inhibitors (Davies et al., 2000; Murray et al., 2004; Bain et al., 2007), which enable investigators to utilize a panel of GSK3 inhibitors with differing off-target actions to provide reasonable confidence in ascribing overlapping effects of the inhibitors to their common target GSK3. The kinase specificity studies (Davies et al., 2000; Murray et al., 2004; Bain et al., 2007) identified CT99021 (Wagman et al., 2004) as the most specific GSK3 inhibitor of those tested. This information is also valuable for finding if GSK3 may be an off-target effect of inhibitors of other kinases. Also valuable is an important report of the *in vivo* CNS penetration and actions of a panel of GSK3 inhibitors (Selenica et al., 2007). Thus, many tools are available for pharmacologically identifying actions of GSK3.

Molecular approaches also have begun to be used to test if increased GSK3 activity has effects opposite to lithium treatment, and if reducing GSK3 molecularly has outcomes similar to lithium treatment. Increased GSK3 activity has been studied by overexpressing GSK3 and by using GSK3 knockin mice. However, although overexpression of GSK3 has provided a wealth of information about the actions of GSK3 in cells, *in vivo* CNS studies are hampered because GSK3 overexpression can cause neurodegeneration (Lucas et al., 2001; Spittaels et al., 2002). However, targeted overexpression of GSK3β in the nucleus accumbens induced a depression-like phenotype in multiple behavioral measurements (Wilkinson et al., 2011). Instead of overexpression of GSK3, another approach was taken by Alessi and colleagues to increase GSK3 activity by developing GSK3 knockin mice (McManus et al., 2005). The two GSK3 isoforms, GSK3α and GSK3β, are mainly regulated by inhibitory phosphorylation on Ser21–GSK3α and Ser9–GSK3β (**Figure 1**). This is normally maintained by signaling pathways, such as serotonergic activity (Li et al., 2004), that may be deficient in mood disorders, resulting in inadequately inhibited GSK3. The importance of inhibitory control of GSK3 can be

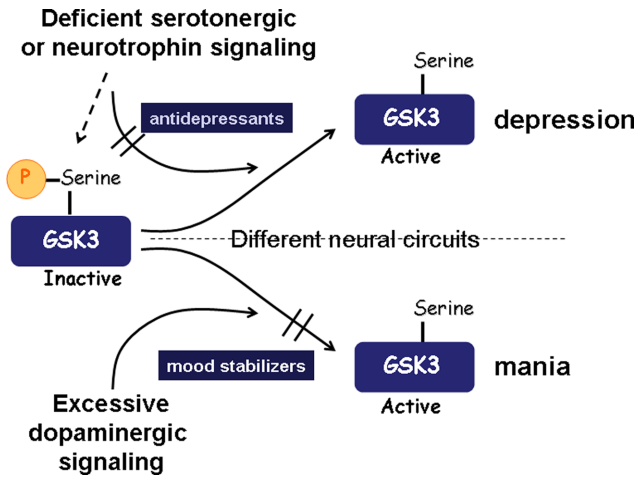

FIGURE 1 | Summary of the involvement of GSK3 in mood disorders. A simplified scheme is shown to represent examples of how GSK3 may be dysregulated in mood disorders. In depression, deficiencies in signals that normally maintain inhibition of GSK3, such as signaling induced by serotonin or neurotrophins, can cause up-regulation of GSK3 activity, which is capable of promoting susceptibility to depression. Mania may involve excessive dopaminergic signaling, which induces activation of GSK3. Part of the therapeutic actions of antidepressants and mood stabilizers may be derived from their direct or indirect effects that cause inhibition of GSK3.

studied using GSK3$\alpha^{21A/21A}/\beta^{9A/9A}$ knockin mice, with the regulatory serines of one or both GSK3 isoforms mutated to alanines (McManus et al., 2005). These mutations maintain GSK3 maximally active, but importantly within the physiological range since both GSK3 isoforms are expressed at normal levels. GSK3 knockin mice develop and reproduce normally with no overt phenotype. For studying the outcomes of molecular deficiencies in GSK3, the Woodgett laboratory has provided invaluable leadership by first producing all of the transgenic mice available for these studies (MacAulay and Woodgett, 2008; Force and Woodgett, 2009). They found that GSK3β knockout mice cannot be used because they are embryonically lethal (Hoeflich et al., 2000), but GSK3$\beta^{\pm}$ heterozygote knockout mice (O'Brien et al., 2004) and GSK3α knockout mice (Kaidanovich-Beilin et al., 2009) have been used to study the effects of reduced GSK3 expression. Thus, there are now multiple pharmacological and molecular tools available to modify GSK3 activity *in vivo* in order to examine effects on behavior, which are discussed below.

DEPRESSION AND GSK3 IN RODENTS

Many of the neuromodulators that are widely thought to be deficient in depression, such as serotonin, BDNF (brain-derived growth factor), and VEGF (vascular endothelial growth factor), normally stimulate signaling pathways that maintain inhibitory control of GSK3. Thus, we hypothesize that deficiencies in these signals in depression leave GSK3 inadequately inhibited, and that restoration of the inhibitory control of GSK3 by therapeutic drugs is an important part of their therapeutic mechanism of action (**Figure 1**).

The concept that dysregulated GSK3 promotes depression-like behavior in rodents has been extensively supported by multiple pharmacological approaches. Early studies indicated that lithium, when administered properly, attenuated several measures of depression in rodents and enhanced the antidepressant effects of serotonin reuptake inhibitors (Faria and Teixeira, 1993; Nixon et al., 1994; Redrobe and Bourin, 1999). Following the 1996 discovery that lithium inhibits GSK3 (Klein and Melton, 1996; Stambolic et al., 1996), many findings supported the conclusion these responses to lithium were likely due to inhibition of GSK3. These include findings of clear antidepressant effects in rodents of a variety of new small molecule inhibitors of GSK3 in addition to lithium (Gould et al., 2004; Kaidanovich-Beilin et al., 2004; O'Brien et al., 2004; Shapira et al., 2007; Beaulieu et al., 2008a; Silva et al., 2008), including on depressive behavior exhibited by tryptophan hydroxylase 2 mutant mice with deficient serotonin (Beaulieu et al., 2008b). Other studies have shown that classical antidepressants, as well as atypical antipsychotics, also inhibit GSK3 in mouse brain after *in vivo* administration of clinically relevant doses (Li et al., 2004, 2007a; Alimohamad et al., 2005; Roh et al., 2007; Beaulieu et al., 2008b; Okamoto et al., 2010). Furthermore, blocked inhibitory serine-phosphorylation of GSK3 in GSK3 knockin mice abrogated the neurogenesis-stimulating effect of fluoxetine and lithium administration, suggesting that at least some of the responses to fluoxetine and lithium depend on their induction of inhibitory serine-phosphorylation of GSK3 (Eom and Jope, 2009). Additionally, inhibition of GSK3 is required for the rapid antidepressant effect of ketamine in the learned helplessness model of depression in mice (Beurel et al., 2011b). In addition to inhibiting GSK3 via serine-phosphorylation, antidepressants also increase signaling by Wnt2, which inhibits GSK3 in the Wnt signaling pathway (Okamoto et al., 2010). Expressing or depleting disheveled, a protein capable of inhibiting GSK3 in the Wnt signaling pathway, induced multiple behavioral outcomes consistent with the concept that inhibiting GSK3 counteracts disrupted mood-relevant behaviors with the important emphasis that these effects resulted from alterations in the nucleus accumbens (Wilkinson et al., 2011). Conversely, GSK3 was found to be activated via decreased inhibitory serine-phosphorylation in the brains of mice exhibiting the learned helplessness model of depression (Polter et al., 2010). This activation of GSK3 during depression-like behavior was further shown to occur in the nucleus accumbens of mice exhibiting social defeat stress (Wilkinson et al., 2011). Taken together, these multiple approaches support the concept that impaired inhibition of GSK3 promotes depression-like behavior, and inhibition of GSK3 promotes resistance to depression.

In conjunction with these pharmacological studies, strategies using molecular modifications of GSK3 have firmly established that GSK3 promotes depression in rodents. The reduced GSK3β level in heterozygote GSK3$\beta^{\pm}$ knockout mice was demonstrated to be sufficient to reduce depression-like immobility in the FST (O'Brien et al., 2004) and the TST (Beaulieu et al., 2008a), and reduced immobility in the TST in tryptophan hydroxylase 2 mutant mice (Beaulieu et al., 2008b). Decreasing GSK3β levels by bilateral intra-hippocampal injections of lentivirus-expressing short-hairpin RNA targeting GSK3β decreased depression-like immobility times in both the forced swim and TSTs (Omata et al., 2011). Decreased immobility times in the FST and the

TST also were displayed by mice lacking a functional *GSK3α* gene (Kaidanovich-Beilin et al., 2009). These studies demonstrate that lowered expression of either isoform of GSK3 reduces vulnerability to depression-like behaviors in rodents. Additionally, overexpression of Wnt2, which inhibits GSK3 in the Wnt signaling pathway, reduced susceptibility to depression-like behavior in the learned helplessness paradigm (Okamoto et al., 2010). Conversely, increased GSK3 activity in GSK3 knockin mice was associated with increased susceptibility to the learned helplessness model of depression and increased immobility time in the FST and TST (Polter et al., 2010). Definitive evidence that GSK3 is the causal target of lithium in modifying behaviors in mice was shown by the finding that transgenic expression of GSK3β in mouse brain rescued lithium-sensitive behaviors, including immobility in the FST, exploratory behavior, and open arm time in the elevated zero maze (O'Brien et al., 2011).

Studies of mice expressing mutant DISC1 or with deficient DISC1 expression have further supported the concept that impaired inhibitory control of GSK3 promotes susceptibility to depression. DISC1 mutations have been implicated as a risk factor for schizophrenia, bipolar disorder, and recurrent major depression. Mice deficient in functional DISC1 exhibit depression-like behavior, as well as other behavioral abnormalities (Clapcote et al., 2007; Hikida et al., 2007; Li et al., 2007; Pletnikov et al., 2008; Shen et al., 2008). Dysregulated GSK3 may contribute to some of these behaviors because wild-type DISC1 directly binds and inhibits GSK3, actions lost with mutated DISC1 (Mao et al., 2009; Lipina et al., 2011a). Importantly, administration of GSK3 inhibitors normalized depression-like behavior in the FST and improved the impaired neurogenesis in DISC1 deficient mice (Mao et al., 2009; Lipina et al., 2011b), and genetic inactivation of GSK3α rescued spine deficits in DISC1 mutant mice (Lee et al., 2011). These are exciting findings because they provide a specific mechanism whereby a molecular variant associated with susceptibility to mood disorders may cause inadequate inhibitory regulation of GSK3.

MANIA AND GSK3 IN RODENTS

As noted above, basal locomotor hyperactivity is a commonly used measure of manic-like activity in rodents, although this is likely only a marginally adequate model. Increased locomotor activity was exhibited by mice postnatally overexpressing constitutively active S9A–GSK3β in neurons (Prickaerts et al., 2006) and GSK3 knockin mice displayed increased locomotor activity in a novel open field (Polter et al., 2010), whereas decreased locomotion was displayed by mice lacking a functional *GSK3α* gene (Kaidanovich-Beilin et al., 2009). Mice deficient in functional DISC1 that impairs its ability to inhibit GSK3 also exhibit spontaneous hyperactivity in the open field (Hikida et al., 2007; Pletnikov et al., 2008) that was normalized by reducing GSK3 activity (Mao et al., 2009; Lipina et al., 2011a,b). These findings are consistent with the concept that increased GSK3 activity is directly correlated with locomotor hyperactivity.

Locomotor hyperactivity induced by drugs, particularly amphetamine, is also widely used to model manic behavior in rodents. Particularly interesting are the studies by Beaulieu et al. (2004) that identified an important role for GSK3 in the locomotor response to amphetamine. They demonstrated that amphetamine administration activated cortical and striatal GSK3 (Beaulieu et al., 2004, 2005, 2008a), and GSK3 was activated in mouse striatum in dopamine transporter knockout mice (DAT-KO) due to reduced Akt activity (Beaulieu et al., 2004). Locomotor hyperactivity displayed by mice lacking the dopamine transporter was reduced by administration of five different GSK3 inhibitors (Beaulieu et al., 2004). GSK3β$^{\pm}$ heterozygote knockout mice displayed attenuated locomotor activation after amphetamine administration compared with wild-type mice, while basal locomotor activity of the two cohorts was equivalent (Beaulieu et al., 2004). Others have also shown that inhibitors of GSK3 reduce amphetamine-induced locomotor hyperactivity, further strengthening the conclusion that active GSK3 is a critical mediator of this response (Kozikowski et al., 2007; Kalinichev and Dawson, 2011). Increased amphetamine-induced locomotor hyperactivity was exhibited by mice deficient in functional DISC1, which eliminates its inhibition of GSK3 (Lipina et al., 2010), and by GSK3 knockin mice (Polter et al., 2010). Conversely, overexpression of β-catenin, which partially models reduced GSK3 activity, attenuated amphetamine-induced hyperactivity in mice (Gould et al., 2007a). These findings clearly demonstrated that GSK3 is activated after amphetamine administration and that GSK3 mediates some of the behavioral effects of dopamine, supporting a relationship between GSK3 activity and certain manic-like behaviors.

GSK3 IN SUBJECTS WITH MOOD DISORDERS

It is a challenging task to confirm in humans, hypotheses that are developed from *in vitro* and animal studies. This is particularly true for mood disorders because the target tissue is inaccessible and there is no clear pathological parameter or biomarker that can be assessed. Nonetheless, significant progress has been made investigating potential alterations of GSK3 in humans with mood disorders. The most direct assessment reported is the elevated GSK3 activity, associated with decreased Akt activity, in postmortem samples from ventral prefrontal cortex from patients with major depression disorder (Karege et al., 2007, 2011). In contrast, lower GSK3β expression was reported in prefrontal cortex of teenage suicide victims (Pandey et al., 2009). There is also evidence of fluctuations in the inhibitory serine-phosphorylation of GSK3 in peripheral blood mononuclear cells, generally with decreases associated with disease and increases following therapy (Li et al., 2007b, 2010). These findings suggest that it may be possible to develop measurements of phosphorylated GSK3 as a biomarker to reflect disease state and/or treatment responses. Thus, limited information is available concerning the functional status of GSK3 in humans with mood disorders. However, these findings generally support the concept developed in animal studies that GSK3 is inadequately inhibited in association with mood disorders and is inhibited in humans treated with lithium. This is an area in great need of further research in order to evaluate whether new GSK3 inhibitors should be tested as therapies in mood disorder patients.

Genetic studies have explored GSK3 and related genes in patients with mood disorders. Associations that have been identified include an increase in copy number variations affecting the

*GSK3*β gene locus in bipolar disorder (Lachman et al., 2007), and GSK3β polymorphisms linked to the age of onset of bipolar disorder and of major depressive disorder and the therapeutic responses to lithium (Benedetti et al., 2004a,b, 2005; Szczepankiewicz et al., 2006a; Saus et al., 2010) or to antidepressants (Tsai et al., 2008), and to gray matter volume in patients with major depressive disorder (Inkster et al., 2009). However, others have not found GSK3β polymorphisms associated with bipolar disorder (Lee et al., 2006; Nishiguchi et al., 2006) or lithium response (Szczepankiewicz et al., 2006b). Since cellular signals regulating GSK3, rather than GSK3 expression, have been implicated in most studies of mood disorder models, examinations of signaling pathways linked to GSK3 may prove most informative. This approach is exemplified by a recent report that polymorphisms in several genes encoding proteins directly related to the function of GSK3 are associated with regional gray matter volume changes in major depressive disorder patients (Inkster et al., 2010).

ACTIONS OF GSK3 THAT MAY CONTRIBUTE TO MOOD DISORDERS

Discussions of the role of GSK3 in mood disorders inevitably lead to the question: What is the target phosphorylated by hyperactive GSK3 that causes increased susceptibility to mood disorders? Ten years ago this was a relevant question because relatively little was known about the actions of GSK3 and few substrates had been identified. However, this question may now be less pressing considering our greater understanding of the regulatory roles of GSK3 in cellular functions and the numerous substrates of GSK3 that are known, and projections of many more that remain to be verified (Pilot-Storck et al., 2010; Taelman et al., 2010). Thus, it may be more pertinent to identify cellular processes regulated by GSK3 that are also dysregulated in mood disorders, rather than identifying individual substrates abnormally phosphorylated by GSK3 in mood disorders. This is somewhat analogous to studies of how GSK3 promotes intrinsic apoptotic signaling (Beurel and Jope, 2006). Although the mechanisms mediating apoptotic signaling are much better understood than mechanisms regulating mood disorders, a single target of GSK3 that underlies its promotion of apoptosis has not been identified. Instead a variety of actions appear to account for GSK3 lowering the threshold for apoptosis that are, in part, related to the initial insult. In this regard, studies of abnormal cellular functions that may contribute to mood disorders and studies of the actions of GSK3 have converged on several common themes. These include, but are not limited to, cellular stress response mechanisms, neurogenesis, and immune system abnormalities, particularly inflammation. There is evidence that alterations in each of these may promote the onset or severity of mood disorders, and that GSK3 has a strong regulatory role in each.

Substantial evidence indicates that mood disorders are associated with neuronal stress, such as oxidative stress and endoplasmic reticulum (ER) stress (Kato and Kato, 2000; Wang, 2007; Andreazza et al., 2008; Ng et al., 2008; Steckert et al., 2010). Initiating causes for increases in neuronal stress associated with mood disorders remain largely undetermined. However, it is well-established that many cell stressors can increase the activity of GSK3 in specific cellular compartments, such as insults causing ER stress (Song et al., 2002) or causing DNA damage (Watcharasit et al., 2002). Furthermore, a well-established characteristic of hyperactive GSK3 is its promotion of detrimental cellular responses to multiple types of insults, including oxidative stress and ER stress, which can be alleviated by GSK3 inhibitors (Beurel and Jope, 2006). Thus, these insults may contribute to abnormal activation of GSK3, and hyperactive GSK3 may contribute to reduced neuronal resilience in stressful environments. However, it is also important to consider that such stresses also affect glia cells, and there is growing evidence for glia abnormalities in mood disorders (Rajkowska and Miguel-Hidalgo, 2007), which may be associated with the increased inflammatory markers associated with mood disorders, as discussed below.

Neurogenesis, the proliferation, and neuronal differentiation of neural precursor cells, may be impaired in mood disorders (Lie et al., 2004). This is supported by findings that antidepressants (Malberg et al., 2000; Manev et al., 2001; Malberg and Duman, 2003; Santarelli et al., 2003; Warner-Schmidt and Duman, 2007; David et al., 2009) and lithium (Chen et al., 2000; Hashimoto et al., 2003; Silva et al., 2008; Wexler et al., 2008) increase neurogenesis in mice, and chronic stress associated with depression-like behaviors decreases neurogenesis (Malberg and Duman, 2003; Dranovsky and Hen, 2006; McEwen, 2008). Dysregulated GSK3 in mood disorders may contribute to deficient neurogenesis because neurogenesis is impaired by 40% in GSK3 knockin mice (Eom and Jope, 2009), neurogenesis was increased by GSK3 deletion (Kim et al., 2009), and GSK3 overexpression inhibited, and the GSK3 inhibitor SB216763 increased, neural precursor cell proliferation that is impaired in mice with DISC1 mutations (Mao et al., 2009). Furthermore, the stimulatory actions of fluoxetine and lithium on neurogenesis were blocked in GSK3 knockin mice in which the drugs could not increase the inhibitory serine-phosphorylation (Eom and Jope, 2009). These results indicate that hyperactive GSK3 impairs neurogenesis and that fluoxetine and lithium need to inhibit GSK3 by serine-phosphorylation to promote neurogenesis. These findings raise the possibilities that impaired neurogenesis by dysregulated GSK3 contributes to increasing susceptibility to mood disorders, and that the rescue of neurogenesis contributes to responses to therapeutic drugs.

Substantial evidence has accumulated demonstrating that mood disorders are associated with activation of the inflammatory system and other alterations of the immune system (Chourbaji et al., 2008; Dantzer et al., 2008; Miller et al., 2009; Rivest, 2009; Miller, 2010). Additionally, inflammatory cytokines impair glucocorticoid responsiveness, raising the possibility that the chronic inflammation associated with mood disorders contributes to reduced glucocorticoid responses in these disorders (Pace et al., 2007). A crucial role for GSK3 in promoting inflammation was first established by the finding that GSK3 promotes the production of several pro-inflammatory cytokines following stimulation of multiple types of Toll-like receptors in human monocytes (Martin et al., 2005). GSK3 deficiency induced with GSK3 inhibitors or by molecular means greatly reduced the production of several pro-inflammatory cytokines. Remarkably, GSK3 regulates oppositely the anti-inflammatory cytokine IL-10, so GSK3 inhibition increased IL-10 levels (Martin et al., 2005). Lithium and other GSK3 inhibitors also reduced by >90% inflammatory

cytokine production by mouse primary astrocytes (Beurel and Jope, 2009a) and microglia (Yuskaitis and Jope, 2009), similarly to peripheral cells (Beurel and Jope, 2009b), and GSK3 counteracts down-regulation of inflammation (Beurel and Jope, 2010). Mechanisms for these actions include the findings that GSK3 is required for activation of NF-κB (Hoeflich et al., 2000; Martin et al., 2005) and STAT3 and STAT5 (Beurel and Jope, 2008), critical transcription factors in inflammation. *In vivo*, chronic administration of a therapeutically relevant dose of lithium rescued 70% of mice from an otherwise 100% lethal inflammatory response to lipopolysaccharide (Martin et al., 2005). *In vivo* chronic lithium treatment also markedly suppressed EAE (experimental autoimmune encephalomyelitis), an animal model of multiple sclerosis that involves substantial neuroinflammation, and the production of inflammatory Th17 cells that contribute to EAE pathogenesis (De Sarno et al., 2008; Beurel et al., 2011a). These results demonstrate that a therapeutically relevant dose of lithium effectively ameliorates CNS inflammatory diseases *in vivo*. These and other immune-regulating actions of GSK3 (Beurel et al., 2010) demonstrate that dysregulated GSK3 may contribute to immune system alterations that are associated with mood disorders and suggest that the immune system actions of lithium may contribute to its therapeutic effects in mood disorders.

Thus, stress response mechanisms, neurogenesis, and immune system abnormalities, are examples of processes strongly regulated by GSK3 that may be disrupted in mood disorders. There are multiple additional cellular functions regulated by GSK3 that may be crucial in mood disorders, such as circadian rhythm alterations, mitochondrial function, neurotransmitter synthesis and receptor-induced signaling, that are equally important potential targets. These three are only presented as representative examples of the concept that focusing on GSK3-regulated processes, rather than individual substrates of GSK3, may be most informative for advancing the understanding of how dysregulated GSK3 promotes susceptibility to mood disorders.

SUMMARY

Figure 1 presents a simplified summary of how GSK3 is proposed to be involved in mood disorders. Clusters of symptoms define these disorders, which are undoubtedly induced by multiple combinations of genetic and environmental influences. An important outcome is impaired inhibitory regulation of GSK3, albeit not globally, but in particular circuits, cells, and signaling pathways. Substantial evidence now supports the concept that depression is associated with reduced signaling that otherwise would maintain GSK3 inhibited, which may involve deficient serotonin or BDNF, for example, and genetic changes, such as diminished functional DISC1 that otherwise contributes to GSK3 inhibition. Increased activation of GSK3 also may occur in mania, although in this case it may not result from deficient inhibitory signaling to GSK3 but from excessive activating signaling, such as can be mediated by increased dopaminergic signaling through dopamine D2 receptors. Because many signals converge on GSK3, which integrates these to modulate cellular responses, pharmacologically bolstering the inhibition of GSK3 can compensate for multiple combinations of genetic and environmental influences to promote the re-establishment of mood stability, thus counteracting conditions that would otherwise induce extremes in mood fluctuations. Therefore, although alterations in GSK3 activity may not constitute a primary insult in mood disorders, the role of GSK3 as an integrator of multiple signals allows therapies directed toward inhibiting GSK3 to compensate for a variety of genetic and environmental conditions that disturb mood homeostasis.

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AKT/GSK3 signaling pathway and schizophrenia

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Schizophrenia is a prevalent complex trait disorder manifested by severe neurocognitive dysfunctions and lifelong disability. During the past few years several studies have provided direct evidence for the involvement of different signaling pathways in schizophrenia. In this review, we mainly focus on AKT/GSK3 signaling pathway in schizophrenia. The original study on the involvement of this pathway in schizophrenia was published by Emamian et al. in 2004. This study reported convergent evidence for a decrease in AKT1 protein levels and levels of phosphorylation of GSK-3β in the peripheral lymphocytes and brains of individuals with schizophrenia; a significant association between schizophrenia and an AKT1 haplotype; and a greater sensitivity to the sensorimotor gating-disruptive effect of amphetamine, conferred by AKT1 deficiency. It also showed that haloperidol can induce a stepwise increase in regulatory phosphorylation of AKT1 in the brains of treated mice that could compensate for the impaired function of this signaling pathway in schizophrenia. Following this study, several independent studies were published that not only confirmed the association of this signaling pathway with schizophrenia across different populations, but also shed light on the mechanisms by which AKT/GSK3 pathway may contribute to the development of this complex disorder. In this review, following an introduction on the role of AKT in human diseases and its functions in neuronal and non-neuronal cells, a review on the results of studies published on AKT/GSK3 signaling pathway in schizophrenia after the original 2004 paper will be provided. A brief review on other signaling pathways involved in schizophrenia and the possible connections with AKT/GSK3 signaling pathway will be discussed. Moreover, some possible molecular mechanisms acting through this pathway will be discussed besides the mechanisms by which they may contribute to the pathogenesis of schizophrenia. Finally, different transcription factors related to schizophrenia will be reviewed to see how hypo-activity of AKT signaling pathway may impact such transcriptional mechanisms.

Keywords: schizophrenia, neuronal signaling, phosphorylation, AKT, PKB, GSK3

INTRODUCTION

Schizophrenia is a severe mental disorder with a lifetime prevalence of 1% throughout the world population (Stilo and Murray, 2010). This highly prevalent mental disorder is typically associated with progressive functional decline, lifelong disability and tremendous suffering (Sadock et al., 2009). Unlike other neuropsychiatric disorders such as Parkinson's disease, no single anatomical abnormality is consistently observed in schizophrenia, nor there are any biochemical tests that can confirm the clinical diagnosis. Case identification usually begins with the observation of positive symptoms such as delusional ideas, hallucinations, disordered thinking, often with a later emergence of negative symptoms, including low levels of emotional arousal, mental activity and social drive (Sadock et al., 2009). Furthermore, there is increasing recognition of several cognitive impairments in this disorder, particularly in attention, memory, and executive functions, which equally contribute to schizophrenia's disability. Although it is believed that multiple pathological processes can lead to schizophrenia, we have neither identified them nor linked them to the various clinical manifestations of the disorder (Sadock et al., 2009).

Several linkage and association studies, including a few genome-wide association studies (GWAS), have been performed during the past decade to identify genetic susceptibilities to the disease. A few review articles have nicely reviewed recent association studies on large schizophrenia cohorts (Tiwari et al., 2010). Such studies identified and replicated further loci in well-powered cohorts (Tiwari et al., 2010). Moreover, over the past 4 years there has been a significant interest in human Copy Number Variants (CNVs). Several deletions that were recently identified in schizophrenia, such as 1q21.1; 2p16.3; 15q11.2; 15q13.3, have also been found in other neurodevelopmental disorders (Tiwari et al., 2010).

Despite identification of several genetic variations that have been repeatedly reproduced in several populations, many studies have been inconclusive. The limited success in detection of genetic factors has led scientists to come to a general consensus that the disease is not caused by the dysfunction of a specific

molecule or pathway. Most likely schizophrenia is caused by the altered function or expression of many genes, which may individually contribute only to a small risk, but their collective dysfunctional effects interfere with the function of several biological pathways that eventually produce the clinical picture we know as schizophrenia (Williams et al., 2009).

AKT AND HUMAN DISEASES

Deregulation of AKT signaling pathway is directly associated with some of the most prevalent and incurable human diseases (Blain and Massague, 2002; Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002; Chen et al., 2003; Emamian et al., 2003, 2004; Brazil et al., 2004; Colin et al., 2005; Griffin et al., 2005). It is now well established that the hyperactivity of AKT is part of the pathologic process in several types of the most common human malignancies (Brazil et al., 2004), including breast cancer, prostate cancer, lung cancer, gastrointestinal tumors, pancreatic cancer, hepatocellular carcinoma, thyroid cancer, and CNS malignancies (such as glioblastoma or gliomas). Moreover, impairment of AKT signaling pathway in a number of neurodegenerative brain disorders such as Alzheimer disease, Huntington disease, spinocerebellar ataxia type 1 (SCA1) and amyotrophic lateral sclerosis (ALS), have been reported during the past few years (Saudou et al., 1998; Chen et al., 2003; Emamian et al., 2003; Kaspar et al., 2003; Colin et al., 2005; Griffin et al., 2005).

In Alzheimer disease, evidence for an increased AKT activation and the subsequent increase in the phosphorylation of several AKT substrates in the brain tissue of patients has been reported (Griffin et al., 2005). In Huntington disease, *in vitro* studies have shown that huntingtin is a substrate of AKT, and the phosphorylation of huntingtin by AKT is crucial for mediating the neuroprotective effects of IGF-1. Furthermore, changes in AKT signaling in an animal model of Huntington disease, as well as in patients with Huntington disease, are also reported (Saudou et al., 1998; Colin et al., 2005). In SCA1, Chen et al. (2003) reports a regulatory role of AKT in the association of ataxin-1 with 14-3-3 through the phosphorylation of Ser-776 of ataxin-1, which modulates the neurotoxicity of ataxin-1 (Emamian et al., 2003). In ALS, IGF-1 stimulates the activity of AKT in the spinal cord and prolongs the lifespan of the mouse model of ALS, by increasing the survival of motor neurons (Kaspar et al., 2003).

Using a reverse protein-to-gene approach, we reported convergent evidence for the impairment of AKT/GSK3 signaling pathway in schizophrenia (Emamian et al., 2004), including a significant association between schizophrenia and an *AKT1* haplotype in U.S. families (Caucasian of Northern European origin). The genetic association of *AKT1* with schizophrenia was later confirmed in several other populations (Ikeda et al., 2004; Schwab et al., 2005; Bajestan et al., 2006). Taken together, it seems that the genetic association of AKT with schizophrenia is not limited to a specific genetic background and is reproducible in populations with different genetic backgrounds.

The main advantage of studies on signaling molecules in complex human disorders with unknown etiology, such as schizophrenia and autism, is their potential for discovering the abnormal function of proteins involved in the pathogenesis regardless the actual causative factor, whether it is genetic or environment or the interplay between them. Another important advantage of studies on signaling proteins such as AKT is their capacity for identification of *sequence specific* activation or inhibition sites on molecules involved in the disease process. Such specific sites could be ultimately used as selective targets for high throughput screening of small molecules or chemical entities, to discover novel therapeutics for several disorders. Therefore, despite the fact that schizophrenia is complex and extremely difficult to understand at the molecular level, dissecting the role of signaling molecules such as AKT or GSK3 in such a complex disorder has obvious therapeutic implications.

AKT IN NON-NEURONAL CELLS

AKT (also known as PKB) is a relatively new member of the AGC kinase family. It was discovered in early 1990s as a major regulator of cell cycle (Brazil et al., 2004). After almost two decades, over 30,000 papers have been published (~7000 in the past year) that show its major regulatory role in multiple aspects of cellular function. **Figure 1** summarizes the molecular targets of AKT signaling pathway.

AKT has emerged as the focal point for many signal transduction pathways, regulating multiple cellular processes such as glucose metabolism, transcription, apoptosis, cell proliferation, angiogenesis, and cell motility (**Figure 1**, www.cellsignal.com, Kandel and Hay, 1999). Stimulation of its catalytic activity is triggered by PI3 kinase and results from the PtdIns(3,4,5)P_3-dependent recruitment of AKT from the cytoplasm to the membrane, as well as the phosphorylation of two regulatory residues: Thr-308 and Ser-473. Phosphorylation of Thr-308, catalyzed by PDK-1, is required for the AKT activity, and this activity is augmented, ~10-fold, by Ser-473 phosphorylation (Alessi et al., 1996; Brazil et al., 2004). Besides functioning as a kinase toward many substrates involved in these processes, AKT forms complexes with other proteins that are not substrates, but rather act as the modulators of the activity and function (Brazil et al., 2004).

AKT is highly expressed in different cell types (Brazil et al., 2004). AKT has three isoforms in mammalian cells: AKT1, AKT2, and AKT3, which have distinct roles in a variety of processes, including development and metabolism (Dummler and Hemmings, 2007). AKT1 is the most highly expressed isoform and is the isoform reported so far to be associated with schizophrenia (Emamian et al., 2004). AKT2 is mainly involved in regulation of metabolism primarily through insulin-regulated glucose homeostasis. AKT2 is expressed at a lower level than AKT1 and significant expression of AKT2 occurs in insulin-responsive tissues, such as skeletal muscle, liver, heart, kidney, and adipose tissue (Altomare et al., 1995; Chong et al., 2005). During development of central nervous system (CNS), the expression of AKT1 and AKT2 is increased during development, but is gradually decreased during postnatal development (Owada et al., 1997). In the adult brain, expression of AKT1 and AKT2 is initially weak. Unlike AKT1 and AKT2, AKT3 is only expressed in specific tissues, such as in the brain and testes, with lower expression evident in skeletal muscle, pancreas, heart, and kidney (Chong et al., 2005). Although AKT3 is expressed in the brain and has a role in postnatal brain development (Birnbaum, 2004; Tschopp et al., 2005; Gonzalez and McGraw,

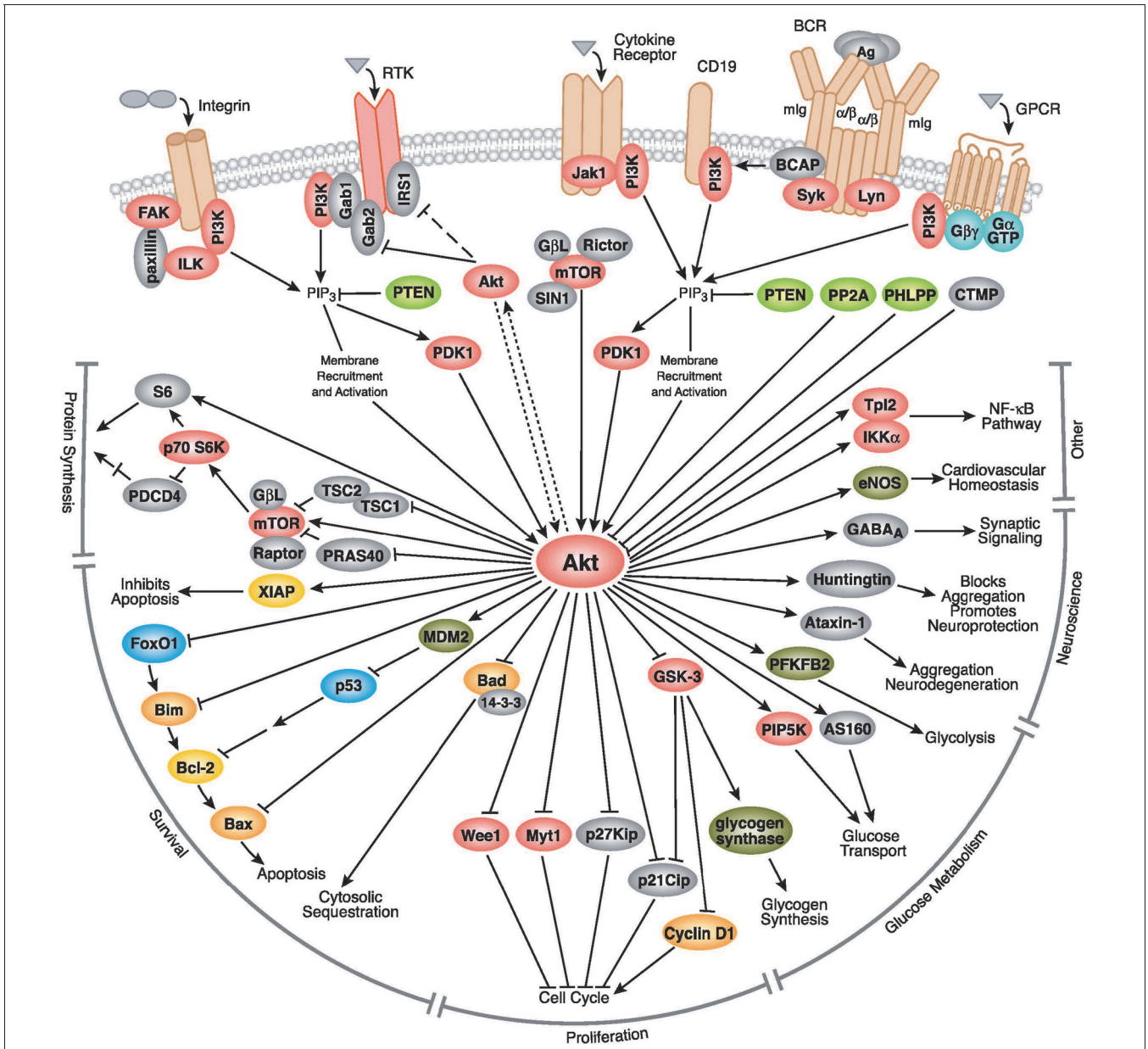

FIGURE 1 | The AKT signaling pathway. AKT is activated by PI3K, which itself is activated by several upstream signaling pathways such as insulin receptors, receptor tyrosine kinases, G protein coupled receptors, cytokine receptors, etc. After activation, it targets several downstream molecules and change their activity by phosphorylation or complex formation. AKT is involved in cell proliferation, glucose metabolism, cell survival, cell cycle, protein synthesis, and in neuronal morphology and plasticity by regulation of several downstream molecules shown in this figure. AKT is involved in cell proliferation through interaction with a number of proteins involved in cell cycle, including Cyclin D1, p21Cip, p27Kip, Myt1, and Wee1. AKT also plays a crucial role in cell survival through interaction with Bad, MDM2 and the subsequent regulation of p53, FoxO1, Bcl-2, and Bax. AKT regulates glucose metabolism through regulation of GSK-3, PFKFB2, PIP5K, and AS160. It could also affect protein synthesis through interaction with S6 kinase, TSC1, and mTOR signaling. In more recent years, AKT's role in neurodegeneration was uncovered, mediated through phosphorylation of Huntingtin and Ataxin-1. AKT also has a role in synaptic plasticity, by phosphorylation of $GABA_A$ receptor. Pathway diagram reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).

2009), there is not that much know about its association with brain disorders. The focus of most studies related to schizophrenia is on AKT1, however, we cannot rule out the possible role of other isoforms of AKT in schizophrenia (Birnbaum, 2004; Freyberg et al., 2010).

AKT also plays a crucial role in cell survival (Brazil et al., 2004). Enhanced AKT activity can foster cell survival against several toxic insults such as matrix detachment (Rytomaa et al., 2000), DNA damage Henry et al., 2001; Kang et al., 2003a,b; Chong et al., 2005, anti-Fas antibody administration (Suhara et al., 2001),

oxidative stress (Kang et al., 2003a,b; Chong et al., 2005), and transforming growth factor-ß application (Conery et al., 2004). Furthermore, many agents or growth factors can prevent apoptotic cellular injury through the activation of AKT. In the vascular system, angiopoietin-1 is an endothelium-specific ligand essential for embryonic vascular stabilization, branching, morphogenesis, and post-natal angiogenesis. Angiopoietin-1 also supports endothelial cell survival and prevents apoptosis through the activation of AKT (Papapetropoulos et al., 2000; Chong et al., 2005). In the cardiovascular system, myocardial protection by insulin during myocardial ischemia/reperfusion is eliminated by inhibition of PI3-K, suggesting that cardioprotection of insulin is mediated by AKT activation (Jonassen et al., 2001; Chong et al., 2005). Other trophic factors and cytokines, such as erythropoietin, may depend upon AKT to offer cellular protection (Chong et al., 2005).

AKT IN NEURONS

Signaling through protein phosphorylation is of importance for the normal development and function of the CNS. Protein phosphorylation is one of the most important mechanisms used in the nervous system for the regulation of the specificity and efficacy of neurotransmitter release from the presynaptic terminal in response to the nerve impulse (Greengard et al., 1993; Greengard, 2001).

AKT has a basic role in regulating neuronal cell size and survival (Franke, 2008; Niizuma et al., 2009; Freyberg et al., 2010). AKT is a critical survival factor that can modulate cellular pathways involved in apoptosis. Over-expression of AKT in cerebellar granule neurons prevents apoptosis during growth factor withdrawal (Dudek et al., 1997; Chong et al., 2005). However, expression of a dominant-negative AKT or inhibition of PI3-K attenuates neuronal cell survival normally supported by growth factors (Maiese and Chong, 2004; Chong et al., 2005). Several studies illustrated an important role for AKT for the survival of various neuronal cell types during cell injury. AKT promotes cell survival during free radical exposure in primary hippocampal neurons (Matsuzaki et al., 1999; Chong et al., 2005), neuronal cell lines (Kang et al., 2003a,b), and cerebral vascular endothelial cells (Chong et al., 2005).

During the past decade, there has been emerging evidence indicating several novel functions of AKT signaling pathway in the brain. In the CNS, AKT prevents injury-induced neuronal death and accelerates axonal regeneration (Namikawa et al., 2000). Moreover, it is shown that AKT is required for NGF-induced axon elongation and enhances axon branching (Markus et al., 2002). Intriguingly, AKT also plays a role in neuronal migration. Reelin, a large extracellular matrix protein responsible for positioning of cortical neurons, contributes to final neuron positioning in the mammalian brain by local modulation of AKT kinase activity (Beffert et al., 2002). The interesting study by Lin et al. (2001) provides a key piece in discovery of the roles played by AKT in the adult CNS. Their study shows AKT plays a crucial role in synaptic plasticity and memory formation. Another study by Mizuno et al. (2003) also shows that phosphorylation of BDNF receptor tyrosine kinase B (TrkB), PI3-K and its target AKT in the hippocampus increased in parallel with spatial memory formation (Mizuno et al., 2003). The study by Wang et al. (2003) uncovered a novel function of AKT in CNS. They discovered that AKT phosphorylates, both *in vitro* and *in vivo*, the type A GABA receptor, the principal receptor mediating fast inhibitory synaptic transmission, leading to increase in the number of GABA receptors on the plasma membrane surface, thereby increasing inhibitory fast synaptic transmission in neurons. Another suggested mechanism for AKT's role in synaptic plasticity and memory is through regulation of the mammalian target of rapamycin (mTOR). AKT is known to activate mTOR via phosphorylation and inhibition of the TSC2 subunit of the tuberous sclerosis complex (TSC). Interestingly, TSC2 heterozygous mice, in which the mTORC1 signaling pathways is constitutively active, exhibit impaired hippocampus-dependent memory that is reversible by rapamcyin treatment (Klann and Dever, 2004; Costa-Mattioli et al., 2009). Finally, the elegant study of Beaulieu et al. (2005) showed that AKT in complex with beta-arrestin 2 and PP2A mediates the dopaminergic neurotransmission and behavior.

Some other studies provided more interesting evidence showing the direct role of AKT is synaptic function and plasticity. AKT modulates synaptic plasticity and affects brain functions such as long-term potentiation, working memory, and fear conditioning (Lin et al., 2001; Lai et al., 2006; Sui et al., 2008; Niizuma et al., 2009; Freyberg et al., 2010). AKT also plays a role presynaptically through intracellular trafficking of biogenic amine transporters including the dopamine and norepinephrine transporters (Freyberg et al., 2010). Dopamine-mediated changes in AKT activity are thought to be mediated by postsynaptic dopamine D_2 receptors (Beaulieu et al., 2007). In Drosophila, a study by Guo and Zhong (2006) shows the requirement of AKT for mediation of long-term depression. Moreover, in the mouse hippocampus, it is been shown that activation of the PI3-kinase and AKT is required for metabotropic glutamate receptor-dependent long-term depression (Hou and Klann, 2004). More evidence in support of this finding is provided by Lee et al. (2005), which shows stimulation of postsynaptic density-95 (PSD-95) protein translation by insulin via the PI3-kinase and AKT signaling pathway. Prior to this study, another report by Akama and McEwen (2003) shows that estrogen can also stimulate PSD-95 rapid protein synthesis via AKT signaling pathway.

AKT SIGNALING IN SCHIZOPHRENIA

Since the initial report in 2004 (Emamian et al., 2004), many other studies have been published supporting that impaired AKT signaling plays an important role in the pathogenesis of schizophrenia. This study and several subsequent ones uncovered association between *AKT1* genetic variants and schizophrenia in case/control samples and family cohorts (Emamian et al., 2004; Ikeda et al., 2004; Schwab et al., 2005; Bajestan et al., 2006). The original study reported a significant association between schizophrenia and an *AKT1* haplotype in U.S. families (Caucasian of Northern European origin). The genetic association of AKT1 with schizophrenia was later confirmed in European (Schwab et al., 2005), Japanese (Ikeda et al., 2004), Middle Eastern (Bajestan et al., 2006), Chinese (Xu et al., 2007) and Irish (Thiselton et al., 2008) populations. Taken together, despite the presence

a negative report in a case-control Japanese population (Ohtsuki et al., 2004), it seems that the genetic association of AKT with schizophrenia is not limited to a specific genetic background and is reproducible in populations with different backgrounds.

Some studies have tried to study the correlations of the original SNPs reported in Emamian et al. (2004) with specific domains of human brain function. For example, Tan et al. (2008) examined the relationship of five original *AKT1* SNPs to cognition in healthy subjects. They found that variants of the gene are associated with differences in specific domains of cognitive function including IQ, executive functioning, and processing speed. The executive functioning domain of brain is one of cognitive domains affected in schizophrenia (Sadock et al., 2009). Interestingly, Tan et al. also found that the single SNPs that is associated with reduced expression of AKT1 in peripheral lymphocytes, is also associated with brain volume reductions in caudate and right prefrontal cortex (Tan et al., 2008).

AKT1 protein level is significantly reduced in different tissues from patients with schizophrenia. Lymphocytes from individuals with schizophrenia expressed 68% less AKT1 relative to control subjects (Emamian et al., 2004). More importantly, AKT1 was reduced in the hippocampus and frontal cortex in postmortem brain samples from those with schizophrenia relative to comparison subjects. This decrease in protein level in the brain was specific to AKT1 isoform and AKT2 and AKT3 levels were unaffected (Emamian et al., 2004). Although some microarray studies failed to find changes in AKT1 mRNA levels in the brain tissue of schizophrenics (Horváth et al., 2011), a number of studies provided convergent evidence of a decrease in AKT1 mRNA, protein, and activity levels in the prefrontal cortex and hippocampus, as well as in peripheral blood of individuals with schizophrenia (Emamian et al., 2004; Zhao et al., 2006; Thiselton et al., 2008). The activity of major AKT1 targets such as GSK3 was also found to be altered in individuals with schizophrenia (Emamian et al., 2004). Several studies, described below, have since been published that uncover specific molecular mechanisms underlying the involvement of AKT signaling pathway in schizophrenia.

GSK-3 SIGNALING IN SCHIZOPHRENIA

Some studies have reported changes in the protein or activity levels of GSK-3β in different tissues obtained from schizophrenia samples (Koros and Dorner-Ciossek, 2007). Yang et al first reported decreased levels of GSK-3α proteins in lymphocytes of individuals with schizophrenia (Yang et al., 1995). We saw a statistically insignificant decrease in GSK-3β total protein levels in lymphocyte of patients with schizophrenia (Emamian et al., 2004). Further analysis of postmortem brains from patients with schizophrenia confirmed decreased phosphorylation levels and GSK-3β protein levels in the frontal cortex, as well as GSK-3β mRNA levels in the dorsolateral prefrontal cortex (Emamian et al., 2004; Kozlovsky et al., 2004; Koros and Dorner-Ciossek, 2007).

Psychopharmacological studies provide the most promising evidence in support of the involvement of GSK-3β signaling in schizophrenia. Several studies have reported that chronic administration of antipsychotics phosphorylates GSK-3β at the Ser-9 residue, and inhibits its activity (Emamian et al., 2004; Koros and Dorner-Ciossek, 2007). More specifically, we saw chronic treatment of mice with haloperidol can increase phosphorylation of GSK-3β at Ser-9, which is associated with increased phosphorylation levels of AKT on both Thr-308 and Ser-473 phosphorylation sites in the frontal cortex (Emamian et al., 2004). Other studies also reported that chronic administration of clozapine, risperidone or haloperidol can enhance protein levels of β-catenin and GSK-3 as well as the inhibitory phosphorylation of GSK-3β in the rat prefrontal cortex and striatum (Alimohamad et al., 2005; Kozlovsky et al., 2006). Atypical antipsychotics after acute administration can increase phosphorylation of GSK-3β in the mouse brain (Li et al., 2007). Besides antipsychotics, lithium, and electroconvulsive shocks used to augment antipsychotic treatment in schizophrenic patients have been shown to lead to activation of AKT and concomitant inhibition of GSK-3β in rats (Kang et al., 2004; Gould, 2006).

Modulation of the activity of receptors of several neurotransmitters involved in schizophrenia can affect GSK-3β activity (Koros and Dorner-Ciossek, 2007). Administration of NMDA ligand can dephosphorylate GSK-3β at Ser-9 which leads to its activation (Luo et al., 2003). Consistent with this finding, non-competitive antagonists of NMDA receptors can inhibit GSK-3β by phosphorylation of Ser-9 site (Gould and Manji, 2005). Dopamine receptor antagonists as well as chemicals enhancing serotonergic transmission have been found to increase the expression and inhibitory phosphorylation of GSK-3β and activation of AKT in the mouse brain (Li et al., 2004, 2007).

A BRIEF REVIEW OF OTHER SIGNALING PATHWAYS INVOLVED IN SCHIZOPHRENIA

Besides the AKT/GSK3 signaling pathway described above, several other signaling molecules have been identified during the last decade to play an important role in schizophrenia. Signaling molecules generally act through a network of proteins to convey a signal from cell membrane to downstream molecules, such as transcription factors in the nucleus. Therefore, signaling molecules are functionally connected to each other and disruption in the function of one molecule could interfere with the efficacy and efficiency of signal transduction from cell membrane to the nucleus, which ultimately can result in the failure of cellular function. In this section DISC1, NRG1, and calcineurin are described as examples of intracellular molecules that are directly associated with schizophrenia through both genetic and functional studies. However, several other known signaling molecules are reported to be involved in schizophrenia but they are not discussed in this review due to the limitation of space.

DISRUPTED IN SCHIZOPHRENIA-1 (DISC1)

DISC1 is now widely accepted as one of the most important candidate genes for schizophrenia. A wide range of genetic, proteomic, and biochemical studies, have provided strong evidence in support of the involvement of this gene in the development of schizophrenia. This gene was initially reported in a pedigree of a large Scottish family with many individuals displaying a variety of psychiatric diagnoses including schizophrenia, bipolar disorder, recurrent depression, and conduct disorder (St Clair et al., 1990). Genomic analysis of the pedigree revealed the presence

of a t(1;11)(q42;q14) balanced chromosomal translocation which cosegregated with mental illness in affected individuals, and lead to a disruption of two genes, Disrupted In Schizophrenia-1 (DISC1) and Disrupted In Schizophrenia-2 (DISC2) (Millar et al., 2000; Kvajo et al., 2010). Linkage and association studies linked DISC1 variants to schizophrenia, schizoaffective disorder, and major depression (Allen et al., 2008). There are four splice variants of the DISC1 gene including the L (Long; 90 kDa), Lv (Long Variant; 95 kDa), S (Short; 70 kDa), and Es (Extremely Short; 41 kDa) (Taylor et al., 2003). The Scottish translocation occurs between exons 8 and 9 of the gene, truncating the C-terminal region of the major (L, Lv, and S) isoforms (Millar et al., 2000). The extremely short DISC1 isoform is present in the Scottish translocation carriers (Taylor et al., 2003; Kvajo et al., 2010).

At the protein level, DISC1 has an N-terminal domain with no clear structure, and a long helical C-terminal domain containing multiple regions with high coiled-coil forming potential, which can bind multiple proteins involved in different cellular processes (Millar et al., 2000). Recent studies provide evidence for the connection between DISC1 and AKT signaling pathways. For example, it has been recently shown that DISC1 regulates new neuron development in the adult brain via modulation of AKT-mTOR signaling through KIAA1212 (Kim et al., 2009). More importantly, it has been shown that DISC1 binds phosphodiesterase 4b (PDE4b), an enzyme which inactivates cAMP, and is believed to be the only way of cAMP degradation in the brain (Millar et al., 2005).

In terms of subcellular localization, DISC1 has been reported to localize in different compartments including the centrosome (Morris et al., 2003), mitochondria (James et al., 2004), spines and dendrites (Millar et al., 2005; Bradshaw et al., 2008), growth cones and axon terminals in neurons and neuronal like cells (Taya et al., 2007), stress granules (Ogawa et al., 2005) and the nucleus (Sawamura et al., 2005). This pattern of localizations lead to a suggestion that DISC1 may act as a molecular scaffold and serve as an assembly point for large protein complexes (Kvajo et al., 2010). During the development of the brain, DISC1 mRNA can be detected in all hippocampal subfields, the cortex, and the thalamus. In the adult brain, however, DISC1 displays a more restricted localization with a high expression only in the dentate gyrus of the hippocampus, and, to a lesser degree in the CA1 region of hippocampus (Austin et al., 2004).

A number of studies in human showed that healthy subjects and patients with schizophrenia carrying DISC1 risk alleles display changes in gray matter volume in the hippocampus, prefrontal cortex, and other brain regions (Callicott et al., 2005; Cannon et al., 2005; Di Giorgio et al., 2008; Szeszko et al., 2008). DISC1 risk alleles are also associated with deficits in working memory, short-term memory, and long-term memory (Burdick et al., 2005; Cannon et al., 2005; Hennah et al., 2005), attention (Hennah et al., 2005; Liu et al., 2006), and hippocampal and prefrontal cortical activation (Callicott et al., 2005; Prata et al., 2008).

Deficiency in DISC1 expression in mice caused region-specific phenotypes, including altered organization and complexity of newly born and mature neurons of the dentate gyrus, as well as changes in short-term plasticity in CA3/CA1 synapses (Kvajo et al., 2008). DISC1 deficient mouse shows highly selective impairment in working memory as well (Koike et al., 2006; Kvajo et al., 2008). Other mouse models of DISC1 have been generated that show selective cognitive behavioral phenotypes (Clapcote et al., 2007; Hikida et al., 2007; Pletnikov et al., 2007, 2008). Overexpression of truncated versions of the human DISC1 also provided evidence for anatomical and behavioral abnormalities (Hikida et al., 2007; Pletnikov et al., 2007, 2008). Moreover, mice carrying point mutations in the DISC1 gene demonstrated that allelic heterogeneity at the DISC1 locus can lead to distinct behavioral phenotypes (Clapcote et al., 2007; Kvajo et al., 2010).

NEUREGULIN 1 (NRG1)

Several review articles have been already published that discuss the genetic, neurobiological, and pathophysiologic studies of NRG1 and ErbB4 in schizophrenia (Mei and Xiong, 2008; Talmage, 2008; Jaaro-Peled et al., 2009; Banerjee et al., 2010). NRG1 is also one of the most important schizophrenia susceptibility genes, for which risk genotypes or haplotypes have been identified and its associations with the illness have been replicated in different populations (Mei and Xiong, 2008; Banerjee et al., 2010).

NRG1 has diverse neurobiological functions, some of which have been implicated in the pathophysiology of schizophrenia (Mei and Xiong, 2008; Talmage, 2008; Jaaro-Peled et al., 2009; Banerjee et al., 2010). These functions include neuronal migration, neuro-glial trophic effects, myelination and modulation of glutamatergic or GABAergic neurotransmitter systems (Mei and Xiong, 2008; Talmage, 2008; Jaaro-Peled et al., 2009; Banerjee et al., 2010). Moreover, there is genetic evidence for ErbB4, tyrosine kinase receptor, as a candidate susceptibility gene as well as a positive epistatic interactions between NRG1 and ErbB4 in schizophrenia. ErbB4 interacts with NRG1 and influences glutamatergic and GABAergic and neurotrophic transmissions. These neurotransmitter systems have all been implicated in the pathogenesis of schizophrenia. Therefore, there is a significant body of literature supporting the role of NRG1–ErbB4 signaling pathway in the development of schizophrenia (Mei and Xiong, 2008; Talmage, 2008; Jaaro-Peled et al., 2009; Banerjee et al., 2010).

While genetic, neurobiological and endophenotypic investigations support the role of the NRG1–ErbB4 pathway in schizophrenia, there are recent evidence showing that this pathway could act along with other signaling molecules involved in schizophrenia (Banerjee et al., 2010). Positive association of NRG1 with schizophrenia has been replicated in various populations. Some genotype–phenotype association studies have demonstrated links between risk genotypes of NRG1 and some of the disease manifestations of schizophrenia (Banerjee et al., 2010). However, most disease endophenotypes were not associated with the NRG1 or ErbB4 risk haplotypes, but rather with a single nucleotide polymorphism within the hapblocks (Mei and Xiong, 2008; Talmage, 2008; Jaaro-Peled et al., 2009; Banerjee et al., 2010). Interestingly, recent studies by Nicodemus et al. (2010) show evidence of increased schizophrenia risk with NRG1, ErbB4, and AKT1 epistasis via functional neuroimaging in healthy controls (Nicodemus et al., 2010). The same group also reported epistatic and

functional interactions of catechol-O-methyltransferase (COMT) and AKT1 on NRG1-ErbB signaling (Sei et al., 2010).

CALCINEURIN (PROTEIN PHOSPHATASE 2B OR PP2B)

Calcineurin is a calcium/calmodulin dependent Ser/Thr phosphatase and is widely expressed in the brain and other tissues. It dephosphorylates a large number of signaling proteins and, therefore, is involved in the regulation of several neuronal processes (Shibasaki et al., 2002). Calcineurin was first linked to schizophrenia by the study of Gerber et al. (2003). Gerber et al. studied a subset of calcineurin-related genes that map to loci previously implicated in schizophrenia by linkage studies and detected association of the PPP3CC gene, which encodes the calcineurin gamma catalytic subunit, with schizophrenia. Some studies could reproduce this finding (Goto et al., 1986; Horiuchi et al., 2007; Liu et al., 2007; Yamada et al., 2007) and some failed to show an association, possibly due to the power of the studies and the level of risk associated with altered calcineurin signaling (Kinoshita et al., 2005; Xi et al., 2007; Sanders et al., 2008; Kvajo et al., 2010). Regardless of genetic association, calcineurin is functionally important because it can affect the activity of several other signaling molecules involved in schizophrenia (described below).

MOLECULAR MECHANISMS UNDERLYING THE ROLE OF AKT/GSK3 PATHWAY IN SCHIZOPHRENIA

AKT is recruited to the cell membrane by binding to lipids generated by PI3 kinase (Brazil et al., 2004). In response to ligand binding to plasma membrane receptors of different tyrosine kinases, PI3K generates phosphatidylinositol 3,4,5 trisphosphate (PIP_3) at the cell surface, which leads to recruitment of AKT to the plasma membrane through its association with the newly generated PIP_3 (Brazil et al., 2004). AKT is subsequently activated by phosphorylation at Thr-308, catalyzed by PDK1. A second phosphorylation at Ser-473, by an unknown kinase, can augment its activity up to 10-folds (Brazil et al., 2004). Activated AKT phosphorylates a number of other molecules including the clinically relevant target, GSK3 (**Figure 1**, Brazil et al., 2004). GSK3 plays several roles in glucose metabolism, differentiation and development, intracellular trafficking, apoptosis, and regulation of gene transcription (Kockeritz et al., 2006). In the brain, some studies suggest that GSK3, like AKT, could modulate synaptic plasticity (Peineau et al., 2008). Unlike AKT that has to be phosphorylated before activation (Brazil et al., 2004), GSK3 is constitutively active in resting cells, requiring phosphorylation by kinases such as AKT to inactivate it (Doble and Woodgett, 2003).

Signaling through Wnt pathway has been also reported to be involved in schizophrenia. The Wnt signaling plays a critical role in a variety of developmental processes during embryonic development but recent studies have also suggested a role for the Wnt pathway in synaptic modulation and plasticity during the adult life (Hall et al., 2000). GSK-3β is an intermediate cytoplasmic component in Wnt pathway (Nelson and Nusse, 2004). GSK-3β forms a complex with multiple proteins such as β-catenin, APC and Axin (Lovestone et al., 2007). GSK-3β constitutively phosphorylates β-catenin, which results in its degradation and the subsequent inhibition of β-catenin-mediated gene transcription. Activation of Wnt receptor facilitates the physical separation of GSK-3β from β-catenin (Lovestone et al., 2007). This separation blocks phosphorylation of β-catenin by GSK-3β, which prevents degradation of β-catenin and causes its nuclear localization. In the nucleus, β-catenin regulates transcription and expression of specific proteins in the cell (Lovestone et al., 2007). Postmortem studies showed decreased in β-catenin levels (Lovestone et al., 2007) and an increased expression of Wnt-1 in the brain samples from patients with schizophrenia (Miyaoka et al., 1999). Taken together, many reports consistently showed the involvement of AKT-GSK3-Wnt pathway in schizophrenia, which makes them an attractive target for development of novel therapeutics for treatment of schizophrenia and other psychiatric disorders (Freyberg et al., 2010).

An interesting study by Mao et al. (2009) reported an interaction between DISC1 and GSK-3β that plays a role in neurogenesis during brain development and adulthood (Mao et al., 2009). Reduction of the expression of DISC1 in neurons decreases neurogenesis mediated by β-catenin signaling (Mao et al., 2009). More specifically, DISC1 can directly bind to GSK-3β and inhibit its activity. This inhibition interferes with its ability to phosphorylate β-catenin. It also affects GSK-3β's auto-phosphorylation at Y216. Interestingly, DISC1-mediated inhibition of GSK-3β affects a selective set of GSK-3β substrates and some of the well-characterized targets apparently are not affected. As discussed above, GSK-3β is immediate downstream molecule to the AKT and a key player of Wnt signaling pathways (Freyberg et al., 2010). This new interaction adds another interesting dimension in support of the convergent evidence for the involvement of AKT/GSK3 signaling pathway in schizophrenia that needs to be dissected out with further studies.

Dopamine receptors have been the center of attention of several groups to investigate how AKT is involved in schizophrenia. Dopamine D_2 class receptors (D_{2-4} receptors) can modulate the activity of AKT (Beaulieu et al., 2007). Activation of dopamine D_2 receptor by dopamine leads to recruitment of β-arrestin 2, a scaffolding protein, which facilitates receptor desensitization and the subsequent internalization (Beaulieu et al., 2005). AKT is also recruited into this complex along with the phosphatase PP2A. PP2A dephosphorylates and inactivates AKT, leading to an increased GSK3 activity (Beaulieu et al., 2004, 2005; Klewe et al., 2008; Skinbjerg et al., 2009). Beaulieu et al. also showed that activation of dopamine D_2 receptor can inhibit AKT activity through an arrestin-dependent but G protein-independent pathway (Beaulieu et al., 2005, 2007). This interesting observation shed new light on the mechanisms of antipsychotic action of the D_2 receptor blockers. In other words, antipsychotic medications may prevent β-arrestin 2 recruitment through blocking dopamine D_2 receptor (Klewe et al., 2008; Masri et al., 2008) and the subsequent activation of AKT. The same group has shown similar function for lithium, which is essentially a mood stabilizer not an antipsychotic drug. Lithium has been shown to disrupt the β-arrestin 2/AKT/PP2A complex, thereby preventing dopamine-induced dephosphorylation of AKT, which is associated with reversal of amphetamine-induced locomotion (Beaulieu et al., 2005). Moreover, amphetamine-induced locomotion is greatly diminished in β-arrestin 2 knockout mice, suggesting that this pathway is critical to at least some psychostimulant

effects (Beaulieu et al., 2005). Several studies have shown that schizophrenia and bipolar mood disorder (BMD) share several genes and pathways that are critically involved in both disorders. However, the clinical phenotypes of these two disorders are very different. It is not clear how two entirely different classes of psychotropic drugs, i.e., antipsychotics and mood stabilizers, act through similar signaling pathways but produce completely different clinical pictures.

TRANSCRIPTION FACTORS RELATED TO SCHIZOPHRENIA

AKT and GSK3 can modulate the activity of several transcription factors; therefore, they can change the expression patterns of many genes that are regulated by those transcription factors. Some transcription factors are reported to be directly associated with schizophrenia. Association between Transcription Factor 4 (TCF4) and schizophrenia has been recently reported, which influences verbal learning and memory functioning in schizophrenia patients (Lennertz et al., 2011; Williams et al., 2011). Variants of the v-rel avian reticuloendotheliosis viral oncogene homolog A (RELA) gene are associated with schizophrenia and their startle responses (Hashimoto et al., 2011). RELA encodes the major component of the nuclear factor kappa B (NF-κB) complex that has important roles in the immune and inflammatory responses. The association between RELA gene and schizophrenia was evident in male subjects but not in female subjects (Song et al., 2009; Hashimoto et al., 2011). Postmortem studies showed decreased in β-catenin levels (Lovestone et al., 2007) and an increased expression of Wnt-1 in the brain samples from subjects with schizophrenia (Miyaoka et al., 1999). Georgieva et al. (2006) provided convergent evidence for the involvement of transcription factor oligodendrocyte lineage 2 (OLIG2) in schizophrenia. They show variation in OLIG2 confers susceptibility to schizophrenia alone and as part of a network of genes implicated in oligodendrocyte function (Georgieva et al., 2006).

Some important signaling molecules that show association with schizophrenia could affect transcriptional activity of other transcription factors as well. For example, calcineurin (PP2B) can directly regulate the activity of CREB and Nuclear Factor of Activated T cells (NFATc), which are both involved in cognitive functions known to be impaired in schizophrenia. CREB not only is known to be involved in depression (Carlezon et al., 2005), but also some variants in the promoter region of CREB in patients with schizophrenia have been reported as well (Kawanishi et al., 1999). Kawanishi and colleagues analyzed the promoter region of the human CREB in schizophrenics and healthy subjects and found two novel variants (−933T–>C and −413G–>A) only in schizophrenics. Calcineurin can activate the transcription factor NFAT, which has a role in neuronal growth and axonal guidance during development *in vitro*. Interestingly, NFAT mutant mice shows severe neurodevelopmental defects *in vivo* (Graef et al., 2001).

CONCLUSION

Review of the literature published during the past few years provide strong support for the involvement of AKT/GSK3 signaling pathway in the development of schizophrenia. A number of studies strongly suggest targeting this pathway is a promising approach for development of novel psychotropic drugs for treatment of schizophrenia and mood disorders. At the same time, there are several other signaling molecules and pathways that show conclusive evidence for being involved in pathogenesis of schizophrenia. Despite many differences in the approach of scientists working on schizophrenia, they have come to a general consensus that schizophrenia is most likely caused by the altered function or expression of many genes. Such genes may individually contribute only to a small risk, but their cumulative effects cause the dysfunction of brain, which manifests itself by the clinical picture we call schizophrenia. Although there has been significant progress in identification of the role of several signaling molecules in schizophrenia, we still do not know how much each gene contributes to the development of pathology. Therefore, we need to develop novel systems biology tools that can quantify the role of individual or multiple genes in disease development. The recently developed fault diagnosis engineering technology in molecular networks is a promising tool that has such capabilities and can model complex trait disorders such as schizophrenia (Abdi et al., 2008, 2009; Emamian and Abdi, 2009; Abdi and Emamian, 2010).

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Glycogen synthase kinase-3: a promising therapeutic target for fragile X syndrome

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Recent advances in understanding the pathophysiological mechanisms contributing to fragile X syndrome (FXS) have increased optimism that drug interventions can provide significant therapeutic benefits. FXS results from inadequate expression of functional fragile X mental retardation protein (FMRP). FMRP may have several functions, but it is most well-established as an RNA binding protein that regulates translation, and it is thought that by this mechanism FMRP is capable of affecting numerous cellular processes by selectively regulating protein levels. The multiple cellular functions regulated by FMRP suggest that multiple interventions may be required for reversing the effects of deficient FMRP. Evidence that inhibitors of glycogen synthase kinase-3 (GSK3) may contribute to the therapeutic treatment of FXS is reviewed here. Lithium, a GSK3 inhibitor, improved function in the Drosophila model of FXS. In mice lacking FMRP expression (FX mice), GSK3 is hyperactive in several brain regions. Significant improvements in several FX-related phenotypes have been obtained in FX mice following the administration of lithium, and in some case other GSK3 inhibitors. These responses include normalization of heightened audiogenic seizure susceptibility and of hyperactive locomotor behavior, enhancement of passive avoidance learning retention and of sociability behaviors, and corrections of macroorchidism, neuronal spine density, and neural plasticity measured electrophysiologically as long term depression. A pilot clinical trial of lithium in patients with FXS also found improvements in several measures of behavior. Taken together, these findings indicate that lithium and other inhibitors of GSK3 are promising candidate therapeutic agents for treating FXS.

Keywords: glycogen synthase kinase-3, GSK3, fragile X syndrome, FXS, autism

INTRODUCTION

During the last few years there has been tremendous progress in understanding the pathological mechanisms underlying fragile X syndrome (FXS). This knowledge has provided several leads of potential interventions that may be therapeutic in FXS. One of these is lithium and possibly other inhibitors of glycogen synthase kinase-3 (GSK3), and this evidence is discussed in this review.

FRAGILE X SYNDROME: ETIOLOGY AND PATHOLOGY

Fragile X syndrome is the most common hereditary form of mental retardation caused by a single genetic defect, the loss of expression of the *fragile X mental retardation 1* (*fmr1*) gene (Pieretti et al., 1991; Kooy et al., 2000; Bardoni and Mandel, 2002). FXS is caused by expansion of a trinucleotide CGG repeat in the 5′ UTR of the *fmr1* gene. This expansion appears as a weak, or fragile-like, end on the X chromosome. Normally there are ∼5 to ∼44 CGG repeats containing occasional AGG triplets, with 29 or 30 being most common (Maddalena et al., 2001). Alleles in the range of 45–54 repeats are considered to be in a gray, or inconclusive, zone; premutation alleles range from 55 to 200–230 CGG repeats, which may reduce translation efficiency of the *FMR1* gene (Feng et al., 1995); and full mutations associated with FXS have over 200–230 CGG repeats, typically containing several hundred or thousand triplet repeats (Maddalena et al., 2001). The extended CGG repeats in FXS are hypermethylated, silencing gene transcription and resulting in loss of the fragile x mental retardation protein (FMRP). FMRP plays important roles in RNA binding and translation regulation, as well as regulation of extracellular transport and sodium-activated potassium channels (Brown et al., 1998, 2010; Bardoni et al., 2000; Laggerbauer et al., 2001).

Since FXS is an X-linked developmental disorder, its incidence is higher in males than females, affecting ∼1 in 4000 males and ∼1 in 7000 females (Crawford et al., 2001). Transmission of the affected *fmr1* allele may occur to female offspring from an affected male and to both male and female offspring from affected females. FXS is characterized by several physical, mental, and behavioral abnormalities. Prominent physical characteristics include overly pronounced ears, an elongated jaw, double-jointed/hyperextensible fingers, flat feet, low muscle tone, and macroorchidism. Sleep disturbances, inattentiveness, hyperactivity, impaired cognition, seizure susceptibility, and autistic-like behaviors, including developmental delays, communication impairments, and anxiety, are common characteristics of patients with FXS.

ANIMAL MODELS OF FXS

The most common animal models used to study FXS include mouse models (Bakker et al., 1994) and Drosophila models (Wan et al., 2000; Zhang et al., 2001). The first mouse model was developed by Bakker et al. (1994), who generated mice with an inactive *Fmr1* gene (FX mice). With these and other FMRP knockout

mice, FX mice have been shown to display characteristics with some similarities to patients with FXS, including macroorchidism, certain features of behavior, and some cognitive impairments. However, the impairments in measures of cognition that have been assessed in FX mice are modest compared to patients with FXS, although a recent report identified a significant impairment in prefrontal cortex-dependent cognition in FX mice (Krueger et al., 2011). FX mice also exhibit increased dendritic spine length and number, but reduced maturation of spines, compared to wild-type littermates (Comery et al., 1997; Irwin et al., 2001, 2002). Autistic-like behaviors characteristic of patients with FXS, and increased susceptibility to audiogenic seizures also occur in FX mice (Musumeci et al., 2000; Yan et al., 2004; Bernerdet and Crusio (2006). The use of Drosophila to study FXS was initiated by Wan et al. (2000), who identified *dfmr1* as the invertebrate family member of the FMR1/FXR gene family. Over-expression of RNA binding-deficient mutant *dfmr1* in Drosophila caused wing deformities, including loss of anterior cross-veins longitudinal veins, and severe rough eye, suggesting altered cell fate determination and proliferation, and increased apoptosis. Drosophila FXS models lacking expression of dFMRP or over-expressing a loss-of-function dFMRP mutant were characterized by Zhang et al. (2001). Over-expression of loss-of-function mutant dFMRP produced an abnormal wing span or up-held wings, impaired coordination, caused early death, and dFMRP-null flies displayed exaggerated synaptic outgrowth (Zhang et al., 2001). Knockout and mutation of *dfmr1* also affected neurotransmission and increased peripheral synaptic transmission while decreasing central synaptic transmission. Thus, both the mouse and fly models of FXS have provided valuable information about the pathology of FXS and contributed to recent advances in developing potential therapeutic interventions for FXS.

GLYCOGEN SYNTHASE KINASE-3

Glycogen synthase kinase-3 is a serine/threonine kinase that exists in two isoforms, GSK3α and GSK3β (Woodgett, 1990). Regulation of GSK3 is primarily mediated by inhibitory serine-phosphorylation, specifically at ser21 of GSK3α and ser9 of GSK3β. The inhibitory serine-phosphorylation of GSK3 is induced by a wide variety of signaling pathways that converge on GSK3. Impairments in these pathways can lead to inadequate inhibition of GSK3, causing hyperactive GSK3, which may contribute to a number of diseases, such as Alzheimer's disease, diabetes, and mood disorders (Jope and Johnson, 2004; Mines et al., 2011). Studies of the actions of GSK3 were accelerated by the discovery that lithium, the classical treatment for bipolar disorder, is a selective inhibitor of GSK3 (Klein and Melton, 1996; Stambolic et al., 1996). Lithium both directly inhibits GSK3 activity and also increases the inhibitory serine-phosphorylation of GSK3 (Jope, 2003). Studies of the effects of lithium contributed immensely to the identification of many of the known actions of GSK3 and to revelations that inadequately inhibited GSK3 is linked to a number of diseases, which includes FXS, as is reviewed here. Connecting GSK3 to pathological processes promoted the development of many new, small molecule selective inhibitors of GSK3 (Martinez et al., 2006). Some GSK3 inhibitors that have been used fairly extensively in experimental studies include indirubin derivatives (Leclerc et al., 2001), L803-mts (Plotkin et al., 2003), SB216763 (Coghlan et al., 2000), TDZD derivatives (Martinez et al., 2002), paullone derivatives (Leost et al., 2000), AR-A014418 (Bhat et al., 2003), and CT99021 (Wagman et al., 2004), the most specific GSK3 inhibitor that has been described (Bain et al., 2007). Of these, only a TDZD derivative called tideglusib is currently in trials in humans (Martinez et al., 2011), and none has yet been tested in patients with FXS.

AMELIORATION OF FXS-ASSOCIATED BEHAVIORAL ABNORMALITIES BY LITHIUM AND OTHER GSK3 INHIBITORS

McBride et al. (2005) reported that lithium treatment ameliorated impairments in courtship behavior in the Drosophila model of FXS. This was a critical finding because it first raised the possibility that lithium may be therapeutic for FXS. A subsequent report confirmed the rescue of FXS-associated impairments in the Drosophila model of FXS by lithium treatment and demonstrated that this rescue was sustainable throughout the aging process (Choi et al., 2009). Since lithium both inhibits GSK3 and modulates phosphoinositide signaling (Jope, 1999), the key target for its effects in the Drosophila model of FXS has yet to be established.

Direct evidence that GSK3 may be involved in the pathology of FXS and be a target for the development of treatments for FXS was obtained in studies of the regulation of GSK3 in brain regions from FX mice. Assessments of the levels of serine-phosphorylation of GSK3 revealed that adult FX mice had lower levels of inhibitory phospho-ser21-GSK3α and phospho-ser9-GSK3β in several brain regions compared to wild-type littermates (Min et al., 2009; Yuskaitis et al., 2010a). However the total protein levels of GSK3α and GSK3β were equivalent in FX and wild-type mice, demonstrating that GSK3 expression is normal in FX mouse brain, but the inhibitory control of GSK3 is impaired. The impairment in the regulation of GSK3 was evident in adult FX mice on both the FVB and C57Bl6 backgrounds, thus this is a robust change not dependent on mouse strain, and reduced inhibitory serine-phosphorylation of GSK3 in FX mice was also found in an analysis of whole brain GSK3 phosphorylation levels (Liu et al., 2011). Moreover, impaired inhibitory serine-phosphorylation of GSK3 in adult FX mice was corrected by acute or chronic treatment with lithium (Min et al., 2009; Yuskaitis et al., 2010a; Liu et al., 2011).

The effects of lithium, and in some cases other GSK3 inhibitors, have been tested on several behavioral characteristics of FX mice. Increased susceptibility to audiogenic seizures, which often evolve to lethal status epilepticus, is a well-established phenotype of FX mice (Musumeci et al., 2000). Treatment with lithium dose-dependently reduced audiogenic seizure susceptibility in 4-week-old FX mice, but did not alter the responses in wild-type mice (Min et al., 2009). The occurrence of status epilepticus was also dose-dependently reduced by lithium in FX mice (Min et al., 2009). To assess whether decreased audiogenic seizure susceptibility was a result of lithium-mediated inhibition of GSK3, the effects of two additional GSK3 inhibitors were investigated. Treatment with the selective GSK3 inhibitors AR-A014418 (Bhat et al., 2003) or SB216763 (Coghlan et al., 2000) normalized audiogenic seizure susceptibility in FX mice similarly to the effect of lithium treatment. The finding that three structurally diverse GSK3 inhibitors

were able to reduce audiogenic seizure susceptibility in FX mice, without affecting audiogenic seizure susceptibility in wild-type mice, suggested that the hyperactive GSK3 identified in FX mouse brain is likely mediating the phenotypic seizure abnormalities in FX mice (Min et al., 2009).

Locomotor hyperactivity is a hallmark characteristic of FXS that is also evident in FX mice (Bakker et al., 1994). Therefore, the effects of lithium were examined in FX mice to test if locomotor hyperactivity was ameliorated. Untreated FX mice displayed increased hyperactivity in the open field paradigm (Min et al., 2009; Yuskaitis et al., 2010a; Liu et al., 2011), including significant increases in total line crosses, in center square duration, and in center square entries, and a significant decrease in outer zone line crosses compared with wild-type mice (Min et al., 2009). Administration of SB216763 to inhibit GSK3 did not alter the activity of wild-type mice in the open field, but normalized the total line crosses, center square entries, center square duration, and outer zone line crosses in FX mice (Min et al., 2009). Chronic lithium administration at a therapeutically relevant dose also normalized to wild-type levels the total ambulatory distance traveled by FX mice in the open field, without altering the distance traveled by wild-type mice (Yuskaitis et al., 2010a; Liu et al., 2011). The correction of locomotor hyperactivity of FX mice by two GSK3 inhibitors suggests that hyperactive GSK3 in FX mice makes a significant contribution to this phenotype.

The effect of lithium treatment on the behavior of adult FX and wild-type mice on the elevated plus maze paradigm also has been assessed. This behavioral paradigm uses a cross shaped apparatus consisting of two open arms and two closed arms. Typically, this apparatus is often used to estimate anxiety displayed by hesitation to explore the open arms resulting in increased time spent in the closed arms, although interpretations of the results remain complicated, especially with mice that exhibit locomotor hyperactivity. FX mice spent significantly more time in the open arms and less time in the closed arms than wild-type mice, which would classically be interpreted as exhibition of less anxiety (Yuskaitis et al., 2010a; Liu et al., 2011), but FX mice also displayed an increase in closed arm entries, classically interpreted as increased anxiety (Yuskaitis et al., 2010a). Thus, the locomotor hyperactivity of FX mice may preclude clear interpretations of the measurements using the elevated plus maze paradigm. Despite the difficulty in interpreting the behaviors, administration of lithium normalized behavior in the elevated plus maze paradigm in FX mice to the behavior of wild-type mice (Yuskaitis et al., 2010a; Liu et al., 2011). The elevated zero maze was also used to assess anxiety-like behaviors in FX mice (Liu et al., 2011). The elevated zero maze has two closed quadrants and two open quadrants, and the time spent in the open or closed portions is indicative of the presence or absence of anxiety or fearfulness versus exploratory behaviors. FX mice spent more time in the open quadrants than wild-type mice, and lithium treatment reduced the time spent in the open quadrants, eliminating the difference between FX mice and wild-type mice (Liu et al., 2011). Thus, FX mice display abnormal behavior in each of these tests that is normalized by lithium, but relating these behaviors to the behavior of subjects with FXS remains difficult.

Another common characteristic of patients with FXS is the presence of autistic-like behaviors (Hagerman et al., 2005; Belmonte and Bourgeron, 2006; Hatton et al., 2006). FXS is the most common known genetic cause of autism spectrum disorders (ASDs), which can involve developmental delays, communication impairments, anxiety, and impaired social behaviors. Accordingly, social behavior deficits have been extensively described in FX mice (Mineur et al., 2002, 2006; Spencer et al., 2005; Bernerdet and Crusio, 2006; McNaughton et al., 2008; Liu and Smith, 2009; Moy et al., 2009). The effects of lithium treatment on social behavior were studied using the two-phase social interaction behavior paradigm (McNaughton et al., 2008). This test consists of a sociability phase 1, the introduction of one novel stimulus mouse (S1), and a social preference phase 2, the introduction of a second stimulus mouse (S2). Although during the sociability phase FX mice generally behaved as wild-type mice, lithium treatment increased sociability measures in both wild-type and FX mice (Mines et al., 2010; Liu et al., 2011), increasing the time in the socializing chamber (Mines et al., 2010; Liu et al., 2011) and increasing social approach assessed by the number of nose contacts (Mines et al., 2010) and by the time spent sniffing the stimulus mouse (Liu et al., 2011). In the social preference phase 2, wild-type mice display preference for S2 over S1, but FX mice lacked this preference and spent equivalent times with S1 and S2 (Mines et al., 2010; Liu et al., 2011) and displayed a lower number of nose contacts with S2 and time sniffing S2 than wild-type mice (Mines et al., 2010; Liu et al., 2011). These abnormal behaviors in the social preference assessment of FX mice were significantly normalized by chronic lithium treatment (Mines et al., 2010; Liu et al., 2011). Lithium treatment also appeared to modestly reduce displays of social anxiety by FX mice (Mines et al., 2010). To test if hyperactive GSK3 might contribute to the altered social behaviors exhibited by FX mice, in addition to administering lithium to inhibit GSK3, another model of hyperactive GSK3 was used, GSK3 knockin mice. These mice have serine-to-alanine mutations in the regulatory serines of GSK3α and GSK3β, so the mice express constitutively active GSK3 that cannot be inhibited by serine-phosphorylation (McManus et al., 2005). These mice also displayed no altered behavior in the sociability phase 1 test, but displayed impairments in the social preference phase 2 similar to those of FX mice (Mines et al., 2010). Collectively, these studies suggest that GSK3 inhibition may be useful in reducing impairments in social behaviors and social anxiety in FXS.

GSK3 AND MORPHOLOGY IN FX MICE

In addition to improving abnormal behaviors in FX models, lithium treatment also modified structural and anatomical characteristics of FX mice. Macroorchidism is a common feature of FXS that is replicated in FX mice (Bakker et al., 1994; Comery et al., 1997). Chronic administration of lithium significantly reduced testicular weight in adult FX mice, but not in adult wild-type mice (Yuskaitis et al., 2010b). Increased dendritic spine length with altered morphology are characteristic of FXS and replicated in FX mice (Comery et al., 1997). Liu et al. (2011) confirmed increased apical and basal dendritic spine length in FX mice as compared to wild-type littermates. Furthermore, they found that lithium treatment normalized dendritic spines in FX mice (Liu et al., 2011). Reactive astrogliosis has been reported in postmortem brains of patients with ASDs (Laurence and Fatemi, 2005; Vargas et al., 2005). Yuskaitis et al. (2010b) reported that adult FX mice

displayed increased levels of glial fibrillary acidic protein (GFAP), a classical marker of astrogliosis, in several brain regions, including the striatum, hippocampus, and cortex, and that chronic lithium treatment reduced GFAP levels in both adult FX and wild-type mice. Although the mechanism for this effect of lithium was not examined, the transcription factor signal transducer and activator of transcription-3 (STAT3) promotes GFAP expression and is inhibited by inhibitors of GSK3, including lithium (Beurel and Jope, 2008), raising the possibility that this action accounted for the reduced levels of GFAP *in vivo* after lithium treatment.

GSK3, mGLuRs, AND SYNAPTIC PLASTICITY

Alterations in synaptic plasticity have been a focus of studies of FXS since early reports found that FMRP may be important for normal maturation of synaptic connections (Weiler et al., 1997; Weiler and Greenough, 1999; Antar et al., 2004) and FX mice displayed enhanced metabotropic glutamate receptor (mGluR)-dependent long term depression (LTD) in the CA1 region of the hippocampus (Huber et al., 2002). These and other reports (e.g., McBride et al., 2005; Yan et al., 2005; Dölen et al., 2007) have strengthened the mGluR theory of FXS (Bear et al., 2004), which proposes that many of the protein synthesis-dependent functions of metabotropic receptors are exaggerated in FXS. This has led to the development of mGluR5 antagonists as potential therapeutics for FXS. The mGluR5 antagonist MPEP (2-methyl-6-phenylethynyl-pyridine) has often been used in studies of FX mice, and MPEP treatment rescues a number of impairments, such as heightened audiogenic seizure susceptibility (Yan et al., 2005). Surprisingly, administration of MPEP also increased the inhibitory serine-phosphorylation of GSK3 in FX mouse brain, while having little effect in wild-type mice (Min et al., 2009; Yuskaitis et al., 2010a). This finding indicated that mGluR5 signaling to GSK3 is abnormal in FX mice, and demonstrated an overlap in the effect of MPEP with GSK3 inhibitors. Furthermore, Choi et al. (2011) found that lithium treatment ameliorated enhanced mGluR-mediated LTD at CA1 synapses in FX mice. Although the mechanism underlying this action of lithium was not examined, that it might be due to inhibition of GSK3 is consistent with the finding that GSK3 promotes LTD (Peineau et al., 2007). Taken together, these studies lend further support to the possibility that inhibition of GSK3 may be a beneficial therapeutic intervention for FXS.

CLINICAL TRIALS

The promising reports that lithium corrects FX-associated abnormalities in flies and mice was corroborated in a pilot clinical trial of lithium in patients with FXS (Berry-Kravis et al., 2008). The use of lithium in humans is well-established because it has been used clinically for 60 years in the treatment of mood disorders, especially bipolar disorder (Jope, 1999). Berry-Kravis et al. (2008) assessed the clinical effects of lithium in patients with FXS given lithium carbonate orally with doses adjusted to obtain a serum level of 0.8–1.2 mEq/L for the final 4 weeks of a 2-month trial. This pilot trial found that aggression, anxiety, mood swings, tantrums, and abnormal outbursts were improved in lithium-treated patients. Care-giver ratings indicated decreases in hyperactivity and inappropriate speech. Improvements in lethargy and stereotypy were also observed in patients given lithium, as compared to baseline behaviors previously recorded (Berry-Kravis et al., 2008). Overall, these findings further bolster the possibility that lithium, and perhaps other GSK3 inhibition, may provide therapeutic benefits in FXS.

POTENTIAL TARGETS OF GSK3 THAT MAY CONTRIBUTE TO FXS ABNORMALITIES

Since GSK3 was only recently found to be hyperactive in FX mouse brain and to be a potential therapeutic target for FXS, identification of the mechanisms by which GSK3 may contribute to FXS phenotypes awaits future investigations. However, several possibilities can be suggested representative of the cellular functions regulated by GSK3 and the abnormalities that have been identified in FX mice.

Regulation of cellular cytoskeleton dynamics provides one potential mechanism by which hyperactive GSK3 could contribute to abnormalities in FXS brain. Prevalent among GSK3 substrates are microtubule-associated proteins (MAPs) that regulate dynamic changes in neuronal plasticity, which is thought to be abnormal in FXS. Microtubules are polymers of the protein tubulin composing networks that maintain the structure and spatial organization of cells and that provide mechanisms for transporting organelles and protein complexes within cells. Microtubules are dynamic structures capable of rapid changes mediated by increased tubulin polymerization or, oppositely, by microtubule depolymerization. These changes occur in response to signals that regulate MAPs, which reversibly bind microtubules and regulate microtubule stability, allowing cells to change shape, extend or retract processes, and to move. Among the MAP family, the protein tau is the most widely studied substrate of GSK3 (Hong et al., 1997; Cho and Johnson, 2004), primarily because of its links to Alzheimer's disease (Johnson and Bailey, 2002). There are many phosphorylation sites on tau, and in general tau phosphorylation reduces its binding to microtubules, whereas dephosphorylated tau tends to bind and stabilize microtubules. Tau binding stabilizes microtubules, and phosphorylation by GSK3 causes tau to dissociate from microtubules, which destabilizes microtubules. Thus, hyperactive GSK3 in FX brains may reduce tau binding to microtubules. Also, a dramatic ∼50% loss of tau protein has been reported in FX cortical neurons (Liao et al., 2008), which could result from its degradation following phosphorylation by GSK3. Considering together the possibilities of tau hyperphosphorylation by GSK3, which dissociates tau from microtubules, and the lower level of tau available to bind and stabilize microtubules, these alterations could contribute to deficits in structural plasticity that may contribute to impaired neuronal plasticity in FXS. Additionally, cyclin-dependent kinase-5 (Cdk5) levels are low in FX cortical neurons (Liao et al., 2008) and normally Cdk5 suppresses GSK3 activity through neuregulin and Akt signaling to reduce tau phosphorylation (Wen et al., 2008). Thus, the reduced Cdk5 levels in FX brain may contribute to hyperactive GSK3 and abnormal phosphorylation of tau. Similarly to tau, GSK3 also phosphorylates and regulates the function of other proteins that bind and regulate microtubules, including MAP1B (Garcia-Perez et al., 1998; Lucas et al., 1998). Notably, MAP1B was one of the earliest targets found to be regulated by FMRP, and aberrantly regulated MAP1B leads to abnormally increased microtubule stability in FX neurons

(Zhang et al., 2001; Lu et al., 2004). Thus, abnormal phosphorylation by GSK3 of tau, MAP1B, or other known cytoskeletal protein substrates of GSK3, such as kinesin (Morfini et al., 2002) and collapsin response mediator protein-2 (Cole et al., 2007), could lead to abnormal microtubule dynamics and function that may contribute to abnormal neural plasticity in FXS.

Glycogen synthase kinase-3 is known to regulate by phosphorylation the activity of a relatively large number of transcription factors (Grimes and Jope, 2001a). Thus, altered transcription caused by aberrant transcription factor regulation by hyperactive GSK3 in FXS brain may exacerbate abnormal translation caused by loss of FMRP. Two representative examples that may be important in FXS are cyclic AMP response element binding protein (CREB) and Heat Shock Factor-1 (HSF-1). CREB regulates many critical processes, such as formation of long term memory and maintenance of synaptic plasticity (Alberini, 2009; Benito and Barco, 2010), both key processes that are impaired in FXS. CREB is active when phosphorylated on serine-133, and this phosphorylation creates a site for GSK3 to bind and phosphorylate serine-129, which inactivates CREB (Wang et al., 1994; Grimes and Jope, 2001b). Thus, hyperactive GSK3 in FXS brain may reduce the learning and neural plasticity functions of CREB by abnormally inactivating CREB. FMRP over-expression increases cyclic AMP production (Berry-Kravis and Ciurlionis, 1998), cyclic AMP production is defective in platelets from patients with FXS (Berry-Kravis and Sklena, 1993), induced levels of cyclic AMP are substantially reduced in platelets and in brains of *Fmr1* knockout mice and in human FXS neural cells (Kelley et al., 2007), and using the drosophila model of FXS, Dockendorff et al. (2002) reported that *dfmr1* is required for normal CREB activity. Thus, CREB activation may be impaired in FXS brain and further impaired by the inhibition induced by GSK3. The transcription factor HSF-1 is activated in response to many stressors because it is a crucial component of cellular mechanisms responding to abnormal or misfolded protein accumulation. Since FMRP is thought to suppress translation of many proteins, its deficiency in FXS may result in aberrantly expressed proteins. Activation of HSF-1 in response to abnormal protein accumulation is important because it controls the expression of heat shock proteins, such as hsp70, which chaperone proteins to prevent their accumulation and aggregation within cells. The action of HSF-1 may be impaired by hyperactive GSK3 in FXS brain because GSK3 phosphorylates HSF-1, resulting in its inactivation (Chu et al., 1996; Bijur and Jope, 2000; Xavier et al., 2000). Thus, these two transcription factors exemplify mechanisms by which hyperactive GSK3 in FXS brain may exacerbate mechanisms contributing to the impairments associated with FXS.

The amyloid precursor protein (APP) regulates synaptic function in neurons and also is proteolyzed to amyloid-β peptides (Aβ) that can be toxic, particularly in the realm of Alzheimer's disease. FMRP was found to repress APP translation (Westmark and Malter, 2007; Lee et al., 2010), and large increases in APP levels were found in cortical extracts from 14-day-old *Fmr1* knockout mice (Liao et al., 2008). Examination of the levels of soluble amyloid-β peptides demonstrated elevated levels in multiple strains of *Fmr1* knockout mice (Westmark and Malter, 2007), which have been suggested to contribute to impairments associated with FXS (Malter et al., 2010). These findings may be associated with the detrimental actions of GSK3 in FXS because APP is phosphorylated by GSK3 (Aplin et al., 1996; Wen et al., 2008). Importantly, GSK3 has been reported to promote the processing of APP to amyloid-β peptides (Phiel et al., 2003; Su et al., 2004). Since GSK3 inhibitors reduce the production of amyloid-β peptides, this may contribute to their therapeutic effects if amyloid-β peptides contribute to impairments in FXS.

Although investigations of neuronal characteristics greatly predominant in the field of FXS research, altered functions of other cell types due to FMRP deficiency undoubtedly contribute to FXS-associated phenotypes and may provide additional targets for therapeutic interventions for normalizing some characteristics of FXS. For example, GSK3α was found to be hyperactivated in testis from FX mice, which display the macroorchidism evident in patients with FXS, and this was significantly reduced by only 4 weeks of lithium treatment to inhibit GSK3 (Yuskaitis et al., 2010b). Alterations in FMRP-deficient astrocytes may be particularly important, as indicated by the remarkable findings that incubation of primary hippocampal neurons from FX mice with wild-type astrocytes led to normalization of some abnormalities in the FX neurons, while FX astrocytes induced abnormalities in co-cultured wild-type neurons (Jacobs and Doering, 2010; Jacobs et al., 2010). FX mouse brains also exhibit elevated levels of GFAP, a marker of astrogliosis, and lithium treatment reduced GFAP levels (Yuskaitis et al., 2010b). GFAP expression is induced by activation of the transcription factor STAT3, and GSK3 promotes STAT3 activation (Beurel and Jope, 2008), raising the possibility that hyperactive GSK3 in FX mouse brain drives STAT3-mediated GFAP expression, which can be reduced by GSK3 inhibitors.

Thus, these examples represent only a few of the many potential mechanisms by which hyperactive GSK3 may contribute to the pathology of FXS. Identification of the abnormalities in FX mice that are corrected by administration of GSK3 inhibitors may clarify which of the targets of GSK3 is important in the phenotypes displayed by FX mice.

SUMMARY

Altogether, lithium has proven to be beneficial for a surprisingly large number of different phenotypes in FX mice and a pilot trial supported the possibility that this may translate into contributing to the treatment of patients with FXS. Improving upon lithium's actions in FXS likely will require clarifying to what extent they result from inhibition of GSK3. In FX mice, heightened susceptibility to audiogenic seizures is almost certainly controlled by inhibiting GSK3, since three structurally dissimilar GSK3 inhibitors normalized this susceptibility without affecting responses of wild-type mice. Evidence also suggests that locomotor hyperactivity can be reduced by targeting GSK3 based on the normalizing actions of the GSK3 inhibitor SB216763 as well as lithium. However, for the other identified therapeutic actions of lithium in FX mice, the information available is largely correlative: GSK3 is hyperactive in the brains of FX mice, lithium inhibits GSK3, and MPEP also inhibits GSK3 in FX brain regions. Thus, it remains to be established if other highly specific inhibitors of GSK3 will prove more beneficial than lithium in the treatment of FXS. If this is established, it would open the way for the application to FXS treatment of the many new selective inhibitors of GSK3 that

have been developed in recent years. Since GSK3 influences many cellular processes, it is unlikely that complete inhibition of GSK3 should be an objective or would be tolerated. Rather, similar to the partial inhibition of GSK3 that is achieved by therapeutic levels of lithium in the treatment of bipolar disorder (Li and Jope, 2010), as well as in FX mice, dampening of the abnormally active GSK3 associated with FXS with new GSK3 inhibitors may be a reasonable therapeutic goal.

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Regulation by glycogen synthase kinase-3 of inflammation and T cells in CNS diseases

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Elevated markers of neuroinflammation have been found to be associated with many psychiatric and neurodegenerative diseases, such as mood disorders, Alzheimer's disease, and multiple sclerosis (MS). Since neuroinflammation is thought to contribute to the pathophysiology of these diseases and to impair responses to therapeutic interventions and recovery, it is important to identify mechanisms that regulate neuroinflammation and potential targets for controlling neuroinflammation. Recent findings have demonstrated that glycogen synthase kinase-3 (GSK3) is an important regulator of both the innate and adaptive immune systems' contributions to inflammation. Studies of the innate immune system have shown that inhibitors of GSK3 profoundly alter the repertoire of cytokines that are produced both by peripheral and central cells, reducing pro-inflammatory cytokines, and increasing anti-inflammatory cytokines. Furthermore, inhibitors of GSK3 promote tolerance to inflammatory stimuli, reducing inflammatory cytokine production upon repeated exposure. Studies of the adaptive immune system have shown that GSK3 regulates the production of cytokines by T cells and the differentiation of T cells to subtypes, particularly Th17 cells. Regulation of transcription factors by GSK3 appears to play a prominent role in its regulation of immune responses, including of NF-κB, cyclic AMP response element binding protein, and signal transducer and activator of transcription-3. *In vivo* studies have shown that GSK3 inhibitors ameliorate clinical symptoms of both peripheral and central inflammatory diseases, particularly experimental autoimmune encephalomyelitis, the animal model of MS. Therefore, the development and application of GSK3 inhibitors may provide a new therapeutic strategy to reduce neuroinflammation associated with many central nervous system diseases.

Keywords: glycogen synthase kinase-3, GSK3, inflammation, cytokines, EAE, multiple sclerosis, mood disorders, neurodegenerative diseases

INTRODUCTION

The immune system responds to infection or damage to attempt to restore homeostasis. Immune system responses are classified as adaptive or innate based on the activation timing and antigen presentation. Innate immune cells provide the first line of defense against pathogens, acting as sentinels to detect signs of distress. Upon the first signs of distress, activated sentinel cells of the innate immune system release soluble mediators of inflammation, such as cytokines and chemokines, to clear the pathogen. On the other hand, adaptive immunity is a slower response dependent on T cells and B cells reacting with antigen-presenting cells. Together, the innate and adaptive immune systems contribute to the process of inflammation, and dysregulation of either system can contribute to a variety of pathological conditions. Importantly, immune cell activation is usually rapidly reversed by anti-inflammatory and immunosuppressive functions in order to avoid damaging the host. Although the immune responses are mainly thought to be triggered in the periphery, there are also organ specialized immune cells, which exhibit unique immune function.

There is growing recognition that inflammatory signaling molecules have profound influences on many functions of the central nervous system (CNS). These effects include actions of both the innate and adaptive immune systems, as well as glia within the CNS (Raison et al., 2006; Dantzer et al., 2008; Miller et al., 2009; Miller, 2010). While it is important to remember that many of these neuroimmune actions are beneficial, as well as necessary, for a healthy CNS, research has been particularly focused on detrimental effects of neuroinflammation in association with psychiatric and neurodegenerative diseases. This has raised awareness that much remains to be learned about signaling mechanisms that regulate neuroinflammation, and that targeting regulators of neuroinflammation may prove to be a useful therapeutic strategy capable of affecting a diverse array of CNS disorders. Recent evidence is reviewed here demonstrating that glycogen synthase kinase-3 (GSK3) is an important regulator of both innate and adaptive immune system mediators that can profoundly affect the CNS.

NEUROINFLAMMATION

In contrast to other organs, the brain does not display a classical immune response but is capable of mounting its own inflammatory responses. The blood brain barrier serves to separate the brain's local inflammatory responses from systemic inflammation by limiting, but not entirely preventing, the penetration of

peripheral inflammatory mediators and immune cells (Lampson, 1987; Fuchs and Bullard, 1988). This unique protection from systemic inflammation likely developed to protect neurons, since they are long-lived cells with limited capacity to recover from noxious insults. However, although for many years the immune privileged status of the CNS was misinterpreted to mean exclusion of immune cells, it is now evident that "immunosurveillance is an integral part of the functioning brain" (Schwartz and Shechter, 2010). In the CNS, microglia are the sentinel cells that constantly survey the environment and become activated to produce an inflammatory response to infection or damage (Kim and De Vellis, 2005). In conjunction with microglia responses, astrocytes are also well-established to contribute to neuroinflammatory responses (Lieberman et al., 1989; Hamby and Sofroniew, 2010). Acutely or chronically, these glial responses could be beneficial or detrimental because they allow microglia to migrate to afflicted sites where they produce cytokines and growth factors that can contribute to the recovery, or be deleterious, for affected neurons, and may contribute to many diseases of the nervous system (Matyszak, 1998; Correale and Villa, 2004; Campbell, 2005; Cartier et al., 2005; Hauwel et al., 2005; Kim and De Vellis, 2005; Imitola et al., 2006; Kielian, 2006; Schwartz et al., 2006). Thus, it is crucial to identify mechanisms that induce and regulate neuroinflammation. The prevalence of markers of inflammation associated with psychiatric and neurodegenerative diseases suggest there is prolonged activation and impaired down-regulation of neuroinflammation in these conditions, including influences by the peripheral immune system. Thus, it remains difficult to dissociate distinct contributions of the peripheral and CNS inflammatory systems, making it challenging to identify therapeutic targets to control neuroinflammation.

GSK3 PROMOTES NEUROINFLAMMATION

Inflammatory responses were first found to be promoted by GSK3 following stimulation of several types of toll-like receptors (TLR) in monocytes and peripheral blood mononuclear cells (Martin et al., 2005). This demonstrated that GSK3 is necessary for full stimulation of the production of several pro-inflammatory cytokines, such as interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α (TNFα), and inhibitors of GSK3 greatly reduced the production of pro-inflammatory cytokines. Remarkably, *in vivo* administration of GSK3 inhibitors provided protection from endotoxin shock sufficiently enough to allow the survival of most mice from an otherwise lethal (LD_{100}) dose of lipopolysaccharide (LPS; Martin et al., 2005). This study showed for the first time the powerful ability of GSK3 inhibitors to shift the balance of the inflammatory response from pro-inflammatory to anti-inflammatory, and revealed the therapeutic potential for these drugs in inflammatory conditions (Martin et al., 2005). These findings raised the novel possibility that inhibitors of GSK3 may prove to be beneficial in conditions involving inflammation (Jope et al., 2007).

The pro-inflammatory action of GSK3 and anti-inflammatory actions of its inhibitors have been demonstrated with a variety of inflammatory molecules and extended to several cell types (Gao et al., 2008; Wang et al., 2009a,b, 2011a; Gurrieri et al., 2010; Kao et al., 2010; Klamer et al., 2010; Baarsma et al., 2011; for review Beurel et al., 2010), including cells in the CNS that contribute to neuroinflammation. In LPS-stimulated microglia, GSK3 promotes the production of cytokines and other inflammatory molecules, such as IL-1β, TNFα, IL-6, IL-8, RANTES, CXCL-10, and nitric oxide (NO; Luna-Medina et al., 2005; Hashioka et al., 2007; Beurel and Jope, 2009b; Cheng et al., 2009; Huang et al., 2009; Yuskaitis and Jope, 2009). As in the periphery, NF-κB is thought to be a critical transcription factor targeted by GSK3 for promoting neuroinflammation (Yuskaitis and Jope, 2009; Wang et al., 2010), as discussed below. In addition to microglia, GSK3 also promotes cytokine production by astrocytes (Park et al., 2006; Beurel and Jope, 2010), in particular IL-6, and promotes the IL-6/signal transducer and activator of transcription-3 (STAT3)-dependent activation of glial fibrillary acidic protein (GFAP), which is a critical marker of astrogliosis (Beurel and Jope, 2008, 2009b). Tolerance is a mechanism whereby cells dampen their response to two consecutive identical stimuli, and the promotion of IL-6 production by GSK3 was shown to also involve GSK3 counteracting LPS-induced tolerance for IL-6 production in astrocytes (Beurel and Jope, 2010).

Besides regulating cytokine production in glia, GSK3 also promotes migration and activation of glial cells (Beurel and Jope, 2008; Yuskaitis and Jope, 2009). Inhibition of GSK3 promotes microglial survival during oxygen–glucose deprivation (Chong et al., 2007) and treatment with erythropoietin both inhibited GSK3 and supported microglia survival (Li et al., 2006), actions that may contribute to minimizing permanent CNS damage.

Recently, inhibition of GSK3 was suggested to promote stabilization of the brain blood barrier (Ramirez et al., 2010). This was based on findings in cultured brain microvascular endothelial cells that GSK3 inhibition reduced the production of several inflammatory molecules and monocyte adhesion to and migration across cytokine-stimulated cells. Furthermore, *in vivo* inhibition of GSK3 reduced leukocyte adhesion to brain endothelium under inflammatory conditions.

PRO-INFLAMMATORY MECHANISMS OF GSK3

GSK3 CAN PROMOTE PRO-INFLAMMATORY CYTOKINE PRODUCTION THROUGH NF-κB ACTIVATION

Regulation of the inflammatory transcription factor NF-κB was found to be key for the pro-inflammatory actions of GSK3 (Martin et al., 2005; Gong et al., 2008a,b; Yuskaitis and Jope, 2009; Wang et al., 2010). Inhibitors of GSK3 reduced TLR-induced production of inflammatory cytokines by inhibiting the transcriptional activation of NF-κB (Martin et al., 2005). This supported previous reports that GSK3 is necessary for the full transcriptional activity of NF-κB (Hoeflich et al., 2000), and that GSK3 supported NF-κB transcriptional activity in a promoter-specific manner (Steinbrecher et al., 2005). This selective action of GSK3β on NF-κB-induced inflammatory gene expression may facilitate the therapeutic anti-inflammatory uses of GSK3 inhibitors since it indicates that inhibition of GSK3 will not interfere with all the actions of NF-κB. The role of GSK3 in the regulation of NF-κB in inflammation was further expanded by the demonstration of a role for GSK3 in the desensitization of LPS-induced inflammatory signaling by TNFα (Park et al., 2011).

GSK3 BLOCKS ANTI-INFLAMMATORY CYTOKINES

Conversely to inflammatory signaling, GSK3 reduces the production of the anti-inflammatory cytokine IL-10, and inhibitors of GSK3 increased anti-inflammatory cytokine production (Martin et al., 2005; Hu et al., 2006; Antoniv and Ivashkiv, 2011; Ren et al., 2011). Inhibition of IL-10 production by GSK3 was reported to involve a competition for limiting amounts of CBP/p300, with GSK3-promoted activation of NF-κB depleting the amount of CBP/p300 available for cyclic AMP response element binding protein (CREB)-induced IL-10 production (Martin et al., 2005; Hu et al., 2006; Hofmann et al., 2010). It was also proposed that the down-regulation of IL-10 production by GSK3 contributes to the synergistic inflammatory signaling induced by co-treatment with interferons and TLR ligands (Hu et al., 2006; Lin et al., 2008). Furthermore, the production of IL-10 by IFNβ, thought to be important for the anti-inflammatory therapeutic properties of IFNβ in multiple sclerosis (MS) patients, was shown to be dependent on inhibition of GSK3 (Wang et al., 2011a).

TGFβ1 also has anti-inflammatory properties. Although the mechanism whereby TGFβ1 suppresses inflammation remains unclear, it has been proposed that TGFβ1 can antagonize the production of certain pro-inflammatory cytokines in response to inflammatory stimuli by inhibition of GSK3 to promote β-catenin signaling (Dai et al., 2011). Additionally, it is also thought that because TGFβ is required for the induction of regulatory T cells (Tregs), which produce IL-10, the anti-inflammatory action of TGFβ is mediated through its induction of Tregs *in vivo* where Tregs counteract the action of the pro-inflammatory effector $CD4^+$ T cells and of the pro-inflammatory cytokines (Campbell and Koch, 2011). Recently, it was shown that inhibition of GSK3 increases the anti-inflammatory properties of Tregs by increasing β-catenin levels in response to TGFβ (Graham et al., 2010). GSK3 was also shown to regulate activation of Smad transcription factors (Fuentealba et al., 2007; Sapkota et al., 2007; Guo et al., 2008; Liang et al., 2008; Heldin et al., 2009), indicating a direct effect of GSK3 on the TGFβ-induced signaling pathway.

Therefore, it appears that NF-κB activation is a key pro-inflammatory pathway promoted by GSK3, whereas CREB and β-catenin are inhibited by GSK3 to maintain low levels of anti-inflammatory molecules.

COOPERATION OF GSK3 WITH mTOR TO REGULATE CYTOKINE PRODUCTION

A number of inflammatory signaling pathways converge on GSK3 to regulate cytokine production, reinforcing the concept that GSK3 has a central role in the regulation of cytokine production and other inflammatory outcomes. GSK3 promotes the activation of myeloid dendritic cells (DC) and their secretion of cytokines (Ono et al., 2007; Rodionova et al., 2007; Hoarau et al., 2008; Larabee et al., 2011; Liu et al., 2011; Wang et al., 2011b). During DC generation, inhibition of the mammalian target of rapamycin (mTOR) confers resistance to maturation, whereas inhibition of mTOR prior to TLR activation enhanced pro-inflammatory cytokine production. GSK3 activity was shown to be inhibited by the mTOR pathway and modulated the capacity of DCs to produce pro-inflammatory cytokines (Turnquist et al., 2010; Brown et al., 2011; Wang et al., 2011b). The production of cytokines by DCs also influences the differentiation of T helper (Th) cells (Ono et al., 2007) that are highly dependent on cytokines to differentiate toward different lineages (O'Shea and Paul, 2010).

GSK3 INFLUENCES IMMUNE CELL FATE

Glycogen synthase kinase-3 also regulates other transcription factors involved in inflammation besides NF-κB and CREB, particularly STATs. This allows GSK3 to control the amplitude and the duration of inflammatory responses. Thus, it was shown that GSK3 participates in the synergistic action of IFNγ on LPS-induced signaling in part by modulating the downstream signaling induced by IFNγ, the JAK/STAT pathway. Although GSK3 was not involved in the short term activation of IFNγ-induced STAT1 (Beurel and Jope, 2008), inhibitors of GSK3 blocked IFNγ-induced STAT1 activation at longer times in macrophages through SHP2 derepression (Tsai et al., 2009). Furthermore, a differential role of GSK3 in regulating the activation of different STAT subtypes was uncovered, as GSK3 was found to promote STAT3 and STAT5 activation, while GSK3 did not affect STAT6 activation (Beurel and Jope, 2008). This regulation of STAT3 activation by GSK3 may be particularly relevant for the signaling induced by IL-6, as well as for the production of IL-6 in response to both LPS and the combination LPS and IFNγ (Beurel and Jope, 2009a,b).

Although GSK3 has been mainly thought of as a regulator of cytokine production, an unexpected role of GSK3 was found to be in the regulation of the differentiation of Th subsets. Cytokines are key determinants for Th differentiation toward a particular lineage and STAT3 is a crucial instigator of the differentiation toward Th17 cells (O'Shea and Paul, 2010). Thus, it was found that GSK3 is required for Th17 cell production, perhaps due to both promoting IL-6 production and STAT3 activation (Harrington et al., 2005), although other processes likely also contribute to this action of GSK3 on Th17 cells (Beurel et al., 2011). This recent discovery that GSK3 promotes Th17 cell differentiation provides a new step in the understanding of the GSK3-dependent regulatory mechanisms of the immune system. Th17 cells are critical for the pathogenicity of many autoimmune diseases (Langrish et al., 2005; Ivanov et al., 2006; Komiyama et al., 2006), so the finding that inhibition of GSK3 blocked Th17 cell differentiation provides a new avenue of therapeutic intervention that may contribute to controlling Th17-driven diseases.

SURVIVAL ADVANTAGES OF BLOCKING GSK3 ACTIVITY

Glycogen synthase kinase-3 has long been known to promote cell death mediated by the intrinsic apoptotic pathway, whereas it protects from extrinsic apoptosis (Beurel and Jope, 2006; Sun et al., 2008). Several apoptosis-regulating properties of GSK3 have been identified in the immune system. Thus, for example, during the clonal expansion of activated T cells, there is induction of the activated T cell death (ACTD; Kondrack et al., 2003), which is thought to be due to a loss of access to survival signals. ACTD is thought to be critical to prevent autoimmune responses, but need to be tightly regulated because complete elimination of responding T cells would confer increased susceptibility to infections. GSK3 was shown to be required for ACTD and inhibition of GSK3 promotes survival of T cells (Sengupta et al., 2007). GSK3 was also found to be critical to induce apoptosis of DC (Hoarau et al., 2008).

Altogether, GSK3 promotes pro-inflammatory cytokine production by regulating a network of transcription factors. GSK3 also receives signals in order to finely tune this cytokine production. But GSK3 also, intrinsically to immune cells, affects their maturation, differentiation, and survival rendering GSK3 an attractive target for therapeutic interventions.

INFLAMMATION IN PSYCHIATRIC DISEASES

The mood disorders depression and bipolar disorder are prevalent diseases with a high lifetime incidence of ∼20% in the US (Greden, 2001; Belmaker and Agam, 2008), but current treatments are often inadequate, as many patients do not respond or prematurely terminate treatment (Krishnan and Nestler, 2008). Much evidence has shown that mood disorders are associated with alterations of the immune system, especially increased markers of inflammation. For example, extensive evidence has demonstrated that inflammation promotes susceptibility to depression and impairs responses to antidepressants. Elevated serum levels of inflammatory cytokines, such as the cytokines IL-6 and TNFα, have been reported in patients with major depression or bipolar disorder (Anisman and Merali, 2003; O'Brien et al., 2004a, 2006, 2007; Schiepers et al., 2005; Raison et al., 2006; Dantzer et al., 2008; Miller et al., 2009; Miller, 2010). A significant portion of people develop depression following the therapeutic bolstering of immunity by interferon-α administration (Reichenberg et al., 2001; Wright et al., 2005; Prather et al., 2009). Depressive symptoms thought to be due to cytokines also occur after a mild activation of the innate immune system (Raison et al., 2006; Dantzer et al., 2008; Miller et al., 2009). Antidepressants are less effective in patients with activated inflammation, and antidepressant responses are improved in some patients by co-treatment with anti-inflammatory drugs (O'Brien et al., 2007; Roumestan et al., 2007; Dantzer et al., 2008; Miller et al., 2009; Rivest, 2009). Administration of drugs that inhibit the actions of cytokines or their signaling pathways, such as cyclooxygenase inhibitors and etanercept, improve mood in patients with inflammatory diseases (Mendlewicz et al., 2006; Muller et al., 2006; Tyring et al., 2006). These links demonstrating inflammation increases susceptibility to mood disorders have been recapitulated in rodents. For example, administration of individual inflammatory cytokines, or of LPS to induce inflammation, causes depression-like behaviors in rodents that are reversed by antidepressant administration (Dantzer and Kelley, 2007). Increased cytokine levels or administration of cytokines in rodents or humans have been reported to induce behavioral changes with characteristics similar to mood disorders (Licinio and Wong, 1999; Hayley et al., 2005; Simen et al., 2006). Importantly, psychological stress in both humans and rodents is sufficient to activate the production of inflammatory cytokines and to induce depressive symptoms (Anisman and Merali, 2003; O'Brien et al., 2006; Gabbay et al., 2009). Thus, there is much evidence that inflammation can precipitate depression and impair therapeutic responses. While it remains to be determined if inflammatory cytokines contribute to the onset or represent an outcome of mood disorders, it has been suggested that reducing inflammation may contribute to therapeutic interventions based on the depressive effects that cytokines can produce and on evidence that classical antidepressants are anti-inflammatory (Reynolds et al., 2005; Schiepers et al., 2005).

While many investigators have focused on actions of the innate immune system in depression, the impact of the adaptive immune system, especially T cells, remains poorly understood even though the innate and adaptive immune systems are highly integrated. Examinations of the effects of stress and depression on T cell responses demonstrated altered proliferation of peripheral blood mononuclear cells in response to stimulation with T cell mitogens in samples from patients with depression (Bartrop et al., 1977; Kronfol et al., 1983; Schleifer et al., 1983, 1984; Stein et al., 1991). T cell alterations also were indicated by *in vivo* measurements of T cell-mediated immune function in depressed patients (Hickie et al., 1993; Sephton et al., 2009). Studies of mechanisms implicated increased Fas-mediated T cell apoptosis (Eilat et al., 1999; Ivanova et al., 2007; Szuster-Ciesielska et al., 2008), reduced T cell responses to glucocorticoids that are elevated in major depression (Pariante and Miller, 2001), or increased cytokine levels that modulate T cell subtype production and responses (Cope et al., 1994, 1997; Lee et al., 2008). These and additional reports (Miller, 2010) of altered T cells in depression led to demonstrations that T cells mediate the negative impact of depression on health outcomes in depressed patients with infectious diseases or cancer.

Substantial evidence indicates that dysregulated GSK3 contributes to mood disorders (Li and Jope, 2010): brain GSK3 is abnormally active in postmortem human prefrontal cortex from depressed subjects (Karege et al., 2007), GSK3 is activated in mouse brain in the learned helplessness model of depression (Polter et al., 2010), antidepressants inhibit GSK3 (Li et al., 2004), reducing GSK3 activity ameliorates depression-like behaviors in rodents (Gould et al., 2003; Kaidanovich-Beilin et al., 2004; O'Brien et al., 2004b; Beaulieu et al., 2008), and abnormally active GSK3 in GSK3 knockin mice increases susceptibility to depression-like behaviors (Polter et al., 2010). Since inflammation is a candidate cause of increased susceptibility to depression, it is notable that GSK3 strongly promotes inflammation in the periphery (Martin et al., 2005) and CNS (Beurel and Jope, 2009b, 2010; Yuskaitis and Jope, 2009), which is reduced by GSK3 inhibitors. GSK3 knockin mice showed also a mild exacerbation of their depressive-like behavior in response to LPS administration compared to wild-type mice (Polter et al., 2010). Therefore, part of the therapeutic effects of the mood stabilizer lithium, as well as of antidepressants that inhibit GSK3, may come from their anti-inflammatory effects following inhibition of GSK3. However, further investigations are needed to definitively test if promotion of inflammation by GSK3 contributes to mood disorders.

INFLAMMATION IN NEURODEGENERATIVE DISEASES

Inflammatory markers, particularly increased levels of inflammatory cytokines and activated microglia and astrocytes, have been reported in many neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis, as well as MS, which is generally considered an autoimmune disease (Amor et al., 2010; Glass et al., 2010; Philips and Robberecht, 2011). This may be due to impaired mechanisms of inflammation resolution or persistent stimulation of inflammatory responses, such as may be caused by the generation of "danger" signals from degenerating neurons that activate damage-associated molecular patterns (DAMPs). Activation of the

inflammatory system has beneficial effects, such as phagocytosis of dead cells and production of factors bolstering neuronal function and neurogenesis. However, it is generally thought that long-term inflammation associated with neurodegenerative diseases exacerbates the diseases by releasing signaling molecules that impair neuronal function and survival, increasing the impairments associated with the primary disease lesion.

ALZHEIMER'S DISEASE

Alzheimer's disease is a severe, progressive neurodegenerative disease with a growing prevalence and only modestly effective therapies are available. There are two classical neuropathological features of AD: intraneuronal filamentous aggregates of hyperphosphorylated tau and other proteins called neurofibrillary tangles, and extracellular deposits of aggregated amyloid-β (Aβ) peptides called amyloid plaques. The levels of many cytokines, such as IL-1β, IL-6, and TNFα, and chemokines are upregulated in AD, as previously reviewed (Akiyama et al., 2000; Block and Hong, 2005; Sastre et al., 2006). This inflammation and increased cytokine production is also exhibited by transgenic mouse models of AD (Benzing et al., 1999; Matsuoka et al., 2001). Activation of astrocytes and microglia and the ensuing production of inflammatory molecules has been reported to be stimulated by degenerating neurons, neurofibrillary tangles, and Aβ and other cleavage products of amyloid precursor protein, and activated astrocytes and microglia are found surrounding amyloid plaques in AD brain (Bach et al., 2001; Combs et al., 2001; Ho et al., 2005). Inflammation may contribute to the increased risk for AD following traumatic brain injury or diabetes, both of which can cause increased inflammation, as well as to the prevalent co-morbidity of depression with AD, since much evidence links inflammation with depression as discussed above. Many investigations have reported evidence that hyperactive GSK3 in AD contributes to both of its neuropathological hallmarks by phosphorylating tau and promoting Aβ production (Jope and Johnson, 2004; Lesort and Jope, 2010) and to neuronal death (Beurel and Jope, 2006; Mines et al., 2011). These many links between AD and GSK3 have led to a substantial effort to develop GSK3 inhibitors for treating AD (Hooper et al., 2008; Martinez and Perez, 2008; Muyllaert et al., 2008; Hernandez et al., 2010). GSK3 inhibitors reduce inflammatory mediators implicated in AD, such as NO (Yuskaitis and Jope, 2009) and inflammatory cytokines (Beurel et al., 2010). Considering the evidence for inflammation contributing to AD pathology and that GSK3 promotes inflammation, interventions with GSK3 inhibitors may also be beneficial by reducing the inflammation that is thought to exacerbate neurodegenerative processes in AD.

PARKINSON'S DISEASE

Like many other neurodegenerative diseases, substantial evidence indicates that neuroinflammation contributes to PD, and its models in rodents. For example, activated inflammatory processes in the substantia nigra have been implicated in the loss of dopaminergic neurons in PD patients and in animal models of PD (Esposito et al., 2007; Hald et al., 2007; Mcgeer and Mcgeer, 2008; Barnum and Tansey, 2010), and some (Gagne and Power, 2010), but not all (Wahner et al., 2007; Driver et al., 2011), studies have indicated that non-steroidal anti-inflammatory drug use reduces the risk of PD. However, the innate immune system may not be acting alone in promoting PD. Evidence has been reported of activated T cells in the substantia nigra in rodent PD models (Kurkowska-Jastrzebska et al., 1999) and postmortem PD brains (Brochard et al., 2009), and elimination of T cells attenuated dopaminergic neuron loss in rodent PD models (Brochard et al., 2009). As in AD, there is evidence both that GSK3 contributes to neuronal degenerative processes in PD (King et al., 2001; Chen et al., 2004) and that neurotoxic mechanisms, such as α-synuclein in PD, activate GSK3 (Duka et al., 2009; Yuan et al., 2010). Thus, inhibition of GSK3 may protect susceptible neurons from degeneration and reduce the inflammation that appears to promote neurodegenerative processes in PD.

MULTIPLE SCLEROSIS

Multiple sclerosis appears to result from aberrant innate and adaptive immune system actions in the CNS, likely initiated by an autoimmune response that causes inflammation, demyelination, and neuronal degeneration (Weiner, 2008). Thus, in contrast to the other neurodegenerative diseases that appear to initiate with neuronal insults, MS is initiated by aberrant actions of the adaptive immune system. Studies of MS patients and of the animal model of MS (experimental autoimmune encephalomyelitis, EAE) have implicated the Th17 and Th1 subsets of Th cells as key mediators of the disease (Langrish et al., 2005; Ivanov et al., 2006; Tzartos et al., 2008). This first wave of adaptive immune system activity and inflammation seems to be followed by a second wave of neuroinflammation, and both are thought to contribute to the death of oligodendrocytes. Thus, inflammation with activation of microglia and astrocytes are well-established components of MS and targeting the response of these cells has been explored (Kang et al., 2010).

The crucial inflammatory component of MS suggests that inhibitors of GSK3 may provide some amelioration of the disease. Indeed, in EAE, administration of lithium to inhibit GSK3 effectively prevents the disease and almost completely terminates ongoing disease (De Sarno et al., 2008; Beurel et al., 2011). Notably, inhibitors of GSK3 also prevent production of Th17 cells that are critical for the disease (Beurel et al., 2011), suggesting that GSK3 inhibitors provide at least two actions that may contribute to alleviation of MS.

CONCLUSION

Glycogen synthase kinase-3 is now well-established to contribute to inflammatory processes in the periphery, and growing evidence indicates that GSK3 also promotes neuroinflammation. This action appears to be largely the result of GSK3 promoting the activities of transcription factors that mediate inflammatory responses, particularly NF-κB and STAT3. Furthermore, although both paralogs of GSK3, GSK3α and GSK3β, share many common actions, there is increasing evidence that they can also have non-redundant functions (Hoeflich et al., 2000; Liang and Chuang, 2006; Force and Woodgett, 2009). Most of the studies on inflammation focused on GSK3β, mainly because more tools have been developed for studying GSK3β. However, there is a differential role of GSK3α and GSK3β in the regulation of IL-6 production (Beurel and

Jope, 2008). Therefore, identifying differential role for GSK3α and GSK3β may be a promising avenue for future studies. Importantly, GSK3 has been implicated in pathological processes of psychiatric and neurodegenerative diseases that also exhibit increased inflammatory activation, such as mood disorders, AD, PD, and MS. Thus, GSK3 may provide a feasible therapeutic target to control inflammation along with ameliorating other pathological processes in several diseases of the CNS.

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GSK-3 as a target for lithium-induced neuroprotection against excitotoxicity in neuronal cultures and animal models of ischemic stroke

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The mood stabilizer lithium inhibits glycogen synthase kinase-3 (GSK-3) directly or indirectly by enhancing serine phosphorylation of both α and β isoforms. Lithium robustly protected primary brain neurons from glutamate-induced excitotoxicity; these actions were mimicked by other GSK-3 inhibitors or silencing/inhibiting GSK-3α and/or β isoforms. Lithium rapidly activated Akt to enhance GSK-3 serine phosphorylation and to block glutamate-induced Akt inactivation. Lithium also up-regulated Bcl-2 and suppressed glutamate-induced p53 and Bax. Induction of brain-derived neurotrophic factor (BDNF) was required for lithium's neuroprotection to occur. BDNF promoter IV was activated by GSK-3 inhibition using lithium or other drugs, or through gene silencing/inactivation of either isoform. Further, lithium's neuroprotective effects were associated with inhibition of NMDA receptor-mediated calcium influx and down-stream signaling. In rodent ischemic models, post-insult treatment with lithium decreased infarct volume, ameliorated neurological deficits, and improved functional recovery. Up-regulation of heat-shock protein 70 and Bcl-2 as well as down-regulation of p53 likely contributed to lithium's protective effects. Delayed treatment with lithium improved functional MRI responses, which was accompanied by enhanced angiogenesis. Two GSK-3-regulated pro-angiogenic factors, matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor were induced by lithium. Finally, lithium promoted migration of mesenchymal stem cells (MSCs) by up-regulation of MMP-9 through GSK-3β inhibition. Notably, transplantation of lithium-primed MSCs into ischemic rats enhanced MSC migration to the injured brain regions and improved the neurological performance. Several other GSK-3 inhibitors have also been reported to be beneficial in rodent ischemic models. Together, GSK-3 inhibition is a rational strategy to combat ischemic stroke and other excitotoxicity-related brain disorders.

Keywords: lithium, glycogen synthase kinase-3, excitotoxicity, cerebral ischemia, mesenchymal stem cells

INTRODUCTION

Glycogen synthase kinase-3 (GSK-3), an evolutionarily conserved ubiquitous serine–threonine kinase consisting of α and β isoforms, is a multifaceted protein with diverse cellular and neurophysiological functions. The main structural difference between GSK-3α and GSK-3β isoforms lies in the N- and C-terminal regions, while their sequences within the kinase domain are highly homologous. GSK-3 is considered to be constitutively active under non-stimulated basal conditions. A growing body of evidence indicates that GSK-3 is pro-apoptotic and that its dysfunction may be linked to the pathophysiology of mood disorders, schizophrenia, diabetes, and various neurological/neurodegenerative diseases, among others (for review, Meijer et al., 2004; Huang and Klein, 2006; Jope et al., 2007; Chiu and Chuang, 2010; Li and Jope, 2010). GSK-3 inhibition has attracted widespread attention as one of the critical therapeutic targets whereby lithium exerts its effects on mood stabilization, neurogenesis, neurotrophicity, neuroprotection, anti-inflammation, and others (for review, Rowe and Chuang, 2004; Rowe et al., 2007; Beurel et al., 2010). Pharmacological inhibition or gene knockout/knockdown of this kinase mimics the anti-depressant and anti-manic effects of lithium observed in rodent models (Gould et al., 2004; Kaidanovich-Beilin et al., 2004, 2009; O'Brien et al., 2004; Rosa et al., 2008; Omata et al., 2011). The activities of GSK-3 are negatively regulated by phosphorylation of GSK-3α at Ser21 and GSK-3β at Ser9. GSK-3 can be inhibited by lithium through direct binding to the ATP-dependent magnesium-sensitive catalytic site of the enzyme (Klein and Melton, 1996; Stambolic et al., 1996), and/or indirectly through enhanced serine phosphorylation of GSK-3 isoforms by multiple mechanisms (**Figure 1**). Lithium has been shown to enhance GSK-3 serine phosphorylation by activation of protein kinase A (PKA; Jope, 1999; Liang et al., 2008), or phosphatidylinositol 3-kinase (PI3-kinase)-dependent Akt (Chalecka-Franaszek and Chuang, 1999) and protein kinase C-α (Kirshenboim et al., 2004). It has also been reported that lithium can disrupt the β-arrestin-2–PP2A–Akt complex that dephosphorylates/inactivates Akt, thereby enhancing GSK-3 serine phosphorylation (Beaulieu et al., 2005). Moreover, it has been proposed

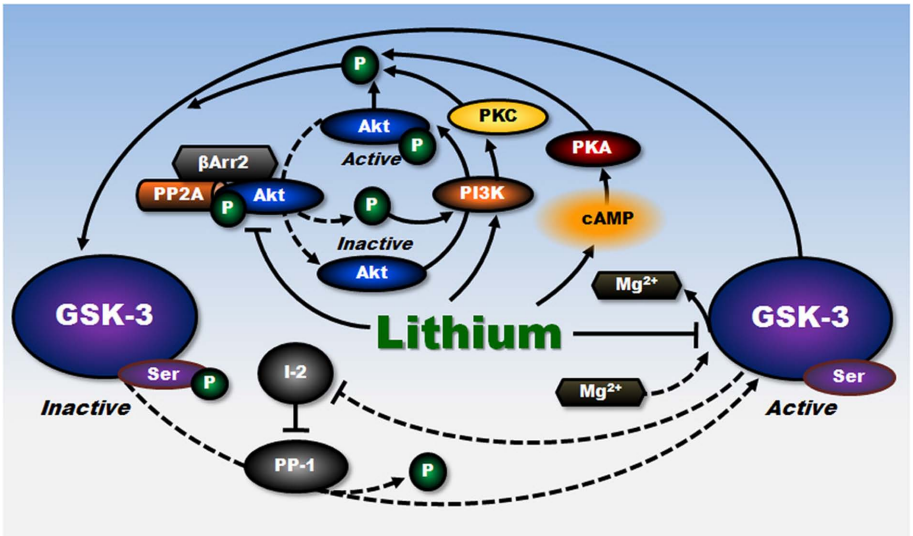

FIGURE 1 | Inhibitory regulation of GSK-3 by lithium. Lithium negatively regulates the constitutively activated GSK-3 activity through multiple mechanisms. Lithium, a competitive inhibitor of magnesium, directly inhibits ATP–magnesium-dependent catalytic activity of GSK-3. The activity of GSK-3 is also reduced by phosphorylation at a specific serine residue. Lithium can indirectly increase this serine phosphorylation of GSK-3 through PI3-kinase-mediated phosphorylation/activation of Akt, PI3-kinase-mediated activation of PKC, and cAMP-dependent activation of PKA. Lithium can also increase the serine phosphorylation of GSK-3 by disrupting the β-arrestin-2 (βArr2)–PP2A–Akt complex that dephosphorylates and inactivates Akt. In addition, by disinhibiting the inhibitory action of inhibitor-2 (I-2) on protein phosphatase-I (PP-1) that dephosphorylates GSK-3 at serine residues, lithium's direct inhibition of GSK-3 interrupts this auto-regulation of GSK-3 and further decreases GSK-3 activity. Lines with solid arrows represent stimulatory connections; lines with flattened ends represent inhibitory connections. Dashed lines represent pathways with reduced activity as a result of lithium treatment. I-2, inhibitor-2; PP-1, protein phosphatase-I. (Modified from Chiu and Chuang, 2010).

that lithium can interrupt auto-regulation of GSK-3 via disinhibition of the inhibitory action of inhibitor-2 complex on protein phosphatase-1 (PP-1; Zhang et al., 2003). This article reviews the findings supporting the role of GSK-3 inhibition in mediating lithium's neuroprotective effects against excitotoxicity in both cultured neurons and animal models of ischemic stroke. Potential down-stream mechanisms underlying lithium's neuroprotection against excitotoxicity are also discussed.

REGULATION AND FUNCTION OF GSK-3 ISOFORMS

We have designed isoform-specific small interfering RNAs (siRNAs) to distinguish the functional and regulatory differences between the two GSK-3 isoforms in rat cerebral cortical neuronal cultures (Liang and Chuang, 2007). Transfection with siRNA for GSK-3α or GSK-3β or with dominant-negative mutants specific for either isoform produced almost complete protection against glutamate-induced, *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity. The siRNA-induced neuroprotection was associated with enhanced N-terminal phosphorylation in both GSK-3 isoforms. Moreover, transfection with α or β isoform-specific dominant-negative mutants of GSK-3 mimicked lithium-induced neuroprotection against glutamate excitotoxicity. These results strongly suggest that both GSK-3α and β are involved in glutamate-induced neuronal death and that both isoforms are the initial targets of lithium-elicited neuroprotection.

GSK-3 has also been implicated in neuronal development, maturation/differentiation, and aging in the mammalian CNS (Spittaels et al., 2002; Kim et al., 2009; Sofola et al., 2010). Substrates phosphorylated by GSK-3 include metabolic, signaling, and structural proteins as well as transcription factors. It is known that inhibition of GSK-3 results in activation, and sometimes suppression, of an array of transcription factors (for review, Grimes and Jope, 2001; Jope and Roh, 2006; Chiu and Chuang, 2010). Among the long list of transcription factors regulated by GSK-3 are cyclic AMP response element binding protein (CREB), nuclear factor-κB (NF-κB), activating protein-1 (AP-1), heat-shock factor-1 (HSF-1), β-catenin, T-cell factor (Tcf)/lymphoid enhancer factor (Lef), and p53. Dysfunction of GSK-3-mediated phosphorylation of transcription factors is believed to relate with the pathophysiology of various pathological conditions (for review, Chiu and Chuang, 2010). We found that GSK-3α silencing activated cAMP response element (CRE)- and NF-κB-responsive transcription more robustly than GSK-3β silencing (Liang and Chuang, 2006). Our protein–DNA array further identified two novel GSK-3-regulated transcription factors, early growth response-1 (EGR-1) and Smad3/4, both of which play important roles in growth, differentiation, survival, and plasticity of brain cells (Harada et al., 2001; Derynck and Zhang, 2003; Lee and Kim, 2004; Droguett et al., 2010). Specifically, the binding activity of EGR-1 was down-regulated by siRNA for GSK-3α, but was up-regulated by siRNA for GSK-3β (Liang and Chuang, 2006). By using siRNAs or dominant-negative mutants specific to GSK-3 isoforms, inhibition of GSK-3α increased the transcriptional activity of Smad3/4, whereas inhibition of GSK-3β reduced it. The differential roles of GSK-3 isoforms are further supported by the opposite effects of GSK-3α and β siRNAs on the protein levels of plasminogen activator inhibitor type-1 (PAI-1), a Smad3/4-regulated gene product. These results demonstrate that selective silencing or

inhibition of the two GSK-3 isoforms could produce different and sometimes opposite effects on the regulation of certain transcription factors including novel GSK-3 targets (Liang and Chuang, 2006).

Differential roles of GSK-3α and β have also been suggested by other investigators. For example, the disruption of GSK-3β in mice is embryonic lethal, despite the normal expression of GSK-3α, indicating that the presence of α isoform can not compensate for the loss of β isoform (Hoeflich et al., 2000). Transfection and siRNA studies suggested that GSK-3α inhibition decreased the processing of β-amyloid (Aβ) precursor protein to form $Aβ_{1–40}$ and $Aβ_{1–42}$, while GSK-3β appeared to have a lesser role (Phiel et al., 2003). In addition, GSK-3β, but not GSK-3α, is required for interferon-γ-induced activation of signal transducer and activator of transcription-3 (Beurel and Jope, 2009). Together, these findings underscore important similarities and differences between the roles of GSK-3 isoforms α and β in cell survival as well as transcription, and suggest that the development of isoform-specific inhibitors may be essential for therapeutic intervention of GSK-3-related neuropathological conditions.

We have also explored lithium's effects on Smad3/4-dependent transcriptional activity and the underlying mechanisms. Smad3/4 is a down-stream mediator of the signaling pathway triggered by transforming growth factor-β (TGF-β), and plays a prominent role in regulating the expression of proteins involved in neuronal survival, differentiation, and synaptic plasticity (for review, Gomes et al., 2005). Treating cultured cortical neurons with therapeutically relevant concentrations of lithium significantly decreased Smad3/4-dependent transactivation and protein levels of PAI-1, a TGF-β-responsive Smad3/4-dependent gene product (Liang et al., 2008). Of particular relevance to the therapeutic efficacy of lithium, PAI-1 has been implicated in the etiology and progression of neurodegenerative diseases and mood disorders (for review, Pawlak et al., 2003; Liang et al., 2008).

Lithium's effects on Smad3/4 likely result from cross-talk of signaling pathways between cAMP/PKA and PI3-kinase/Akt/GSK-3β. We have shown that lithium-induced Smad3/4 suppression involved GSK-3β inhibition through the activation of PKA and cell survival factor Akt followed by the phosphorylation of GSK-3β at Ser9 and CREB at Ser133 (Liang et al., 2008). CREB binding protein (CBP) and p300 are known to be co-activators of CREB. Our data further demonstrated that over-expression of p300, but not CBP, completely antagonized lithium-induced reduction of PAI-1 promoter activity. A series of experimental data support the notion that, in Smad3/4 signaling, the inhibitory effects of lithium are due to complex formation of activated CREB and p300, which results in limited interactions of p300 with the transcription factors/Smad complexes. This in turn prevents efficient Smad3/4-dependent transcription of Smad3/4-dependent genes such as PAI-1 and p21 (**Figure 2**).

INVOLVEMENT OF GSK-3 AND OTHER MOLECULES IN LITHIUM-ELICITED NEUROPROTECTION AGAINST GLUTAMATE-INDUCED EXCITOTOXICITY IN CELLULAR MODELS

Lithium-induced neuroprotection against glutamate excitotoxicity was first noted in rodent primary neuronal cultures of cerebellar granule cells (CGCs), cerebral cortical neurons, and hippocampal neurons (Nonaka et al., 1998). This experimental paradigm was selected because glutamate-related excitotoxicity has been implicated in many neurodegenerative diseases including stroke (for review, Chuang, 2004; Chiu and Chuang, 2010). Our pioneering studies have shown that glutamate-induced, NMDA receptor-mediated excitotoxicity was robustly reduced by extended lithium chloride pretreatment (5–7 days) in cultured rat CGCs and cortical neurons, partly via inhibition of NMDA receptor-mediated calcium influx (Nonaka et al., 1998; Hashimoto et al., 2002a). Moreover, these effects of lithium were likely due to the attenuation of constitutive phosphorylation at Tyr1472 of the NR2B subunit of NMDA receptors, possibly as a result of inhibiting Src tyrosine kinase (Hashimoto et al., 2002a, 2003). Although glutamate-induced excitotoxicity in cultured cortical neurons was blocked by treatment with either lithium or MK-801 (an NMDA receptor antagonist), the Src kinase inhibitor SU6656 only partially diminished this toxicity (Hashimoto et al., 2003), suggesting that other components are involved. In CGCs, lithium-induced neuroprotection against glutamate excitotoxicity was associated with up-regulation of the anti-apoptotic protein Bcl-2, down-regulation of the pro-apoptotic proteins p53 and Bax, and suppressed release of cytochrome *c* from mitochondria (Chen and Chuang, 1999), whereas the involvement of GSK-3 in the regulation of NMDA signaling by lithium treatment is currently unclear and requires further investigations.

Cyclin-dependent kinase 5 (Cdk5) also regulates signaling mediated by NMDA receptors, either directly through phosphorylation of the NR2B subunit or indirectly through phosphorylation of PSD-95 (Morabito et al., 2004; Zhang et al., 2008). Cdk5 activity is primarily regulated by its co-activator p35. However, when it binds to p25 (the product of calpain-mediated cleavage of p35), Cdk5 becomes pro-apoptotic and its activity is dysregulated (Lee et al., 2000; Carmins et al., 2006). Accordingly, p25 accumulation was observed in neurons in response to glutamate or oxidative stress, and also in the brains of several animal models of neurodegenerative diseases. Sustained activation of Cdk5 in neurons has been implicated in many neurodegenerative diseases (Cruz and Tsai, 2004; Dhariwala and Rajadhyaksha, 2008). In cultured rat CGCs, lithium pretreatment prevented colchicine-induced apoptosis and associated increase in Cdk5 expression and fragmentation of p35 into p25 (Jorda et al., 2005). Additionally, pretreatment with lithium also attenuated intracellular calcium increase, calpain activity, Cdk5 activation, and cellular death in primary cultured hippocampal neurons and rat striatum following the treatment of 3-nitropropionic acid (Crespo-Biel et al., 2009), a succinate dehydrogenase inhibitor (for review, Brouillet et al., 1999). Therefore, lithium-induced inhibition of calpain and Cdk5 activation may also contribute to protection against glutamate excitotoxicity.

Prior to changes in gene expression, lithium rapidly and transiently activated the cell survival PI3-kinase and its down-stream target, Akt-1, through phosphorylation at Ser473, thereby reversing glutamate-induced inactivation of this signaling pathway in CGCs (Chalecka-Franaszek and Chuang, 1999). Activated Akt is known to affect several anti-apoptotic targets including Bcl-2 associated death promoter (BAD), CREB, members of the forkhead

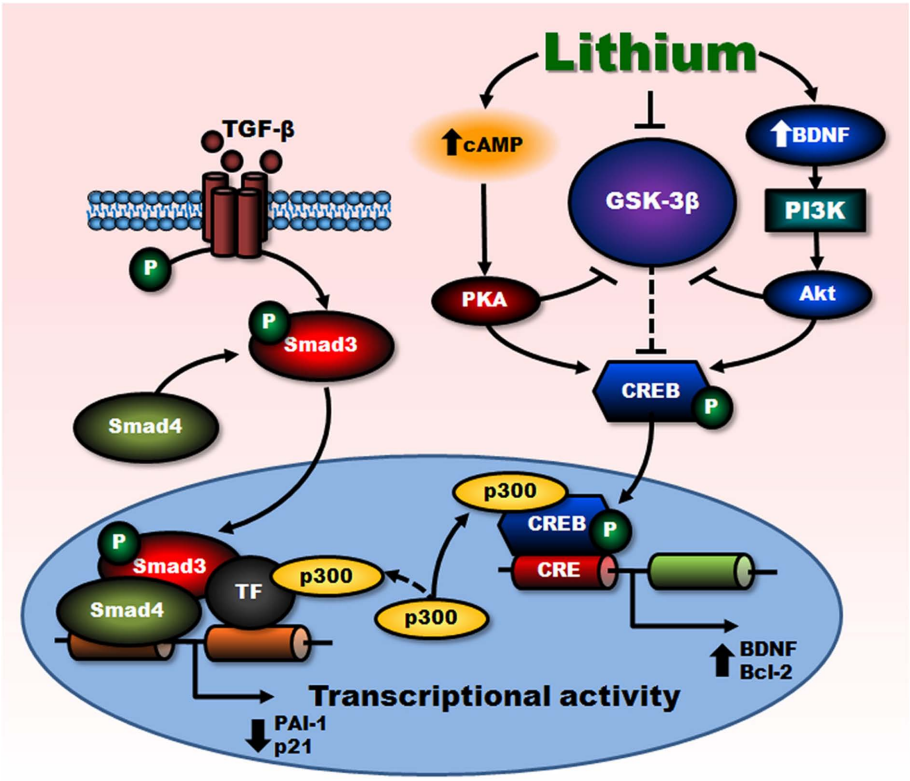

FIGURE 2 | Negative regulation of Smad3/4-dependent transcription by lithium. Transcriptional activations triggered by stimulation of cell surface TGF-β and BDNF receptors are mediated by Smad3/4- and PI3-kinase/Akt-dependent pathways, respectively. Lithium treatment-induced inhibition of GSK-3β, directly and indirectly via cAMP-dependent activation of PKA as well as BDNF-stimulated activation of PI3-kinase/Akt pathways, potentiates BDNF-induced phosphorylation/activation of CREB. This in turn increases CRE-mediated transactivation and expression of survival factors such as BDNF and Bcl-2. Enhanced gene transcription triggered by BDNF, via sequestration of transcriptional co-activator p300, suppresses Smad3/4-dependent transactivation and subsequently decreases the expression of TGF-β-responsive genes, PAI-1, and p21. Lines with solid arrows represent stimulatory connections; lines with flattened ends represent inhibitory connections. Dashed lines represent pathways with reduced activity as a result of lithium treatment. CRE, cAMP response element. (Modified from Liang et al., 2008).

family, and procaspase-9 (for review, Neri et al., 2002; Nicholson and Anderson, 2002; Huang and Reichardt, 2003). In addition, lithium also triggered Ser21 phosphorylation of the α isoform of GSK-3 (and hence resulted in inhibition), and this effect was prevented by a PI3-kinase inhibitor (Chalecka-Franaszek and Chuang, 1999). Another signaling pathway affected by lithium is the mitogen-activated protein (MAP) kinase pathway. One of the down-stream targets of MAP kinase is CREB, a transcription factor that is involved in learning and memory, and promotes the expression of Bcl-2 as well as brain-derived neurotrophic factor (BDNF; for review, Finkbeiner, 2000). In CGCs, toxic concentrations of glutamate-induced an NMDA receptor-dependent decrease in CREB phosphorylation at Ser133 and CREB-driven transcriptional activity (Kopnisky et al., 2003). Concurrent with its neuroprotective effects, long-term (but not acute) lithium treatment suppressed glutamate-induced dephosphorylation of CREB. We also found that glutamate rapidly activated c-Jun-N-terminal kinase (JNK) and p38 kinase in CGCs, resulting in a robust increase in AP-1 binding (Chen et al., 2003a). These two kinases are also activated by a variety of apoptotic insults (for review, Mielke and Herdegen, 2000), and AP-1 has been known to be activated by different stress factors as well. Experiments using lithium and curcumin, a selective AP-1 inhibitor, suggest that NMDA receptor-mediated apoptotic death requires concerted action of JNK and p38 to enhance AP-1 binding, and that lithium's neuroprotection is mediated, at least in part, by suppressing the JNK and p38 kinase pathways.

As one of the major neurotrophins, BDNF is essential for cortical development, synaptic plasticity, and neural survival, and is likely a key mediator of the clinical efficacy of anti-depressants and anxiolytic drugs (for review, Woo and Lu, 2006). The notion that BDNF plays a key role in neuronal survival is supported by our observation that BDNF and neurotrophin-4 (NT-4), but not NT-3, completely protected immature CGCs from apoptosis induced by cytosine arabinoside (Leeds et al., 2005). It was first reported that chronic treatment with lithium increased the expression of BDNF in the rat brain (Fukumoto et al., 2001), and we have documented that BDNF protein levels were increased in cortical neurons following lithium treatment (Hashimoto et al., 2002b). We hypothesized that this BDNF up-regulation and subsequent activation of its receptor TrkB might play a critical role in mediating the neuroprotective effects of lithium. In confirmation of this hypothesis, we

found that lithium's neuroprotection against glutamate excitotoxicity was blocked by a TrkB inhibitor, K252a, or by a neutralizing antibody against BDNF, and was mimicked by exogenous BDNF in rat cortical neurons. In addition, lithium increased intracellular levels of BDNF and this was followed by activation of TrkB. Furthermore, lithium-induced neuroprotection was prevented in cortical neurons from heterozygous (+/−) or homozygous (−/−) BDNF knockout mice (Hashimoto et al., 2002b).

Rodent BDNF has a complex genomic structure that makes it an ideal target for multiple and complex regulation. It contains multiple 5′-untranslated exons and one protein-coding 3′-exon. Each untranslated exon is alternatively spliced to produce various species of BDNF mRNA. We found that treatment of rat cortical neurons with therapeutic concentrations of lithium (e.g., 1 mM) caused a significant increase in the levels of BDNF exon IV-containing mRNA, while levels of exon I, II, or VI-containing mRNA remained unchanged (Yasuda et al., 2009). It is known that exon IV-containing BDNF transcripts are expressed in response to KCl-induced depolarization in rat cortical neurons (Tao et al., 2002). This transcriptional activation requires utilization of the promoter region 80 bp up-stream from the transcription initiation site of exon IV-containing three calcium responsive elements (CaREs; Chen et al., 2003b). We generated various BDNF promoter IV deletion constructs to investigate whether lithium treatment causes an increase in BDNF promoter IV activity, and, if so, which region of promoter IV confers the sensitivity to this drug. We identified that the drug-induced up-regulation of exon IV-containing BDNF transcript was associated with a significant increase in the activity of BDNF promoter IV and total BDNF protein. To our surprise, the lithium-responsive element(s) in promoter IV resides in a region up-stream from the CaREs responsible for depolarization-induced BDNF induction (−170 to −704 bp). Moreover, activation of BDNF promoter IV occurred in cortical neurons depolarized with KCl and depletion of these three CaREs failed to abolish lithium-induced activation. Importantly, we found that lithium-induced activation of promoter IV was mimicked by pharmacological inhibitors of GSK-3 (SB216763, SB415286, inhibitor I, and inhibitor VII) or by transfection with specific siRNA for GSK-3α or GSK-3β. Additionally, their dominant-negative mutants also mimicked lithium-induced activation of promoter IV. These results demonstrate that GSK-3 is the initial target of lithium to selectively activate BDNF promoter IV and that BDNF induction by lithium involves a novel responsive region in promoter IV of the BDNF gene. Lithium-induced, GSK-3-dependent BDNF promoter IV activation could be a part of the molecular mechanisms underlying its neuroprotective effects and as such, possibly accounts for the therapeutic actions in bipolar patients.

It should be noted that in addition to lithium, other GSK-3 inhibitors have been shown to almost completely block glutamate-induced excitotoxicity in rat cortical neuronal cultures (Liang and Chuang, 2007). These include ATP-competitive inhibitors, SB216763 and SB415286, and ATP-non-competitive inhibitors, Inhibitor I and VII. As mentioned in the preceding section, glutamate-induced death of cortical neurons was mitigated by silencing of GSK-3α and/or β, or both isoforms, or inhibition of GSK-3 activity via transfection with dominant-negative mutants of GSK-3α/β isoforms (Liang and Chuang, 2007). Studies from other laboratories also supported the roles of GSK-3 inhibition in protecting neurons from glutamate neurotoxicity. For example, stimulation of NMDA receptors in cultured rat hippocampal or cortical neurons activated GSK-3 by PP-1-mediated serine dephosphorylation of GSK-3β (Szatmari et al., 2005). GSK-3 inhibition reduced the PP-1-mediated serine dephosphorylation of GSK-3 and CREB. Treatment of primary rat cortical neurons with α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), lithium or SB216763 blocked glutamate-induced caspase-3 activation and excitotoxicity, and the protective effects of AMPA required PI3-kinase–Akt-dependent serine phosphorylation of GSK-3β (Nishimoto et al., 2008). Further, in organotypic cultures of chick embryo spinal cord, lithium prevented kainate-induced excitotoxic death of motoneurons by targeting GSK-3β, and this neuroprotection was associated with cytopathological changes (Caldero et al., 2010).

NEUROPROTECTIVE EFFECTS OF LITHIUM IN PRECLINICAL MODELS OF ISCHEMIC STROKE

Stroke is the third leading cause of death in the United States and a major global cause of serious long-term disability in adults. Ischemic strokes represent approximately 87% of all cases, while the rest are hemorrhagic strokes (Roger et al., 2011). In addition to physical deficits, stroke victims also suffer from vascular depression and dementia, both of which are difficult to treat with conventional medicine. It is becoming clear that there is a substantial increase in extracellular glutamate in the brain following cerebral ischemia, and that a significant portion of ischemia-induced brain damage is mediated by over-stimulation of NMDA receptors. Shortly after ischemia, the interruption of cerebral blood flow depletes oxygen and glucose and subsequently prevents ATP production. Inadequate ATP supply will cause the malfunction of ATP-dependent ion pumps and alter the ion concentration gradient across the neuronal membranes. The resulting failure to transport glutamate leads to an accumulation of glutamate in the extracellular space and over-stimulates NMDA receptors, which leads to a toxic influx of calcium and in turn drives the activation of damaging calcium-mediated intracellular enzymes. This cascade of events ultimately results in mitochondrial failure, production of reactive oxygen species, neuroinflammation, and cell necrosis and apoptosis (Allen and Bayraktutan, 2009; Deb et al., 2010).

GSK-3β has been strongly implicated in the neuronal cell death caused by cerebral ischemic insult. One study in rats subjected to transient middle cerebral artery occlusion (MCAO) demonstrated a rapid increase in the expression of cytoplasmic and nuclear GSK-3β protein in ipsilateral lamina I, II, V, and VI in young rat brains, whereas in lamina V and VI in old rat brains (Sasaki et al., 2001). In addition, the distribution of GSK-3β was well correlated with TUNEL-staining. Although the phosphorylation status of GSK-3β was not mentioned, these findings implicate a role of GSK-3β in cerebral ischemic injury. It is well known that GSK-3β can be phosphorylated at serine and tyrosine residues in which Ser9 phosphorylation renders it inactive, while Tyr216 phosphorylation is necessary for its functional activity (Hughes et al., 1993). Discrepancies exist in the literature regarding changes of Ser9 and

Tyr216 phosphorylation levels following cerebral ischemia. The phosphorylation levels of GSK-3β at Ser9 and Akt at Ser473 were reported to be markedly enhanced in the vulnerable hippocampal CA1 region, but not in the ischemia-resistant CA3 region in rats subjected to transient global cerebral ischemia, while there was no change in levels of Tyr216 phosphorylation or total GSK-3β (Endo et al., 2006). Levels of GSK-3β Ser9 phosphorylation were also increased shortly after permanent focal cerebral ischemia and decreased to basal levels or even lower 24 h after ischemic onset (Sasaki et al., 2006; Gao et al., 2008). However, it has also been reported that transient focal cerebral ischemia in rats caused an increase in GSK-3β Tyr216 phosphorylation in degenerating cortical neurons with no alteration in Ser9 phosphorylation (Bhat et al., 2000). This discrepancy may stem, in part, from the difference of ischemic severity and ischemic models across various studies. It appears that transient focal cerebral ischemia tends to activate GSK-3β and subsequently to induce apoptotic cell death. In contrast, GSK-3β is inactivated shortly after permanent focal cerebral ischemia or global cerebral ischemia, which in turn may promote survival of vulnerable neurons. A very recent study showed that following hypoxia–ischemia both GSK-3α and GSK-3β mediated the expression of a lethal protein, neuronal pentraxin 1 (Russell et al., 2011). In light of these findings, GSK-3 inhibition may provide neuroprotective effects against cerebral ischemia-induced injury. The beneficial effects of lithium in rodent cerebral ischemic models demonstrated by us and others support this notion.

In an initial study, long-term lithium pretreatment at therapeutically relevant doses decreased brain infarct volume, reduced apoptotic cell death and improved behavioral performance after permanent cerebral ischemia-induced by MCAO (Nonaka and Chuang, 1998; Xu et al., 2003). In a subsequent study, we demonstrated that subcutaneous injection of rats with lithium at therapeutic doses (e.g., 0.5 and 1.0 mEq/kg) after the onset of transient MCAO markedly decreased infarct volume, reduced TUNEL-positive DNA damage, and suppressed neurological deficits measured by sensory, motor, and reflex tests (Ren et al., 2003). The time window for these beneficial effects was at least 3 h after the onset of ischemia. Heat-shock protein 70 (HSP70), a well-established cytoprotective factor against apoptosis, was induced in the ischemic penumbra where neuronal recovery takes place. Post-insult treatment with lithium increased the DNA binding activity of HSF-1 to the heat-shock element, superinducing HSP70 which inhibits brain ischemia-induced apoptosis (Ren et al., 2003). Lithium-elicited GSK-3 inhibition is likely associated with HSF-1 activation and HSP70 induction (Bijur and Jope, 2000). Notably, post-insult lithium treatment mitigated apoptosis and brain damage by preventing GSK-3β and ERK dephosphorylation, suppressing calpain and caspase-3 activation, and inhibiting mitochondrial release of cytochrome *c* and apoptosis-inducing factor in a neonatal hypoxic–ischemic rat model (Li et al., 2010). These findings suggest that lithium-induced GSK-3 inhibition contributes to its anti-apoptotic effects under ischemic conditions.

In addition, it was found that lithium pretreatment largely suppressed ischemia-induced exploratory behavioral changes and memory impairments in gerbils after global cerebral ischemia (Bian et al., 2007). These behavioral benefits were associated with an increase in the number of viable cells and a decrease in apoptotic cells in the CA1 hippocampal area of ischemic gerbils. Moreover, lithium-induced neuroprotection in the ischemic brain was accompanied by down-regulation of pro-apoptotic p53 in the CA1, and up-regulation of anti-apoptotic Bcl-2 and HSP70, both of which are targets of GSK-3. It is likely that lithium protection against ischemia-induced injury involves multiple mechanisms. In the rat hippocampus, lithium was reported to inhibit ischemia-induced NMDA receptor hyperactivation by inhibiting NMDA subunit 2A tyrosine phosphorylation and its interactions with Src and Fyn through PSD-95 (Ma and Zhang, 2003). Lithium also attenuated hypoxia-induced serine dephosphorylation of GSK-3α and β in the mouse brain (Roh et al., 2005). Additionally, in organotypic cultures of rat hippocampus subjected to oxygen and glucose deprivation, lithium showed neuroprotection in conjunction with HSP27 activation (Cimarosti et al., 2001).

It is widely recognized that neuroinflammation plays a causative role in ischemic stroke injury. Post-ischemic inflammation is a dynamic process involving a complicated set of interactions between inflammatory cells and molecules (Iadecola and Alexander, 2001). A recent study documented the anti-inflammatory effects of lithium in a neonatal rat hypoxic–ischemic model. Post-insult lithium treatment significantly reduced total tissue loss following hypoxia–ischemia, and this beneficial effect of lithium was associated with inhibiting microglia activation and attenuating levels of pro-inflammatory cytokines or chemokines, such as interleukin-1β and chemokine ligand 2 (Li et al., 2011). One possible underlying mechanism is through HSP70 superinduction. HSP70 over-expression can inactivate the key inflammatory transcription factor NF-κB by stabilizing the NF-κB-IκB complex, and thereby preventing nuclear translocation of activated NF-κB subunits in a mouse MCAO model (Zheng et al., 2008).

Besides anti-inflammation, lithium also increased proliferation and differentiation of hippocampal neural progenitor cells in both non-ischemic and ischemic brains without altering the relative levels of neuronal and astrocytic differentiation, and this effect lasted at least 7 weeks after hypoxia–ischemia in neonatal rats (Li et al., 2011). In line with this finding, chronic lithium pretreatment was found to increase the generation and survival of newborn cells in the hippocampal dentate gyrus, and did not affect the neuronal or astrocytic differentiation of these newborn cells in a transient four-vessel occlusion model (Yan et al., 2007). ERK1/2 phosphorylation following ischemia was enhanced by lithium treatment, while ERK1/2 inhibitor U0126 prevented the effects of lithium in increasing BrdU-positive cells and improving spatial learning and memory (Yan et al., 2007). In fact, chronic lithium treatment has been demonstrated to increase activity in the MEK/ERK pathway *in vivo*, and lithium's neuroprotection has been suggested to depend on the induction of this signaling pathway (Einat et al., 2003). In addition, it was reported that activation of ERK associates with and phosphorylates GSK-3β at the Thr43 residue, which primes this kinase for its subsequent phosphorylation at Ser9 by p90RSK, resulting in inactivation of GSK-3β and up-regulation of β-catenin (Ding et al., 2005). Therefore, lithium might affect GSK-3β phosphorylation through the MEK/ERK pathway, which

in turn inhibits this kinase via RSK. GSK-3β is also negatively regulated by the Wnt/β-catenin signaling pathway and, accordingly, activating the canonical Wnt pathway has been shown to contribute to adult hippocampal neural progenitor cell proliferation triggered by lithium treatment (Wexler et al., 2008).

In a collaborative study, the neurohemodynamic aspects of recovery induced by delayed chronic lithium treatment were assessed using functional magnetic resonance imaging (MRI; Kim et al., 2008). Rats were subjected to transient MCAO and then injected with lithium (LiCl, 1 mEq/kg, s.c.) 12 h after the ischemic onset. This delayed lithium injection was followed by daily injections, and on day 15, an MRI scan was performed to monitor changes in blood oxygen level dependence (BOLD) and functional cerebral blood volume (fCBV) responses using electric stimulation of forelimbs. The mean activated volume ratio and total activation magnitude ratio between ipsilateral and contralateral cortices for both BOLD and fCBV were significantly higher in the lithium-treated than in the saline-treated rats. The lithium-induced increase in fCBV in the peri-infarct regions suggests a possible vascular transformation. Indeed, the size and distribution of immunohistochemical staining of CD31, a microvasculature marker, were enhanced by lithium treatment in the peri-infarct regions. Co-localized with CD31, the tissue staining of matrix metalloproteinase-9 (MMP-9) was also much more pronounced following lithium treatment, suggesting MMP-9-dependent neurovascular remodeling in the recovering brain area. Moreover, treatment of cultured rat brain endothelial cells with lithium in a follow-up study was also found to increase the protein levels of vascular endothelial growth factor (VEGF) via a mechanism involving the PI3-kinase and GSK-3 signaling pathways (Guo et al., 2009). Since VEGF has been linked to angiogenesis, neurogenesis, and neuroprotection (for review, Fan and Yang, 2007), VEGF over-expression may contribute to lithium's ability to promote neurovascular remodeling and to induce functional recovery after ischemic stroke.

Ample evidence supports the therapeutic potential of mesenchymal stem cells (MSCs) in several human diseases including stroke. However, it is increasingly recognized that the effectiveness of MSC transplantation is limited by their poor migration toward disease target sites such as ischemic brain regions. In a recent study, we investigated whether treatment of MSCs with lithium and another mood stabilizing drug, valproic acid (VPA), would enhance cell migration (Tsai et al., 2010). We found that treatment of MSCs with lithium (2.5 mM for 1 day) selectively elevated the transcript and protein levels of MMP-9 and its enzymatic activity. These effects were mimicked by pharmacological inhibition or gene silencing of GSK-3β. Lithium treatment also potentiated stromal cell-derived factor-1α (SDF-1α)-dependent MSC migration across the extracellular matrix, which was suppressed by two MMP-9 inhibitors, doxycycline and GM6001. Short-term (3 h) exposure of MSCs to a relatively high concentration (2.5 mM) of VPA markedly increased the transcript and protein levels of CXC chemokine receptor 4 (CXCR4). VPA-induced CXCR4 expression required its ability to inhibit histone deacetylases (HDACs), including the HDAC1 isoform, and involved histone hyperacetylation at the CXCR4 gene promoter. VPA treatment enhanced SDF-1α-mediated MSC migration, which was completely blocked by AMD3100, a CXCR4 antagonist. Notably, combining lithium and VPA treatment further increased MSC migration, and the additive enhancement of migration was completely blocked by the co-presence of AMD3100 and GM6001. Our results suggest that lithium and VPA stimulate MSC migration through distinct targets and mediators: GSK-3β–MMP-9 and HDAC–CXCR4, respectively (Tsai et al., 2010).

In a follow-up *in vivo* study, MSCs were primed with lithium and/or VPA and then injected into the tail vein of transient MCAO rats 24 h after ischemic onset. Priming with lithium or VPA increased the number of MSCs homing to the cerebral infarcted regions such as the cortex and striatum 2 weeks after transplantation, and co-priming with lithium and VPA further enhanced this migratory effect (Tsai et al., 2011). MCAO rats receiving lithium- and/or VPA-primed MSCs showed improved functional recovery, reduced infarct volume, and enhanced angiogenesis in the infarcted penumbra regions. These beneficial effects of lithium and VPA priming were reversed by pharmacological inhibition of MMP-9 and CXCR4, respectively, suggesting that these effects were likely mediated by lithium-induced MMP-9 up-regulation and VPA-induced CXCR4 over-expression. Together, these findings raise the potential utility of using MSCs primed with inhibitors of GSK-3 and HDAC to enhance the migration and homing capacity for transplantation into stroke victims.

In addition to lithium, other pharmacological GSK-3 inhibitors have been shown to exert neuroprotective effects against cerebral ischemia by various groups. A specific GSK-3β inhibitor, Chir025, was demonstrated to protect cultured hippocampal neurons from glutamate excitotoxicity and to attenuate death of cortical neurons following oxygen–glucose deprivation, an *in vitro* model of cerebral ischemia (Kelly et al., 2004). Moreover, Chir025 reduced infarct size in focal cerebral ischemic rats, but did not affect TUNEL-positive neurons or caspase-3/9 activities, although Bcl-2 expression was increased. GSK-3 enzymatic activity was markedly elevated after transient MCAO in rats, and this GSK-3 activation was blocked by jugular vein injection of GSK-3 inhibitor VIII (Koh et al., 2008). Pre- or post- (up to 2 h) MCAO injection with inhibitor VIII also reduced blood glucose levels, infarct size, caspase-3 activity, and water content in the ipsilateral brain hemisphere. Furthermore, ischemia-induced inflammation-related signals such as COX-2 over-expression and neutrophil infiltration were alleviated by this GSK-3 inhibitor. Prophylactic or therapeutic administration of a GSK-3β inhibitor TDZD-8 reduced infarct volume and cerebral injury in the rat hippocampus after transient ischemia (Collino et al., 2008). This was accompanied by suppression of ischemia-induced oxidative stress, apoptosis, and neuroinflammation. Delayed treatment with Compound I, a GSK-3β and Cdk inhibitor, decreased TUNEL-positive cells in the ipsilateral hippocampus and striatum of adult (but not juvenile) mice subjected to hypoxic–ischemic injury (Cowper-Smith et al., 2008). These neuroprotective effects of Compound I were associated with long-lasting functional recovery. Finally, GSK-3 inhibition by SB216763 counteracted oxygen–glucose deprivation-induced mitochondrial biogenesis impairment and reduced mitochondrial reactive oxygen species generation in primary cortical neurons (Valerio et al., 2011). When systematically administrated to permanent MCAO mice, SB216763 decreased infarct volume and

restored the loss of mitochondrial DNA, thus supporting a novel role of GSK-3 inhibitors in stimulating the renewal of functional mitochondria following ischemic stroke.

CONCLUSION

A growing body of evidence supports that lithium, a mood stabilizer used to treat bipolar disorder, has neuroprotective properties

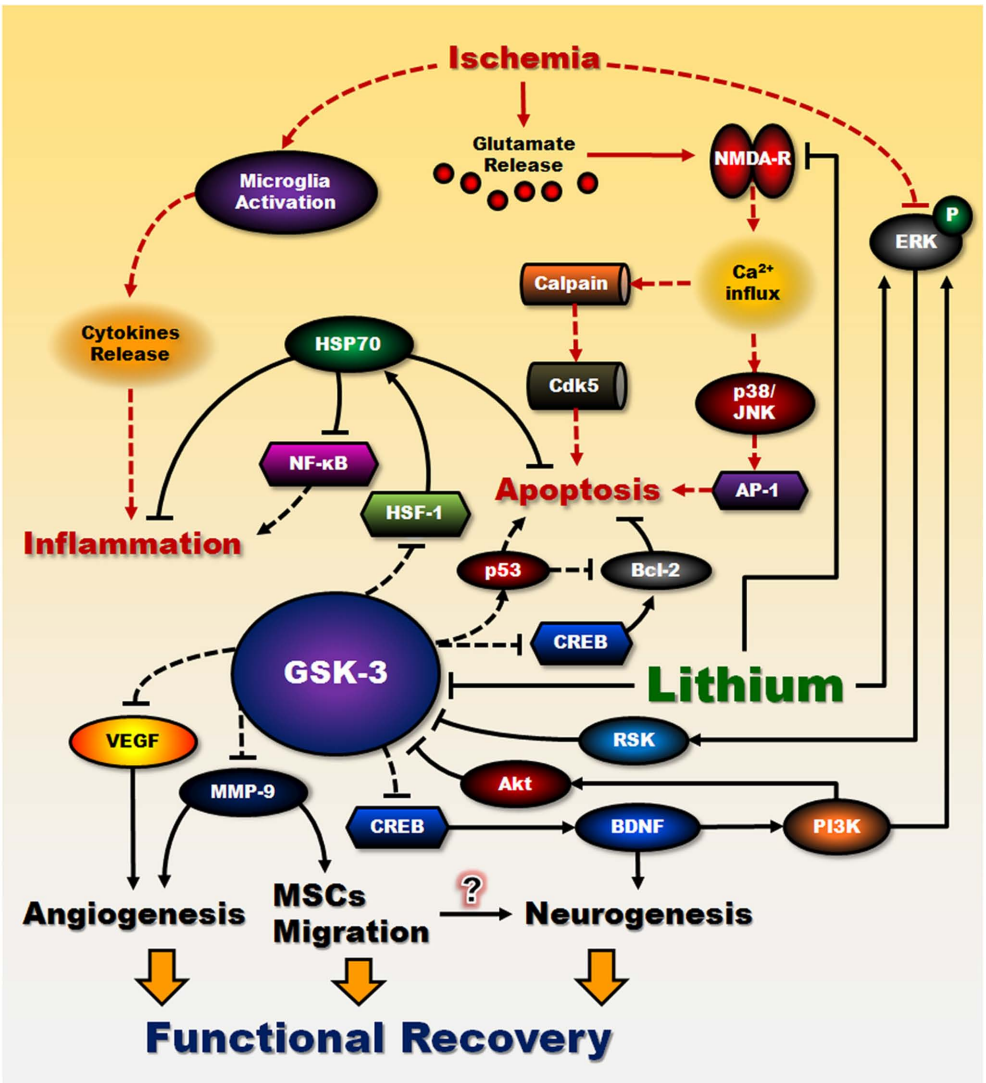

FIGURE 3 | Proposed lithium's neuroprotective effects against cerebral ischemia. The neuroprotective effects of lithium against cerebral ischemia are proposed to result from its interactions with cell survival and apoptotic machinery. A significant portion of brain damage following cerebral ischemia is caused by an increase in extracellular glutamate and subsequent over-stimulation of NMDA receptor-mediated toxic increase in intracellular calcium. This signaling pathway plays a critical role in mediating glutamate-induced caspase activation and apoptosis. Lithium at therapeutically relevant concentrations inhibits NMDA receptor-mediated calcium influx, which in turn decreases subsequent activation of JNK, p38 kinase, and transcription factor AP-1. Inhibition of intracellular calcium increase also attenuates the activity of calpain and calpain-mediated activation of pro-apoptotic Cdk5/p25 kinase. On the other hand, lithium can directly and indirectly reduce the activity of constitutively activated GSK-3 by multiple mechanisms, leading to disinhibition of several transcription factors, such as CREB and HSF-1, and resulting in induction of major cytoprotective proteins such as BDNF, VEGF, MMP-9, HSP70, and Bcl-2. A decrease in GSK-3 activity further reduces the activity of pro-apoptotic protein p53 and its downregulating effect on Bcl-2. BDNF, via activating its cell surface receptor and the down-stream ERK and PI3-kinase/Akt pathways, induces neuroprotective effects in part by inhibiting GSK-3 and stimulating CREB. Induction of BDNF is an early and essential step for neuroprotection and is involved in lithium-induced neurogenesis. In addition, superinduction of HSP70 by lithium treatment not only inhibits brain ischemia-induced apoptosis, but also contributes to the anti-inflammatory effects of lithium through inactivation of NF-κB. Counteraction of GSK-3 inhibition of VEGF and MMP-9 by lithium enhances angiogenesis and neurovascular remodeling. MMP-9 is also a key molecule involved in potentiating MSCs migration by lithium. Improvement in transplanted MSCs migration toward ischemic sites might increase neurogenesis as well. Taken together, these effects of lithium in reducing apoptosis, suppressing inflammation, enhancing angiogenesis and neurogenesis, contribute to behavioral improvement and functional recovery after ischemia. Lines with solid arrows represent stimulatory connections; lines with flattened ends represent inhibitory connections. Dashed lines represent pathways with reduced activity as a result of lithium treatment. NMDA-R, NMDA receptor.

in both cellular and *in vivo* experimental settings. One of the major targets of lithium is GSK-3, a serine/threonine kinase implicated in the pathogenesis of diverse CNS disorders. Lithium inhibits GSK-3 activity by direct binding to the enzyme or indirectly by enhancing serine phosphorylation of both α and β isoforms through multiple mechanisms. Lithium has been used as a prototype drug to seek evidence for the involvement of GSK-3 inhibition in lithium-induced protection against excitotoxicity in cultured neurons and animal models of cerebral ischemic stroke. Lithium at therapeutically relevant concentrations robustly protected primary brain neurons from glutamate-induced, NMDA receptor-mediated excitotoxicity. The neuroprotective effects of lithium were associated with GSK-3 inhibition, and were mimicked by other pharmacological GSK-3 inhibitors, by silencing GSK-3α and/or β isoforms, or by expression of isoform-specific dominant-negative mutants. These results support the roles of GSK-3 inhibition in lithium-elicited protection against excitotoxicity. Lithium rapidly activated the cell survival PI3-kinase–Akt signaling pathway to enhance GSK-3 serine phosphorylation and to block glutamate-induced Akt inactivation as well as apoptosis. Lithium also caused an increase in the expression of cytoprotective Bcl-2 and suppressed glutamate-induced up-regulation of pro-apoptotic p53 and Bax, resulting in blocking cytochrome *c* release from mitochondria. Induction of BDNF and activation of the BDNF–TrkB signaling were prerequisite for lithium's neuroprotection. BDNF promoter IV was selectively activated by GSK-3 inhibition using lithium or other drugs or through gene silencing/inactivation of either isoform. This effect on promoter IV resulted in BDNF transcriptional activation and protein up-regulation. However, there is a gap in the understanding of how GSK-3 inhibition causes an increase in BDNF promoter activity. In addition, lithium's neuroprotective effects were associated with inhibition of NMDA receptor-mediated calcium influx and suppression of p38/JNK and AP-1 activation, thus reducing apoptosis. This effect appears to stem from inhibition of Src/Fyn kinase to suppress NR2B Tyr1472 phosphorylation of the receptor. It remains to be explored as to whether this lithium-induced action on NMDA receptors is related to GSK-3 inhibition. It should be noted that lithium has other direct targets such as inositol phosphatases. The potential roles of these other targets in mediating the neuroprotective effects of this drug also deserve future investigation.

It is well known that glutamate overflow and NMDA receptor hyper-stimulation are early events following cerebral ischemia. In rodent ischemic models, pre- or post-insult treatment with therapeutic doses of lithium decreased infarct volume, caspase-3 activity and apoptotic cells in the injured brain. Importantly, lithium administration ameliorated neurological deficits, and improved functional recovery. The beneficial time window of lithium is at least 3 h after the ischemic onset. Up-regulation of HSP70 and Bcl-2 as well as down-regulation of p53 likely contributed to the protective effects of lithium in the ischemic conditions, thus supporting similar underlying neuroprotective mechanisms in the excitotoxic cellular models and animal models of ischemic stroke. Limited data suggested that lithium might also display anti-inflammatory effects by inhibiting ischemia-induced microglia activation and pro-inflammatory factors release. Delayed and chronic injections of lithium improved functional MRI responses such as increases in BOLD and fCBV. The improved fCBV was concurrent with enhanced angiogenesis and neurovascular remodeling. Indeed, lithium was found to induce two pro-angiogenic factors, MMP-9 and VEGF in a GSK-3-dependent manner. Lithium has also been reported to stimulate ERK1/2 activity and to enhance proliferation of hippocampal neural progenitor cells and memory performance after ischemia. Finally, lithium promoted migration of MSCs *in vitro* by up-regulation of MMP-9 through GSK-3β inhibition and this migratory effect was potentiated by co-treatment with VPA, another mood stabilizer. Notably, transplantation of lithium–VPA co-primed MSCs into ischemic rats markedly increased MSC migration to the injured brain regions, decreased infarct size and improved the neurological performance. Lithium-induced stem cell migration, neurogenesis, and angiogenesis all likely contribute to functional recovery. **Figure 3** illustrates proposed molecular events leading to lithium-induced beneficial effects following cerebral ischemia. It should be noted that several other GSK-3 inhibitors have also been reported to exert beneficial effects in rodent ischemic models and their actions were accompanied by suppression of ischemia-increased GSK-3 activity. Accordingly, GSK-3 inhibitors have therapeutic potential to treat stroke and other excitotoxicity-related neurodegenerative diseases. Lithium has been used in bipolar patients over 60 years and its clinical profiles are well understood. Therefore, lithium is a prime candidate for use in clinical trials of new therapies for stroke victims.

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GSK-3 mouse models to study neuronal apoptosis and neurodegeneration

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Increased GSK-3 activity is believed to contribute to the etiology of chronic disorders like Alzheimer's disease (AD), schizophrenia, diabetes, and some types of cancer, thus supporting therapeutic potential of GSK-3 inhibitors. Numerous mouse models with modified GSK-3 have been generated in order to study the physiology of GSK-3, its implication in diverse pathologies and the potential effect of GSK-3 inhibitors. In this review we have focused on the relevance of these mouse models for the study of the role of GSK-3 in apoptosis. GSK-3 is involved in two apoptotic pathways, intrinsic and extrinsic pathways, and plays opposite roles depending on the apoptotic signaling process that is activated. It promotes cell death when acting through intrinsic pathway and plays an anti-apoptotic role if the extrinsic pathway is occurring. It is important to dissect this duality since, among the diseases in which GSK-3 is involved, excessive cell death is crucial in some illnesses like neurodegenerative diseases, while a deficient apoptosis is occurring in others such as cancer or autoimmune diseases. The clinical application of a classical GSK-3 inhibitor, lithium, is limited by its toxic consequences, including motor side effects. Recently, the mechanism leading to activation of apoptosis following chronic lithium administration has been described. Understanding this mechanism could help to minimize side effects and to improve application of GSK-3 inhibitors to the treatment of AD and to extend the application to other diseases.

Keywords: GSK-3, apoptosis, mouse models, Alzheimer's disease, neurodegenerative disorder

Glycogen synthase kinase-3 (GSK-3) was initially identified more than two decades ago as an enzyme involved in the control of glycogen metabolism (Cohen, 1979; Embi et al., 1980). In mammals two closely related isoenzymes, GSK-3α and GSK-3β are present (Woodgett, 1991). The more conserved isoform in evolution is GSK-3β with a widely expression throughout the animal kingdom, while GSK-3α is only found in vertebrates (Plyte et al., 1992; Alon et al., 2011). GSK-3α and βare ubiquitously expressed in all tissues, with particularly abundant levels in the brain (Woodgett, 1990; Yao et al., 2002; Perez-Costas et al., 2010).

GSK-3 is implicated in several signaling pathways like the insulin and insulin-like growth factor-1 (IGF-1)-mediated signal transduction or the wnt/wingless signaling pathway. GSK-3 phosphorylates and thereby regulates the functions of many metabolic, signaling, and structural proteins. Some metabolic and signaling proteins that are phosphorylated by GSK-3 are glycogen synthase, insulin receptor substrate-1 (IRS-1), cyclin D1, and eIF2B. Examples of structural proteins regulated by GSK-3 are microtubule associated proteins (MAPs) among others. Given the numerous targets and the implication in several signaling pathways, GSK-3 is involved in regulating many processes like cellular structure, function, and survival. Regarding the latter, as we will see below, GSK-3 plays a key role in apoptosis (Grimes and Jope, 2001; Jope and Johnson, 2004). Thus, GSK-3 is now recognized as an important regulator of a large number of cellular processes and, when deregulated, is thought to play a role in the etiology of various diseases. These include diabetes and/or insulin resistance (reviewed in Eldar-Finkelman, 2002), muscle hypertrophy (reviewed in Hardt and Sadoshima, 2002), cancer (reviewed in Manoukian and Woodgett, 2002), bipolar mood disorder (reviewed in Manoukian and Woodgett, 2002), schizophrenia (Emamian et al., 2004; reviewed in Jope and Roh, 2006), and neurodegenerative diseases like Alzheimer's disease (AD; reviewed in Avila et al., 2004) and Huntington's disease (Carmichael et al., 2002; Berger et al., 2005).

DUAL ROLE OF GSK-3 IN APOPTOSIS

Programmed cell death is a major component of both normal development and disease. Apoptosis is a process that takes place with condensation of the nucleus, DNA fragmentation and, finally, disintegration of the cell in small apoptotic bodies that are destined to be phagocytized (Assuncao Guimaraes and Linden, 2004). Two opposing roles have been established for GSK-3 in apoptosis (**Figure 1**). Under certain conditions GSK-3 acts as a strong inhibitor of apoptosis and in other conditions plays a pro-apoptotic role. What determines whether GSK-3 acts in one way or the other is the type of apoptosis that occurs, which may be intrinsic (type I) or extrinsic (type II). Consequently, inhibitors of GSK-3 provide protection from intrinsic apoptosis signaling but potentiate extrinsic apoptosis signaling (Beurel and Jope, 2006). The concept that GSK-3 inhibits apoptosis came from the discovery that GSK-3β knockout mice died during embryonic

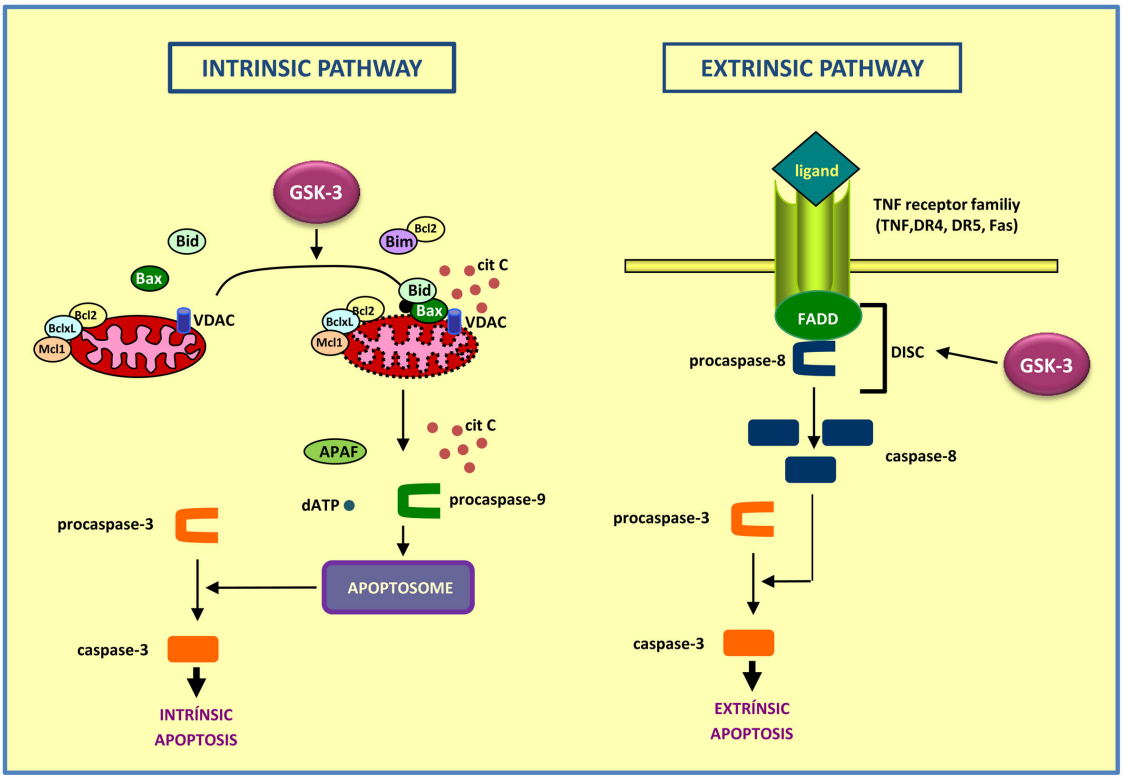

FIGURE 1 | Diagram showing the intrinsic (left) and extrinsic (right) apoptotic pathways. In the intrinsic apoptotic pathway, GSK-3 exerts a pro-apoptotic role, acting on targets as Bax, Bim, and VDAC, thus contributing to the disintegration of mitochondria and the release of cytochrome *c*. On the contrary, the role of GSK-3 is anti-apoptotic in the extrinsic pathway, by preventing DISC complex formation.

development due to massive apoptosis of hepatocytes (Hoeflich et al., 2000), which demonstrated that GSK-3β is an important inhibitor of apoptosis. However, this observation appears to be in direct opposition to the finding that overexpression of GSK-3β is sufficient to induce apoptosis (Pap and Cooper, 1998). It is important to understand this duality, since, among the diseases in which GSK-3 is involved, some are characterized by excessive cell death (neurodegenerative diseases), while in others a deficient apoptosis is occurring (cancer or autoimmune diseases).

(a) The *intrinsic apoptosis* involves the loss of integrity of mitochondria with release of cytochrome *c* leading to cell destruction. In the cytoplasm, released cytochrome *c* binds to APAF, ATP, and procaspase 9, thus forming the apoptosome and leading to activation of caspases which finally induces cell death. GSK-3 participates in the intrinsic apoptosis pathway by acting on targets such as Bax (Linseman et al., 2004), Bim (Hongisto et al., 2003), or VDAC (Pastorino et al., 2002), thus contributing to the disintegration of the mitochondria (Beurel and Jope, 2006).

The stimuli able to trigger this type of cell death are diverse and they include, among others, DNA damage, oxidative stress or endoplasmic reticulum stress. Numerous pieces of evidence have shown that under conditions that activate the intrinsic pathway, GSK-3 plays a pro-apoptotic role. Initially it was found that GSK-3 was involved in apoptosis in response to inhibition of the PI3K pathway (Pap and Cooper, 1998). Later it was found that, in cultures of rat cortical neurons, GSK-3 not only was promoting apoptosis caused by inhibition of the PI3K pathway, but also it was involved in the apoptosis in response to a withdrawal of trophic factors (Hetman et al., 2000, 2002). Moreover, due to the withdrawal of trophic factors in PC12cells, phosphorylation of GSK-3β at Tyr 216 increases (activation), and can be reversed by lithium or insulin, which affects the phosphorylation at Ser 9 (inactivation) but not at Tyr 216 (Bhat et al., 2000). In response to thapsigargin, an inducer of endoplasmic stress, not only caspase-3 is activated, but it also significantly increases GSK-3 activity (Song et al., 2002). Three pro-apoptotic stimuli such as serum withdrawal, staurosporine, or heat shock produced increased nuclear localization of GSK-3β. This change in subcellular localization seemed to be independent of phosphorylation and prior to caspase-9 and -3 activation, thus indicating that nuclear accumulation of GSK-3 is an early event in the intrinsic apoptotic signaling. Nuclear GSK-3 or GSK-3 targets present in the nucleus, as transcription factors, may be promoting intrinsic apoptosis signaling (Bijur and Jope, 2001).

An interesting fact was described in Rat-1 and PC-12 cells where, in the absence of apoptotic stimuli, overexpression of GSK-3 resulted in cell death by itself, being able to reverse apoptosis with

the expression of a dominant-negative form of GSK-3 (K85R-GSK-3; Pap and Cooper, 1998). In most of these studies induction of apoptosis can be reversed by lithium or other inhibitors of GSK-3, establishing a neuroprotective role with a potential therapeutic application of such inhibitors (Cohen and Goedert, 2004; Wada, 2009).

(b) The *extrinsic apoptosis* involves activation of death receptors of the TNF receptor family located in the plasma membrane, such as TNF-R, Fas (also called CD95), DR4, and DR5 (Ashkenazi and Dixit, 1998). Although each of these receptors is activated by its own ligand, they share a common mechanism. The stimulation by the ligand results in receptor trimerization to which the protein Fas-associated death domain (FADD) and procaspase-8 can bind. This promotes the formation of the death-inducing signaling complex (DISC; Peter and Krammer, 2003). Subsequently a self-activation of caspase-8 occurs, which leads to activation of effector caspases and completion of apoptosis (Beurel and Jope, 2006).

The role of GSK-3 as an anti-apoptotic agent of the extrinsic pathway has been less studied. Most of these studies were conducted in tumor cell models, following the hypothesis that inhibition of GSK-3 improves response to chemotherapy. The earliest evidence dating back to 1989 when Beyaert et al. found that LiCl enhanced the toxicity by TNF, in murine and human tumor cells, in a manner similar to the effects of cycloheximide, the actinomycin D, or interferon γ (Beyaert et al., 1989). As a result, a combination of both was proposed as a treatment to improve the response to chemotherapy in cancer patients.

Other studies show potentiation of the extrinsic pathway of apoptosis by selective inhibitors of GSK-3 in tumor cell lines (Liao et al., 2003; Ougolkov et al., 2007). A recent study, also in tumor cell lines, depth in this mechanism, and places GSK-3 complexed with death receptor, thus preventing the formation of DISC. When activated, the death receptor induces changes in the complex and inhibits GSK-3, favoring apoptosis (Sun et al., 2008). In 2004, also the group of R. S. Jope published the first study of GSK-3 as a component of the extrinsic pathway in neuronal cells. This study revealed that apoptosis induced by the extrinsic pathway, specifically by Fas receptor signaling, was enhanced by lithium and other GSK-3 inhibitors (Song et al., 2004).

MOUSE MODELS WITH GENETIC MODIFICATIONS OF GSK-3

In order to investigate the involvement of GSK-3 in cellular processes and to clarify its implications in diseases, a great effort was and is still being made to generate animal models that mimic pathological conditions through the deregulation of GSK-3. Up to date the main effort has been invested in creating animal models with altered expression of GSK-3, especially the evolutionary well conserved isoform GSK-3β.

To achieve this goal, different strategies have been applied: overexpression, suppression, and modulation of the expression of the enzyme. Many of these models direct expression or modification of GSK-3 to the central nervous system (CNS) for the generation of mouse models of AD. Nowadays several other species apart from the mouse are also used as model systems, which include *Drosophila melanogaster* and zebrafish. In this review we will focus on results obtained from mouse models.

MOUSE MODELS WITH INCREASED GLYCOGEN SYNTHASE KINASE-3 ACTIVITY

One of the earliest diseases linked to dysfunctions of GSK-3β was AD. More recently, it has been proposed that the deregulation of GSK-3β might affect other tauopathies like frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17; Perez et al., 2000). Regarding AD, it has been demonstrated that GSK-3β mediates tau hyperphosphorylation, β-amyloid-induced neurotoxicity, and mutant presenilin-1 pathogenic effects (Jope and Johnson, 2004). Increased levels of GSK-3 have also been reported in postmortem analysis of brains from AD patients compared to age-matched control samples (Pei et al., 1997), where as a spatial and temporal pattern of increased active GSK-3β expression correlates with the progression of NFT and neurodegeneration (Leroy et al., 2002). Thus, the first GSK-3 mouse models focused on the study of the implications of GSK-3β overexpression in the CNS on the development of AD and related neuropathological diseases. To achieve this goal, promoters with known neuronal expression like NF-L, thy1, or the more region specific promoter CaMKIIα, were used.

In 1997, Brownlees et al. generated the first two GSK-3 mouse models. One carried the human wild-type GSK-3β isoform as the transgene and the other one the mutant form of GSK-3β in which serine-9 of GSK-3β was mutated to alanine (S9A), thus supposedly resulting in a more active form by preventing inactivation through the phosphorylation on Ser9 (reviewed in Grimes and Jope, 2001). These transgenes were under the transcriptional control of either the ubiquitous murine sarcoma virus (MSV) promoter or the neuronal specific promoter of the murine neurofilament light chain (NF-L; Brownlees et al., 1997b). In spite of detecting the two transgenes mRNAs through reverse-transcribed PCR, no substantial increase in total activity of GSK-3β was observed, neither regarding the wild-type GSK-3β nor the mutated form. They also observed that the expression of the mutated form of GSK-3β (S9A) was much lower, probably due to the fact of its increased activity. Despite no detectable GSK-3 overexpression, Western blot analysis of the two mouse lines with the highest levels of transgenic GSK-3β activity revealed that the phosphorylation status of tau was elevated at the AT8 epitope. The authors postulated that toxicity elicited by GSK-3β overexpression during embryonic and postnatal development of the CNS may explain why they were unable to generate mouse lines with detectable GSK-3β overexpression. Conversely, as it will be commented below, GSK-3β knockout mice die during embryonic life (Hoeflich et al., 2000). From this first model of GSK-3 increased activity we extract the notion of an embryonic lethality but no further analysis regarding the role of GSK-3 in apoptosis was achieved.

The second transgenic animal published also expressed the S9A mutant form of the kinase, i.e., GSK-3β (S9A); this time under the control of the modified murine thy1 gene (Spittaels et al., 2000). The engineered thy1 construct used in this model drives the expression of the transgene only postnatally and only in neurons. This mouse shows a twofold increase in GSK-3β activity.

Accordingly, an increase in phosphorylated tau was demonstrated, but only in older transgenic mice (7–8 months). In a second round of characterization of this mouse line, Spittaels et al. also observed in these mice a significant decrease in overall brain weight and volume, with the largest reduction occurring in the cerebral cortex. This reduction was not due to apoptosis, since there was no increase in TUNEL or in cleaved caspase-3 positive neurons (Spittaels et al., 2002). The resulting compacted architecture was further demonstrated by an increased neuronal density, by reduced neuronal cell bodies and of the somatodendritic compartment of pyramidal neurons in the cortex. The overall reduction in size of the entire CNS induced by constitutive active GSK-3β caused only very subtle changes in the psychomotor ability of adult and aging GSK-3β transgenic mice. Overexpression of GSK-3β in this model also has been shown to up- or down-regulate the expression of numerous proteins in neurons, including down-regulation of MAP2 (Tilleman et al., 2002). In spite of increased tau phosphorylation and decreased brain weight these GSK-3β transgenic mice performed normally in the Morris water maze test (Spittaels et al., 2002).

One of the characteristics of persons affected by FTDP-17 is an increased expression of the longest human protein tau isoform containing the four repeat regions in the carboxyl-terminal part (Heutink, 2000). For this reason Spittaels et al. (1999), in another approach, cross-bred their GSK-3β (S9A) mice with transgenic mice that carry the longest human protein tau isoform expressed under the same promoter (thy1). The authors observed a reduction of the number of axonal enlargements and motor impairments typical of these tau transgenic animals (Spittaels et al., 2000). In these mice, an increase in phosphorylation of human tau was demonstrated, although neither an increase in insoluble tau aggregates nor the presence of paired helical filaments was observed. Spittaels et al. concluded that the improvement of axonopathy and motor problems found in htau40 mice when mixed with GSK-3β (S9A) mice was due to phosphorylation of tau induced by GSK-3β which decreases its affinity for microtubules and consequently rescues axonal transport. The same group crossed this mutant GSK-3β (S9A) mice with a tau model overexpressing Tau (P301L; Terwel et al., 2008) observing an increase in forebrain tau pathology although conversely the bigenic mice survived longer than the parental Tau (P301L).

In a similar approach Li et al. (2004) used the isoform GSK-3β (S9A), isoform resistant to inhibition by phosphorylation, under control of the neuron specific human platelet-derived growth factor (PDGF) β-chain promoter. This promoter drove the expression mainly to the cortex and hippocampus of the transgenic brain. They observed an increase in GSK-3 activity without changes in the total level of GSK-3β. They also observed an increase in tau phosphorylation detected by the AT8 antibody by Western blot analysis. This approach did not show any evidence of apoptosis although they show increased pretangle morphology which could be indicating a neurodegenerative process.

Overall, the GSK-3β (S9A) mice generated by Spittaels and co-workers show decreased brain weight and volume, more pronounced in the cortex that cannot be attributed to an apoptotic effect as there is no evidence of increased TUNEL or caspase-3 levels.

In view of the postulated lethality of embryonic GSK-3β overexpression (Brownlees et al., 1997a) as well as the known role of GSK-3β in development, a further GSK-3β transgenic mouse was generated (Lucas et al., 2001) by using the conditional tetracycline-regulated system. In these mice transgene expression was under the control of the CaM kinase II α-promoter to achieve substantial overexpression of wild-type GSK-3β in forebrain neurons and, therefore, in regions more relevant for AD.

In this transgenic line (Tet/GSK-3β mice), GSK-3β overexpression was restricted to certain cortical neurons and hippocampal neurons. It was in the hippocampal region were a 30% increase in GSK-3β activity was observed by enzymatic assay on tissue homogenate (Hernandez et al., 2002). Hippocampal overexpression of GSK-3β resulted in an increase in the phosphorylation of tau in Tet/GSK-3β animals, as detected with antibodies raised against the phosphorylated tau modified in AD, like PHF-1/AD2. This hyperphosphorylated tau was found in the somatodendritic compartment, not because of an increase in the total amount of tau protein, but exclusively due to the hyperphosphorylation of tau by GSK-3β. In spite of a substantial increase in hyperphosphorylated tau, the aberrant tau aggregation found in AD was not observed in these GSK-3β transgenic mice. In this model, β-catenin, another GSK-3 substrate relevant to AD (Zhang et al., 1998), was also analyzed. GSK-3 is a key enzyme involved in β-catenin stabilization and nuclear translocation (reviewed in Barth et al., 1997; Anderton, 1999). Increased GSK-3β in Tet/GSK-3β mice was seen effective in modifying β-catenin in neurons of the CNS *in vivo*, since nuclear β-catenin was reduced by ∼75% in hippocampal granular cells (Lucas et al., 2001). Considering that the genes transactivated by β-catenin are poorly characterized, these mice may serve as a good tool to identify such genes. Tet/GSK-3β mice also demonstrate neuronal stress and neuronal death as revealed the presence of reactive glia, TUNEL labeling, and cleaved caspase-3 staining. These data are in agreement with the known role of GSK-3β in the survival pathway, as well as supporting the neuroprotective effect of lithium (reviewed in Chuang et al., 2002). The hyperphosphorylation of hippocampal tau in transgenic Tet/GSK-3β mice, despite the lack of filament formation, results in a behavioral impairment that can be measured in the Morris water maze test (Hernandez et al., 2002) and in the object recognition test (Engel et al., 2006a).

Animal models generated by using the tetracycline-regulated system make it possible to explore any potential reversal of their phenotype (Mayford et al., 1996; Yamamoto et al., 2000). Thus the Tet/GSK-3β should be a good tool to test the neuroprotective effect of forthcoming GSK-3β specific inhibitors. In 2006 Engel and co-workers published a study of the effect of silencing transgene expression by the administration of tetracycline to the Tet/GSK-3β mice (Tet-OFF system). They show that transgene shutdown in symptomatic mice leads to normal GSK-3 activity, normal phospho-tau levels, diminished neuronal death, and suppression of the cognitive deficit, thus further supporting the potential of GSK-3 inhibitors for AD therapeutics (Engel et al., 2006b).

The tetracycline-regulated system also gives us the opportunity to overexpress the GSK-3β transgene in other regions than the CNS, using another promoter for the tetracycline-activator. This

would provide the great opportunity to study the implications of a deregulation of GSK-3β in other cell types, other tissues, and to generate animal models for other diseases.

The implication of wild-type GSK-3β in tau pathology was further analyzed by combining this transgenic model with transgenic mice expressing tau with a triple FTDP-17 mutation which develop prefibrillar tau aggregates (VLW mice; Lim et al., 2001). In the VLW single transgenic mice, which express the longest human tau isoform with three FTDP-17 point mutations (G272V, P301L, and R406W, $hTau^{VLW}$) under the control of the modified thy1 mouse promoter, thin filaments with a width of ∼5 nm can be observed. The triple transgenic mice Tet/GSK-3β/VLW showed an increase in tau phosphorylation in the hippocampus, the area where the two transgenes, GSK-3β and $hTau^{VLW}$ were co-expressed and where GSK-3 activity was increased. In addition, hyperphosphorylated tau with an aberrant high molecular weight was present in these triple transgenic mice but not in the single transgenic mice VLW or the Tet/GSK-3β mice. Similarly, thioflavine-S positive neurons were only observed in the hippocampus of Tet/GSK-3β/VLW mice and filaments with a width wider than 10 nm (a width similar to that found in tauopathies) could be purified only from Tet/GSK-3β/VLW mice. This model demonstrates that an increase in GSK-3β activity is a key factor able to induce tau aggregation (sarkosyl-insoluble material as well as tau filaments). Neurodegeneration studies in these mice showed a decrease in the volume of the dentate gyrus of Tet/GSK-3β/VLW mice at 5 months, not seen in Tet/GSK-3β mice of the same age. At the advanced age of 18 months both Tet/GSK-3β and Tet/GSK-3β/VLW exhibited a severe atrophy of the dentate gyrus at a similar level in both transgenic mice. Thus, the neurodegenerative process occurring in the dentate gyrus of Tet/GSK-3β transgenic mice is accelerated by the presence of mutated tau.

As a proof of concept that tau modification by GSK-3 plays a role in neurodegeneration found in the previously exposed models, GSK-3 overexpressing mice were brought to tau knockout (Tau−/−) background. Interestingly in Tet/GSK-3β + Tau−/− mice the toxic effect of GSK-3 overexpression is milder and slower in the absence of tau (Gomez de Barreda et al., 2010).

What we can extract from studies in Tet/GSK-3β is that an increase in GSK-3 activity correlates with an increase in apoptosis which seems to involve tau modification, as seen when mice were combined with models of tau modifications.

Another approach used to study the deregulation of GSK-3 is the generation of knock-in mice with a modified GSK-3 transgene. In 2005, McManus et al. have generated a GSK-3 homozygous knock-in mice in which the protein kinase B (PKB/AKT) phosphorylation sites on GSK-3α (Ser21) and GSK-3β (Ser9) were changed to a non-phosphorylatable Ala residue (McManus et al., 2005). This protein modification would enable studying the role played by the inactivation of GSK-3 through these phosphorylation events in insulin and Wnt signaling pathways. These knock-in mice develop and reproduce normally and were not diabetic, despite insulin being unable to stimulate glycogen synthase in muscle. In double GSK-3 knock-in mice the GSK-3 isoforms are present at normal levels. Analyzing this model it was demonstrated that insulin regulates muscle glycogen synthase mainly through GSK-3β rather than GSK-3α. Regarding the CNS, this model shows altered neurogenesis as well as a deficient production of the supporting vascular endothelial growth factor (VEGF) in the hippocampus (Eom and Jope, 2009). *In vivo* chronic co-administration of lithium and fluoxetine increases proliferation in the dentate gyrus of wild-type but not GSK-3α/β (S21A/S21A/S9A/S9A) knock-in mice. Following these results it was suggested that blockade of the inhibitory control of GSK-3 results in impaired neurogenesis (Eom and Jope, 2009). At this respect, it has been described that GSK-3 is a master regulator of neuronal progenitor homeostasis during embryonic development (Kim et al., 2009). In the specific case of adult neurogenesis taking place in the subgranular zone of the dentate gyrus, a role for tau phosphorylation (by GSK-3) has been found in the migration and differentiation of neuronal precursors into mature neurons (Fuster-Matanzo et al., 2009; Hong et al., 2010). Interestingly, Tet/GSK-3 mouse model of GSK-3 overexpression showed an impairment in proliferation and maturation that together with increased apoptosis, contribute to the atrophy of the dentate gyrus (Sirerol-Piquer et al., 2011).

Overall GSK-3 increased activity induced in homozygous GSK-3α/β (S21A/S21A/S9A/S9A) knock-in mice results in altered neurogenesis, without clear implications in apoptosis.

An additional transgenic model overexpressing wild-type GSK-3β was generated to be crossed with mice overexpressing FAD-mutant APP (Rockenstein et al., 2007). These mice showed an improvement in Morris water maze compared with single mutant APP mice, and strikingly, they had a decrease in tau phosphorylation as well as a decrease in APP phosphorylation and Aβ levels. To explain these unexpected results, the authors argued that, although GSK-3β was overexpressed, this resulted in an increase in the inhibitory domain of the kinase. That was the reason to name this animal model, in a confusing way, as a dominant-negative GSK-3β model. Overall the manuscript demonstrates that GSK-3 is able to modulate the amyloid aspect of AD *in vivo*, but has no implications in apoptosis.

In summary, the role of GSK-3 in apoptosis when explored in mouse models with increased GSK-3 activity seems to be pro-apoptotic. Even if apoptosis has not been studied in detail, a toxic effect seems to be common to these mouse models.

ANIMAL MODELS WITH REDUCED GLYCOGEN SYNTHASE KINASE-3 ACTIVITY

Several genetic approaches have been used to generate mice with a decrease in GSK-3 levels. The first study to suppress its expression in a mouse model was the generation of GSK-3β-KO mice by Hoeflich et al. (2000). In this study the GSK-3β gene has been disrupted by targeted deletion. These investigators showed that GSK-3β is absolutely essential for survival. The generated GSK-3β-knockout mice developed normally to mid-gestation, but died around embryonic day 14 following massive tumor necrosis factor-α (TNF-α)-induced hepatocyte apoptosis, which could be prevented by the injection of antibodies that block the function of TNF-α. Nuclear factor κB (NF-κB) activation is known to counteract TNF-α- induced apoptotic signaling to promote survival by turning on a set of anti-apoptotic genes (Pomerantz and Baltimore, 2000). The intriguing finding made by Hoeflich et al. is that GSK-3β is required for the NF-κB-mediated survival response. This study therefore demonstrates for the first time

an isoform-specific GSK-3 function since GSK-3α was unable to compensate for the loss of GSK-3β.

Although the GSK-3β knockout mice died during mid-gestation, heterozygotes mice were viable and appeared morphologically normal. Mouse embryonic fibroblasts derived from the GSK-3β heterozygotes knockout mice expressed reduced levels of GSK-3β protein without a compensatory increase in GSK-3α protein levels, indicating a partial loss of function in GSK-3β heterozygous. The authors also observed a reduced GSK-3β activity in the brains of the heterozygous mice (Hoeflich et al., 2000).

These GSK-3β heterozygous knockout mice were used by Beaulieu et al. and O'Brien et al. (2004) in two subsequent studies. In the first study Beaulieu et al. (2004) demonstrated in dopamine transporter knockout mice that dopamine can exert its behavioral effects by acting on a lithium-sensitive signaling cascade involving Akt/PKB and GSK-3. In this study increased dopamine neurotransmission resulted in inactivation of Akt and concomitant activation of GSK-3α and GSK-3β. These biochemical changes were effectively reversed by the administration of the GSK-3 inhibitor lithium or when combined with the GSK-3β heterozygous knockout mice, thus establishing this cascade as an important mediator of dopamine action *in vivo*. Interestingly, these DAT-KO mice, with increased GSK-3 activity, also show increased microgliosis, and a small percentage of apoptotic neurons associated with increased tau phosphorylation (Cyr et al., 2003).

O'Brien et al. (2004) compared the behavioral effects of chronic lithium treatment on mice with the behavioral phenotype of the GSK-3β heterozygous knockout mice. In this study the authors observed that lithium treated mice spent less time immobile in the forced swimming test, test widely used as a predictor of antidepressant efficacy. The same result was obtained when they used the GSK-3β heterozygotes knockout mice. In the exploratory test, both, lithium treated and GSK-3β heterozygotes knockout mice, acted in the same way with less exploratory activity as compared to wild-type mice. Molecular targets of GSK-3 dependent signaling, such as β-catenin, are also affected similarly by lithium and GSK-3β haploinsufficience with a substantial increase of ∼30%. These behavioral and molecular correlations strongly support the hypothesis that GSK-3 is an important target for the behavioral effects of lithium.

However, another heterozygous mice generated by homologous recombination in Takashima's lab have no defect regarding learning and memory in Morris water maze test although they show impaired memory reconsolidation in fear conditioning test (Kimura et al., 2008).

In summary, GSK-3β knockout mice present an embryonic lethality promoted by massive apoptosis in hepatocytes. Liver degeneration correlates with excessive TNF-α toxicity. As TNF-α is a member of death receptor family, we could conclude that apoptosis in hepatocytes is taking place by extrinsic pathway of apoptosis. Studies in fibroblasts from −/− embryos revealed decreased NFκB activation which seems to be GSK-3β dependent. No further analysis of apoptosis in CNS has been made in the heterozygous mice generated by Hoeflich et al. (2000) nor in the heterozygous made by Kimura et al. (2008).

Mice without GSK-3α are viable and show an increase in glucose and insulin sensitivity accompanied by liver glycogen accumulation and a reduction in fat mass (MacAulay et al., 2007). Respect to CNS these animals show an increase in cerebellar volume, although normal brain volume, suggesting a role for GSK-3α in this area. In addition, GSK-3α KO mice present alterations in a wide range of behavior tests involving this isoenzyme in normal brain function. Some defects found in exploratory activity, decreased immobility time and anti-aggression behavior remind to those found in GSK-3β ± mice. Abnormal behaviors as decreased locomotion, decreased social motivation, impaired sensorimotor gating, associative memory, and coordination are specific for GSK-3α −/− mice (Kaidanovich-Beilin et al., 2009).

In order to delete GSK-3 specifically from neurons, deletion of GSK-3α/β was carried out. Thus, mice with a GSK-3α null background and also carrying the construction GSK-3β (loxP/loxP) were crossed with nestin-cre line (Kim et al., 2009). These animals showed bigger heads and died at P0. The main finding was a massive hyperproliferation of neural progenitors by expansion of the radial progenitor pool. These effects were linked with β-catenin deregulation, Sonic Hedgehog, Notch, and fibroblast growth factor signaling. This model mainly demonstrates the role of GSK-3β in brain development (reviewed in Hur and Zhou, 2010). Besides, it has been recently demonstrated that GSK-3β is involved not only in embryonic neural development but also in adult neurogenesis. Thus, GSK-3 is able to phosphorylate tau protein in doublecortin positive cells in adult dentate gyrus (Fuster-Matanzo et al., 2009; Hong et al., 2010). In addition, GSK-3α/β (S21A/S21A/S9A/S9A) knock-in mice, described previously (Eom and Jope, 2009), present a drastic impairment in adult neurogenesis *in vivo*. In good agreement, adult neurogenesis is also altered in Tet/GSK-3β mice (Sirerol-Piquer et al., 2011).

From these studies we can extract that decreasing GSK-3 activity would translate in diminished apoptosis as indicated by the phenotype of bigger heads in both mouse models which is similar to the exencephaly observed in knockout mice for caspase-9 and caspase-3 (Haydar et al., 1999).

The study of a long term genetic suppression of the GSK-3 activity was difficult to achieve because of the embryonic lethality reported by Hoeflich et al. (2000). An alternative approach to study neurological consequences of sustained GSK-3 inhibition has been to generate mice with conditional expression of a canonical dominant-negative form of GSK-3(Gomez-Sintes et al., 2007). More precisely the K85R mutant form of GSK-3β (DN-GSK-3) was chosen in view of its previously shown efficacy in decreasing GSK-3 activity (Dominguez et al., 1995). The mouse design is similar to the one used for generating Tet/GSK-3β mice (Lucas et al., 2001). It consists in a transgenic mouse with conditional tetracycline-controlled system (Tet-OFF system), in which the expression of the tTA transgene is driven by CaMKIIα promoter. This promoter addresses the expression of tTA, and consequently of the Myc-K85R-GSK-3β construction, specifically to adult neurons in the forebrain. The transgene expression was evident in striatum, cortex, and hippocampus of Tet/DN-GSK-3 mice. GSK-3 activity measured in cortex and striatum homogenates revealed decreased GSK-3 activity. Subsequently, in these regions both GSK-3α and β showed increased phosphorylation in Ser 9 and 21 respectively and decreased phosphorylation in tau epitopes, PHF-1 and AT8. As a neurological consequence of decreased activity in

postnatal neurons of the forebrain they showed impaired motor coordination. The motor deficit correlates with an increase of neuronal apoptosis, detected by activated caspase-3 immunostaining and TUNEL selectively in areas responsible of motor control, like cortex and striatum. Interestingly these consequences were restored after shutdown expression (doxycycline administration). In this model, an increased apoptosis as a consequence of decreased GSK-3 activity was found. Regarding the paradoxical dual role of GSK-3 in apoptosis exposed previously, these results suggest that apoptosis induced by GSK-3 inhibition is occurring via the extrinsic pathway.

Models with increased GSK-3 activation had revealed a common effect in increasing apoptosis/toxicity. Intriguingly, studies in mouse models with decreased GSK-3 have raised different results regarding the role of GSK-3 in apoptosis. These controversial data are probably dependent on the isoform affected α or β, the cellular type or the developmental state in which GSK-3 activity has been decreased. If the isoform lacking during development is GSK-3α, the consequence is an increase in cerebellar volume which could be indicating a defect in apoptosis. When GSK-3β is decreased at early embryonic stage as in the two knockout mice of GSK-3β, it results in increased apoptosis/lethality. On the contrary, if GSK-3β is deleted specifically in neurons over a GSK-3α −/− background, then has an opposed effect of bigger heads which again indicates decreased apoptosis. Finally, mice with decreased GSK-3β activity occurring once the CNS is formed as Tet/DN-GSK-3β revealed an increased in apoptosis in striatum and cortex where expression of the transgene is more remarkable. This apoptosis seems to act through extrinsic signaling pathway. However, despite these experimental data, the contribution and the physiological role of both GSK-3 isoenzymes in relation to apoptosis are still not clear. Thus, it will be necessary to understand their relative contribution to apoptotic signaling cascades and, as a consequence, their relative contribution to neurodegeneration.

CORRELATE WITH PHARMACOLOGICAL INHIBITION

Deregulation of GSK-3 has been shown to be involved in all mechanisms described for AD neuropathology (Grimes and Jope, 2001). Interestingly, there is indirect evidence of increased activity of GSK-3 in AD patients (reviewed in Imahori and Uchida, 1997; Pei et al., 1997; Blalock et al., 2004; Hye et al., 2005; Leroy et al., 2007; Hooper et al., 2008). Some studies have also implicated GSK-3 in the pathology of type 2 diabetes (Eldar-Finkelman and Krebs, 1997; Eldar-Finkelman, 2002; Jope and Johnson, 2004). Thus, regarding the previous data, GSK-3 inhibitors were presented as a promising therapeutic tool for the treatment of AD, type 2 diabetes and possibly other neurodegenerative diseases (Cohen and Goedert, 2004; Meijer et al., 2004; Martinez and Perez, 2008).

Moreover, taking into account that cancer is characterized by a defect in apoptotic mechanism, inhibition of GSK-3 has been postulated as useful pharmacological approach to improve response to chemotherapy (Beyaert et al., 1989; Beurel et al., 2004; Luo, 2009). This therapeutic strategy would be only effective when directed to certain types of cancer, since directed to other types may have the opposite effect.

A reliable approach to test the validity of GSK-3 inhibitors for the treatment of AD would be the restoration of normal levels of GSK-3 in the Tet/GSK-3β mouse model (Lucas et al., 2001). These mice permit to explore whether the biochemical, histopathological, and behavioral consequences of increased GSK-3 activity are susceptible to revert after the shutdown of transgene expression. In this study Engel et al. (2006b) found that 6 weeks of doxycycline administration were sufficient to lower GSK-3 activity and tau phosphorylation to normal levels, to diminish neuronal cell death, and to improve cognitive deficit, thus further supporting the potential of GSK-3 inhibitors for AD therapy. However, GSK-3 inhibition leading to levels of GSK-3 activity below normal could be counterproductive. This was evidenced by the characterization of Tet/DN-GSK-3 mice carried out in our lab that showed increased levels of apoptosis and motor deficits as a consequence of sustained inhibition of GSK-3 (Gomez-Sintes et al., 2007). Fortunately this toxicity can be reverted when restoring normal activity of GSK-3, which suggests that GSK-3 should be maintained within certain physiological levels. The study performed by Frautschy et al. support this notion. They perform intracerebroventricular infusions of Aβ42 oligomer, which produces an increase in GSK-3 activity, and/or SB 216763, a selective inhibitor of GSK-3. Their results show a beneficial effect of SB 216763 to revert toxicity induced by Aβ42 oligomer. Intriguingly, they have also found a certain toxicity of SB when administered to control animals with normal levels of GSK-3 activity (Hu et al., 2009).

In view of the potential use of GSK-3 inhibitors for the treatment of AD and maybe other diseases in which deregulation of GSK-3 has been involved, many efforts have been directed to the development of selective and effective GSK-3 inhibitors (reviewed in Cohen and Goedert, 2004; Frame and Zheleva, 2006; Gould et al., 2006; Martinez and Perez, 2008). Indeed, two GSK-3 selective inhibitors from the pharmaceutical company Noscira are at the present under clinical trials phase II (Medina and Castro, 2008; Sereno et al., 2009; Del Ser, 2010). Nowadays this company is in the most advanced stage of the development of GSK-3 inhibitors. We must still wait for future studies in analysis of postmortem tissue to evaluate the effect of these inhibitors regarding apoptosis.

A classical non-selective GSK-3 inhibitor, lithium, has been used in clinics for decades (Klein and Melton, 1996; Stambolic et al., 1996). Since its introduction into psychiatric pharmacotherapy 60 years ago, lithium remains the most effective agent in the treatment and prophylaxis of major mood disorders, particularly bipolar disorder (BD; Manji et al., 1999; Baldessarini et al., 2002; Grof and Muller-Oerlinghausen, 2009; Kovacsics and Gould, 2010). Despite the obvious advantages of chronic lithium therapy, its clinical use is often curtailed by its narrow therapeutic index and its devastating overdose-induced toxicity (Macritchie and Young, 2004). Accordingly, patients must be closely monitored not only at the beginning of treatment, but also during treatment maintenance, to keep serum lithium concentrations within a therapeutic window of 0.6–1.4 mM. Even within this therapeutic range, mild neurological side effects such as hand tremor are common, and progressive toxicity to marked neurological impairment correlates with increasing serum levels above 1.5 mM (Macritchie and Young, 2004). The biochemical and cellular basis for lithium's therapeutic efficacy and the precise molecular mechanisms through which

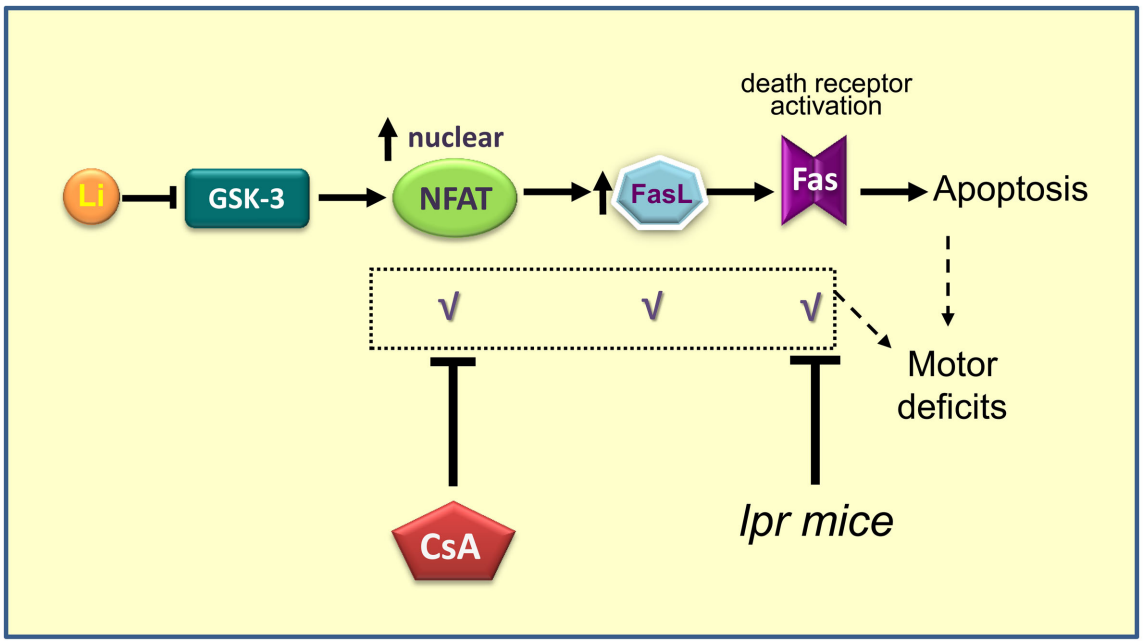

FIGURE 2 | Scheme summarizing the proposed mechanism for lithium induced neuronal apoptosis (Gomez-Sintes and Lucas, 2010). Prolonged administration of lithium produces inhibition of GSK-3, which promotes entry and accumulation of NFAT into the nucleus. Once in the nucleus, NFAT activates the production of FasL. When FasL is secreted outside the cell binds to Fas receptor present in the membrane of the same cell or cells nearby, which triggers death by apoptosis. When NFAT/Fas signaling is blocked by co-administration of Cyclosporin A or when lithium is administered to Fas-deficient mice (*lpr*) motor deficits and apoptosis are absent.

it exerts its unwanted neurological side effects remain to be fully elucidated. In 2008 a study in AD patients was accomplished to test the feasibility of chronic lithium treatment for this disease. It showed that side effects, although mild and reversible, difficult the application to elder people and the discontinuation rates are high (Macdonald et al., 2008).

As lithium inhibits GSK-3 *in vivo* (Klein and Melton, 1996; Stambolic et al., 1996) and we recently reported neuronal apoptosis and motor deficits in dominant-negative GSK-3 transgenic mice (Gomez-Sintes et al., 2007), we hypothesized that therapeutic levels of lithium could also induce neuronal loss through GSK-3 inhibition and that maybe this is the cause of the known extrapyramidal side effects, as hand tremor, produced by chronic treatment with lithium in BD patients. To test this hypothesis chronic lithium was administered to wild-type mice and it was found that apoptosis and motor coordination impairment were occurring, similarly as in Tet/DN-GSK-3β mice (Gomez-Sintes and Lucas, 2010). In this case GSK-3 inhibition was not restricted to forebrain areas as it was not dependent of CaMKII promoter. Thus, increased apoptosis was noticeable in areas like cortex, striatum, globus pallidus, hippocampus, and cerebellum, all of them involved in motor control. Interestingly chronic lithium resulted in a poor performance of motor tasks as evidenced by vertical pole test or DigiGait apparatus, which measures footprint pattern and other parameters of walking regularity. To depth into the mechanism of lithium toxicity, we elaborated a hypothesis involving NFAT/Fas signaling that we proceed to test (**Figure 2**). The results demonstrated that GSK-3 inhibition by lithium increase translocation of nuclear factor of activated T cells c3/4 (NFATc3/4) transcription factors to the nucleus, leading to increased Fas ligand (FasL) levels, and Fas receptor activation, which finally gives rise to extrinsic apoptosis signaling (death receptor dependent; Gomez-Sintes and Lucas, 2010).

Interestingly, apoptosis and motor deficits were prevented when cyclosporin A, that blocks NFAT nuclear translocation, was co-administered with lithium. These side effects could be also reverted by blocking Fas signaling. This was tested by treating *lpr* mice (naturally Fas receptor-deficient) with chronic lithium. These findings leave an open way to combined therapies. To test the validity of these results but providing a genetic inhibition of GSK-3, experiments with Tet/DN-GSK-3 mice in *lpr* background are being undertaken.

All the mouse models with increased or decreased GSK-3 activity described in this review have helped to understand the implication of GSK-3 on neurodegenerative or psychiatric diseases and also to depth in the physiology of GSK-3. Although there is still a lot of work to do in this direction, these models constitute a good tool to achieve these studies.

Understanding the role of GSK-3 in apoptosis would improve the application of GSK-3 inhibitors to diseases as ADs, as apoptosis seems to be responsible of motor side effectsGSK-3 inhibition. The combination of GSK-3 inhibitors and NFAT/Fas signaling blockers could be a good clinical strategy to minimize side effects and extend the use of GSK-3 inhibitors to other neurodegenerative and neuropsychiatric diseases.

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GSK3 and Alzheimer's disease: facts and fiction...

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The physiological functions and pathological roles of the Glycogen synthase kinase-type 3 (GSK3) kinases in peripheral and central systems are diverse and complex, and therefore hard to unravel in molecular detail *in vivo*. Our assignment to review and discuss available data to clarify the actual position of these kinases in the pathology of Alzheimer's dementia (AD) was both ambitious and easy. On the one hand, numerous studies are available in isolated, recombinant, or cell-based systems, which have resulted in very diverse datasets that are hardly informative for the brain *in vivo*. At the other extreme, reliable, and relevant models for the role of GSK3 in CNS are rare, if not lacking. Moreover, (too) many *in vivo* studies used Li^+ as "specific" inhibitor of GSK3, which is factually not valid because lithium ions are neither specific nor potent inhibitors of GSK3 *in vivo*. More specific pharmacological inhibitors of GSK3 have met with considerable problems, and are reviewed by others in this issue or elsewhere. We concentrate here on AD-related aspects of GSK3 in brain *in vivo*, mainly studied in transgenic mice and highlight some of the more important issues, among many remaining: activation of GSK3 by amyloid, phosphorylation of protein tau, effects on or interference with synaptic activity, differentiation between both GSK3 isoforms. These relate directly to brain function, and brain dysfunction in AD, and are to be resolved if we want to understand the molecular pathology of this dreadful disease.

Keywords: GSK3, Alzheimer, tau

ALZHEIMER'S DISEASE

The term dementia stems from latin words meaning "without mind" or "de-humanized," without any doubt the most horrifying condition a human being can experience. Dementia comprises the age-related deterioration of cognitive abilities and memory, associated with severe changes in personality, in character, in interests (or rather lack thereof), in social, and in general behavior. Progressive dementia closely connects to aging and is therefore rapidly becoming a prime problem for the aging populations of any nation where life expectancy exceeds 65 years. In parallel with improved health and medical care, the ever increasing life expectancy promotes age-related dementia as a major medical and socio-economic problem, next to vascular disease and cancer. It is estimated that already over 10 million people suffer dementia in Europe, and about 40 million world-wide (data 2010). These numbers will likely double every next 20 years (Alzheimer's disease International; World Alzheimer Report, 2009). Because dementing patients are to be cared for around the clock, the number of care-takers needed in the future will surpass all other medical needs.

While dementia is the general medical term for various diseases that distort brain functioning, the most common types are Alzheimer's dementia (AD), vascular dementia, various types of frontotemporal dementia (FTD) and dementia with Lewy bodies (DLB). AD is most conspicuously named after – not by – Alois Alzheimer, MD, who described the first case in 1907. It is by far the most frequent dementia, accounting for 50–70% of all cases, with rapidly increasing incidence and prevalence. The prevalence is only a few percent around ages 65–69 but increases to 25–35% in octogenarians (Ferri et al., 2005). Despite the small fraction of cases with early onset (before age 65), these are without doubt the most poignant, with most severe personal, familial, and social impact. Moreover, more women than men are becoming demented, and apparently not only because women live longer. According to some studies the increase is "exponential with age," meaning that all humans will eventually become demented when old enough – a daunting prospect that is fortunately not yet supported by all facts.

Alzheimer's dementia is a chronic, progressive disorder beginning with mild symptoms that are hard to recognize, mostly only in retrospect, and as yet impossible to diagnose objectively. The end-stage comprises very severe cognitive defects and deformed personality, inevitably evolving into complete dependence on external care. The associated physical weakening leads eventually to death from various causes, e.g., lung infections, stroke, coronary disease, and cancer. The entire disease process covers extended periods of 10–20 years or more, suspected to depend on physical, genetic, and epigenetic factors, all to be identified.

Even to date, more than a century after its first description, and despite massive fundamental and clinical research, AD remains hard to diagnose. The only conclusive diagnostic tool is identical to that used by professor Alzheimer: post-mortem silver-impregnation of brain sections, to reveal the two main pathological lesions, the extracellular amyloid plaques and the many neurons filled with tangles. Importantly, and often overlooked, Alzheimer also described the active glial processes, indicative of ongoing brain inflammation, which we believe is an essential element in the neurodegeneration process (Jaworski et al., 2009, 2010a,b, 2011).

In the absence of objective early diagnosis, AD is an exclusion diagnosis by clinical examination, with initial (un-)certainty

around 50%, when the patients' condition is labeled "probable AD." Including biomarkers, i.e., biochemical in CSF or various types of brain imaging, diagnosis is becoming gradually more accurate, but remains statistical, yielding no more than about 80% certainty during life. Post-mortem analysis of the brain according to Alzheimer still remains the only certain diagnosis. Although this might not be the most important problem faced by clinicians in daily practice, the large margin of diagnostic uncertainty in early phases of AD, i.e., in mild cognitive impairment (MCI), poses a tremendous problem for clinical-phase therapeutic studies: stratification of the AD population at risk is needed, by genetic and diagnostic means.

The currently available pharmacological therapy for AD aims largely on increasing brain acetyl-choline levels, which is totally symptomatic. Given the rapidly rising incidence and prevalence, development of disease-modifying therapy is overdue and must go hand-in-hand with improved diagnosis, for reasons outlined above. Fundamental and applied research are to be fostered to understanding the mechanisms that cause and underlie, especially the sporadic forms of AD, to surpass the hurdle of most current studies and models that are largely restricted to familial, purely genetic types of AD.

GENETICS: AMYLOID FIRST

The isolation and sequencing of amyloid peptides from brain of AD and Down's patients (Glenner and Wong, 1984) was the most important biochemical finding in this field and marks the beginning of the molecular era of AD research. It allowed molecular cloning and sequencing of the cDNA coding for their precursor amyloid precursor protein (APP). In close parallel, but less conspicuous, phosphorylated protein Tau was detected as the major component of the intraneuronal tangled fibrils observed and described by Alzheimer (Brion, 1985; Grundke-Iqbal et al., 1986). Both findings boosted research into these enigmatic proteins, although less intense and less prominent for protein Tau than for amyloid.

The majority of AD cases are sporadic, with onset typically in persons over 65 years, and with "age" as the only known determining factor. An experiment of nature turned out "fortunately for science": a small fraction, 1% or less of all AD cases are familial, purely genetic and inherited dominantly, causing early onset familial AD (EOFAD). The responsible mutations have been pin-pointed to the genes coding for the APP (12 families world-wide) and presenilins (PS1, 70+ mutations; PS2, 7 families; Goate et al., 1991; Sherrington et al., 1995; St George-Hyslop, 2000). These familial forms of AD are characterized by an age of onset between 35 and 55 years, and are most aggressive, progressing more rapid than sporadic AD over 5–10 years.

For sporadic AD, aging is a genetically or molecularly largely undefined condition. Only one, indirect genetic factor has been identified and stands out: the ε4 allele of the APOE gene, with an allele frequency of 15% in the general population, but associated to 40–50% of sporadic AD cases. The connection is again statistical and again not diagnostically useful, but the effect is marked: carriers' risk is 5- to 10-fold higher, with an earlier age of onset (Corder et al., 1993).

More recently, three new genes, i.e., CLU, CR1, and PICALM, were found associated with sporadic AD in more global studies (Harold et al., 2009; Lambert et al., 2009). These genes do not, however, reach the statistical significance of the ApoE4 allele, and are yet to be studied as interesting research tools, hopefully helping to understand the overall disease process and their contribution to it. Nevertheless, even 17 years after the discovery of the AD–ApoE4 connection, we still do not understand to the fullest how this lipoprotein operates in brain, and how ApoE4 differs from the main ApoE3 isotype in brain functions. Whether scavenging and transport of ApoE-lipoproteins include removal of amyloid peptides from the interstitial spaces between neurons remains to be analyzed in detail (Castellano et al., 2011).

Genetic analysis of EOFAD cases provided knowledge of the genes that became the starting points for all biochemical, cell-biological and transgenic modeling of the mechanisms underlying familial AD – and by extrapolation sporadic AD, we all hope. These genes, i.e., APP and PS1/2, constitute the basis for the amyloid hypothesis posting that amyloid peptides are the prime cause of AD.

GENETICS: A STRONG CASE FOR TAU IN NEURODEGENERATION

Importantly, the known EOFAD cases are caused by mutations that affect amyloidogenic processing of APP, resulting either in more amyloid peptides, or in longer, less soluble amyloid peptides. Nevertheless, these EOFAD cases are very similar to sporadic AD, and always accompanied by severe tauopathy that is co-diagnostic for all AD cases – even those struck at young age!

Although protein Tau is thereby inherently involved, diagnostically as well as mechanistically, the recognized mutations in the MAPT gene on chromosome 17q21 coding for protein Tau, cause not AD but another type of dementia: FTD with parkinsonism, linked to chromosome 17 (FTDP-17). This sub-type of the FTD spectrum of dementias is clinically and diagnostically close to AD (Delacourte and Buée, 2000; Ingram and Spillantini, 2002). Tauopathies in general are a class of widely varying neurodegenerative diseases, all characterized by pathological aggregation of protein Tau in specified brain regions and types of neurons, the most prominent of which are FTD, corticobasal degeneration and Pick's disease (review Sergeant et al., 2005; Ludolph et al., 2009).

Importantly, the mutations in the MAPT gene that cause the FTDP-17 sub-type are situated not only in exons, but also in introns. The exonic mutations give rise to the expression of mutant protein Tau in the central neurons of patients in these FTD families. Physiologically, Tau is subject to alternative splicing resulting in the generation of six different isoforms, containing three or four microtubule (MT) binding domains (Tau3R or 4R respectively) and 0, 1, or 2 N-terminal repeats. Surprisingly, many mutations in introns surrounding exon 10 (encoding MT-binding domain 2) were located in, or close to the DNA signals that control mRNA splicing. Consequently, more or less – pending the mutation – species of wild-type protein Tau are produced containing MT-binding domain 2, encoded by exon 10, resulting in a distorted Tau3R/4R ratio. We can deduce from data in developing brain with mostly or even only Tau3R species expressed, that the Tau3R/4R ratio defines or allows more axonal morphological modulation

and plasticity. The precise consequences of the Tau3R/4R ratio in adult and aging brain are to be defined, in FTD and eventually in AD. Despite the lack of a direct genetic link of the MAPT gene to AD, the link might be indirect by interaction with the GSK3β gene (Kwok et al., 2008).

In all primary tauopathies, the cognitive demise cannot but be due to the evident progressive Tau pathology – although molecularly it is not clear by what actions or mechanisms. In AD, the co-diagnostic tauopathy is generally described as "secondary" to amyloid pathology. The hypothesis that tauopathy is pathologically responsible in AD is supported by clinical and pathological observations, demonstrating that the spatial and temporal pattern, not of the amyloid but of the Tau pathology correlates most closely with the cognitive decline in AD (Braak and Braak, 1991; Reitz et al., 2009). MCI is the most likely prodrome of AD, but the underlying molecular changes and factors that mediate – or prevent – the conversion MCI to AD are not understood. Unexpectedly, recent pathological data demonstrate beginning tauopathy in CNS already at young age, i.e., decades before other defects, like amyloid deposition become apparent (Braak and Del Tredici, 2011a,b; Duyckaerts, 2011).

In this respect one wonders again why these dementias, even or in particular the familial types, take decades to develop? This can be related to, or even because of, the inherent resilience of neurons and their post-mitotic nature. Alternatively, or in addition, more than one or even more than two hits might be needed to functionally deteriorate, and eventually kill a neuron.

TAU, A NATURALLY UNFOLDED, PHOSPHORYLATED MAP

Protein Tau was first identified in early microtubule (MT) preparations and subsequently described to promote MT assembly, with its designated name (τ) referring to "tubule forming" (Weingarten et al., 1975). The only known function of Tau is as microtubule associated protein (MAP), with binding to MT regulated by a complex interplay of isoform expression and phosphorylation. Alternative mRNA-splicing yields Tau3R or Tau4R isoforms containing three or four MT-binding domains, as stated above (Mandelkow et al., 1996). Phosphorylation of serine and threonine residues is performed by kinases, with most prominent proline-directed kinases [e.g., Glycogen synthase kinase-type 3 (GSK3), cdk5, MAPK], and non-proline-dependent kinases (e.g., MARK, PKA, PKC; reviews Lau et al., 2002; Johnson and Stoothoff, 2004; Hanger et al., 2009).

While protein Tau contains a large number of serine/threonine phosphorylation sites, only about 15 have been demonstrated to be physiologically or pathologically important *in vivo*. Nevertheless, therein hides a major analytical problem: the potential 2^{15} differently phosphorylated sub-forms of protein Tau are impossible to analyze individually. The balance between kinase and phosphatase activities determines the actual level of phosphorylation of protein Tau, like for all proteins in any biological system. In contrast to the relatively small set of kinases, the known phosphatases have been less well-studied with respect to the function of protein Tau in health and disease, although PP2B/calcineurin and PP2A are potentially important (Louis et al., 2011; and references therein).

The main action of phosphorylation of protein Tau as a MAP is in its interaction with MT: decreasing its affinity for, preventing its binding to and provoking its detachment from MT. In AD – and in all primary tauopathies – protein Tau becomes "hyper-phosphorylated": rising from an average 2–5 phosphate groups per molecule, to up to 10 phosphate groups per Tau molecule in diseased and tangled neurons. The resulting pathological defects are mechanistically not well understood, but are presumed to comprise deregulation of MT-mediated transport, not only in axons but also in somata and dendrites, by the aggregated Tau-fibrils.

AMYLOID AND TAU IN AD: A TROUBLED RELATION. . .

Overall, the genetic findings briefly summarized in previous sections, boosted interest in protein Tau, because they made clear that defective Tau is sufficient to cause neurodegeneration and dementia in FTD, not unlike the disease process in AD. On the other hand, the findings also re-fueled the "amyloid-first cascade" hypothesis, albeit a re-phrased version: accumulation and aggregation of amyloid peptides causes "also" tauopathy – by unknown mechanisms – and thereby the clinical symptoms and pathological defects, from mild early cognitive defects to severe dementia – again by unknown mechanisms (Hardy and Allsop, 1991; Hardy and Selkoe, 2002; Duyckaerts et al., 2009; Jaworski et al., 2010).

In the last decade it gradually became clear that not the "cemented" protein-aggregates, but rather smaller, even soluble aggregates of amyloid and Tau are synapto- and neuro-toxic (Moechars et al., 1999; Lesné et al., 2006; Lasagna-Reeves et al., 2011; review Jaworski et al., 2010). Indeed, the earliest cognitive dysfunction in AD is likely caused by functional and/or structural weakening of synapses in affected limbic regions (Gonatas et al., 1967; Hamos et al., 1989; Dekosky and Scheff, 1990). These precede deposition and formation of solid amyloid plaques and tangles, and neuronal loss, thereby marking MCI, or a sub-type of MCI. We are challenged to understand this sub-type because it is the prodromal stage of AD, and we need to define its cause: amyloid or Tau or – most likely – both.

We now believe that synaptic dysfunction is caused by the accumulation of aggregated amyloid peptides and/or soluble phosphorylated Tau, but their exact relation remains enigmatic and burdened with questions. How do amyloid peptides in the secretory pathway or secreted affect the cytoplasmic protein Tau? Through a receptor for amyloid-aggregates and its downstream kinases? Is this part of a normal physiological mechanism, or merely a pathological problem?

The physiological relation between amyloid and Tau – if any – is not clear in normal brain, while their pathological relation is evident in AD brain, although complicated and still largely to be clarified. The amyloid cascade hypothesis initially posed aggregation of amyloid into plaques to cause synaptic dysfunction and neurodegeneration. Later the Tau pathology became more prominently included, still subordinate to amyloid pathology as outlined above, and defining AD as the combination of amyloid plaques and neurofibrillary tangles, conform the original description by A. Alzheimer.

Interestingly, recent findings in experimental models shed some light on the complex relation between amyloid and Tau, in as far as deficiency of protein Tau prevents amyloid-induced cognitive defects (Roberson et al., 2007, 2011; Ittner et al., 2010; Shipton et al., 2011). Moreover, protein Tau appears to be needed for

amyloid to disrupt axonal transport in primary neurons (Vossel et al., 2010), although *in vivo* confirmation is needed. Additionally, specific phosphorylation of protein Tau could be involved in its mis- or re-localization from axons to the somatodendritic compartments, and perhaps even to dendritic spines (Hoover et al., 2010; Ittner et al., 2010; Kremer et al., submitted). These considerations and hypotheses, although still littered with large open spaces of unknowns, implicate kinases most deeply in the fundamental pathological mechanism that cause AD and related tauopathies.

For one, we showed that early in the amyloid pathology both GSK3 isozymes become activated, as demonstrated by increased tyrosine phosphorylation, in mouse brain *in vivo* (Terwel et al., 2008). Our finding then raises the important issue of the molecular mechanism by which amyloid peptides – and which species – augment the proposedly autocatalytic tyrosine phosphorylation of GSK3 (Cole et al., 2004; discussed below). These and related *in vivo* findings firmly establish GSK3 as essentially contributing to the pathogenesis of AD, linking amyloid to tau phosphorylation and confirming its original designation as Tau kinase I (Ishiguro et al., 1993; Spittaels et al., 2000; Muyllaert et al., 2006, 2008; Terwel et al., 2008; reviews Takashima, 2006; Jaworski et al., 2010). Most interestingly, GSK3 is also becoming intimately implicated in normal physiological mechanisms underlying synaptic plasticity, learning, and memory (Hooper et al., 2007; Peineau et al., 2007; Dewachter et al., 2009; Hur and Zhou, 2010; Smillie and Cousin, 2011). Consequently, we must besides amyloid and Tau consider direct contributions of (activated) GSK3 to synaptic defects in AD (**Figure 1**; Terwel et al., 2008; Jaworski et al., 2010a,b; and references therein).

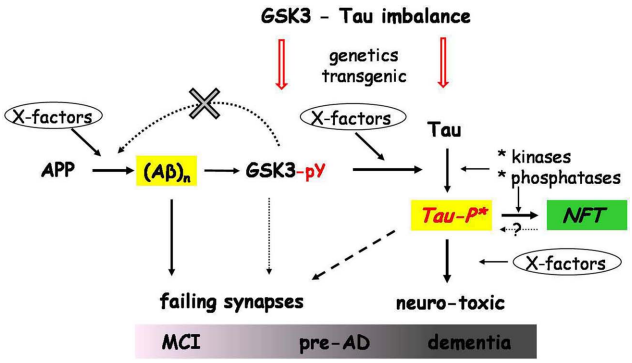

FIGURE 1 | Schematic relations between amyloid, GSK3, protein Tau, and other factors. The scheme depicts the activation by amyloid peptides of GSK3α/β by increasing tyrosine phosphorylation, and leading to increased phosphorylation of protein Tau as the central event in AD pathogenesis. Condensed from *in vivo* observations in transgenic models, both proven (solid arrows) and proposed effects (broken arrows) are represented. The unknown molecular factors (X-factors) and mechanisms behind the relations and connections in this scheme are not yet fully understood as discussed in the text. Our most recent results did not confirm the proposed feedback effect of GSK3 on APP processing (data not shown). The amyloid and pTau species that cause synaptic defects, and eventually neurodegeneration, are not aggregates, but soluble oligomers (marked in yellow boxes). The phosphorylation of Tau by GSK3 and other kinases, produces a neurotoxic species, represented here as Tau-P*. This hypothetical intermediate is a soluble single, dimer, or small aggregate, in a transitional conformational state that can be directed either into aggregation (NFT; green box) or toward synaptic and neuronal toxicity. Tau-P* causes synaptic dysfunction, which in various combinations with amyloid peptides and aberrant activated GSK3 results in various synaptic defects, initiated in the earliest phases MCI or pre-AD, and evolving to dementia, as highlighted in the scheme. The genetic imbalance between GSK3 and Tau genes depicted in the scheme refers to the proposed interaction between the Tau (MAPT) and GSK3β genes in humans, discussed in the text. This interaction might impact on both GSK3 activation or availability and the Tau3R/4R ratio, thereby also contributing to the propensity of Tau phosphorylation. The imbalance is also generated in the various single and bigenic models, discussed in the text. The combination of all actors and factors and their interactions lead to a variety of clinical and pathological symptoms, observed in sporadic AD patients.

GLYCOGEN SYNTHASE KINASE-TYPE 3

Glycogen synthase kinase-type 3 was first described as the major regulator of glycogen metabolism, by phosphorylating and thereby inhibiting glycogen synthase (Embi et al., 1980; Woodgett, 1990). GSK3 denotes the proline-directed S/T kinases that exist as two isozymes, GSK3α and GSK3β encoded by different genes on chromosomes 19 and 3, respectively (Woodgett, 1990; Shaw et al., 1998). The GSK3 isozymes share overall 84% sequence identity, but 98% in the kinase domain indicating similar substrate specificities (Woodgett, 1990). Nevertheless, they are functionally not identical as demonstrated by *in vitro*, cellular, and *in vivo* data (Hoeflich et al., 2000; Kaidanovich-Beilin et al., 2010; Soutar et al., 2010). In addition to the overall similar structure, the α isozyme contains an extended glycine-rich N-terminal region that could define cellular localizations and interactions unique to this isozyme (Azoulay-Alfaguter et al., 2011).

Importantly, total absence of GSK3β is embryonically lethal in mice, implicating that GSK3α cannot compensate for the lack of its counterpart (Hoeflich et al., 2000). In contrast, GSK3α can be completely eliminated without obvious major adverse effects on viability or health, with the possible exception of male sterility (Kaidanovich-Beilin et al., 2010). The very different outcomes in mice lacking either GSK3 isozyme has been attributed to differences in their mediation or regulation of transcriptional activity by CREB, NF-κB, EGR-1, Smad3/4, or others (Liang and Chuang, 2007; Mines et al., 2011). For one, GSK3β deficient mouse pups most likely die because of the inability of NF-κB to regulate liver development in the absence of GSK3β (Hoeflich et al., 2000) and/or from a patterning malformation in the heart (Kerkela et al., 2008). In general, the GSK3 isozymes affect cell survival and death, which is of course essential, or detrimental to genesis and normal functioning of any organ (review Mines et al., 2011).

The molecular basis for the distinct functions of both GSK3 isozymes in normal physiology remains largely unknown, which is evidently not helping to define their similar or different roles in pathology. Differential expression of GSK3α and GSK3β mRNA and proteins indicate, not unexpectedly that they are subject to different regulation in different tissues to fulfill diverse biological roles. Recent advances in mice lacking one or the other GSK3 isoform, completely for GSK3α or in a cell-specific manner for either isoform are becoming known and confirm these general impressions. For instance, mice lacking GSK3α display improved glucose tolerance and increased glycogen synthesis as opposed to

tissue-specific GSK3β knock-out mice (MacAulay et al., 2007; Patel et al., 2008).

Recently, progress is made in understanding the physiology of GSK3 isozymes in CNS. Both GSK3 isozymes differentially regulate neuronal survival, but both appear needed for axon formation, despite acting on different substrates in brain (Kim et al., 2006, 2009; Garrido et al., 2007; Liang and Chuang, 2007; Soutar et al., 2010). GSK3β is located throughout neuronal compartments, as well as in astrocytes, and associated with rough endoplasmatic reticulum, free ribosomes, mitochondria, and interestingly also with the post-synaptic density in dendritic spines (Perez-Costas et al., 2010). This fact links snugly to data that GSK3β mediates interaction between two major forms of synaptic plasticity: NMDA-dependent long-term potentiation (LTP) and long-term depression (LTD). Responsible signaling mechanisms involve the classic PI3K/AKT/GSK3 connection that regulates phosphorylation of S9 on GSK3β, and thereby its kinase activity. This link appears as crucial in the formation of LTD or LTP, and thereby for synaptic plasticity and cognition (Hooper et al., 2007; Peineau et al., 2007, 2008; Dewachter et al., 2009). GSK3 further contributes to NMDA receptor trafficking and functioning in cortical neurons, again important in cognition (Chen et al., 2007). Besides these post-synaptic actions, GSK3 also contributes to pre-synaptic functions in developing and mature nerve terminals (review Smillie and Cousin, 2011).

Several excellent reviews are concerned with various structural and physiological functions of the GSK3 isozymes in peripheral organs and in brain. We therefore concentrate on data – and even more on hypotheses – that eventually link either or both GSK3 isozymes to the pathology in AD. In any case it must be remembered that the GSK3α isozyme is by far the least well-studied isoform, largely because of the historically stronger interest in GSK3β in AD. We impose here an additional restriction by concentrating on data obtained *in vivo*, mainly in genetically modified mice. We believe that *in vivo* models are the only relevant for physiology and pathology alike, however, not without acknowledging that mouse brain will never equal the complexity of human brain.

GSK3 IN AD PATHOLOGY

The overriding hypothesis in AD maintains that amyloid peptides indirectly affect the activity of phosphatases and even more so of kinases, in particular GSK3β but also others that all contribute – in concert – to the increased phosphorylation of protein Tau typical for all tauopathies. Moreover, it is highly likely that changes in kinase activity are an intrinsic aspect of the pathological problem, because they can and will negatively affect, or even disrupt, other synaptic signals essential for learning and memory. Despite considerable technical efforts, it is as yet impossible to separate and define the contributions of amyloid, of GSK3 and other kinases, and of phosphorylated protein Tau on synaptic functions (**Figure 1**).

The central role of tauopathy in AD and of the increased phosphorylation of protein Tau therein, was recognized early (Brion et al., 1986; Grundke-Iqbal et al., 1986). Hyper-phosphorylated protein Tau forms filamentous aggregates that become deposited mainly as neuropil threads in axons and dendrites, and as the better known neurofibrillary tangles in soma of neurons in cortex and hippocampus. The various intracellular deposits of aggregated Tau are proposed to cause problems with all sorts of transport systems, leading to impaired synaptic transmission, dendritic amputation, generalized neuronal dysfunction, and eventually neurodegeneration. Nevertheless, it is by no means certain that the large fibrillar aggregates are responsible, but rather the putative smaller aggregates we termed "Tau-P*" (**Figure 1**; Jaworski et al., 2009, 2010a,b, 2011).

In FTD the eventual hyper-phosphorylation is ascribed to the mutation in Tau or to the distorted Tau3R/4R ratio. Nevertheless, neither of these defects is *a priori* expected to evoke or solicit more phosphorylation than wild-type Tau or than a normal isoform ratio. Obviously, mutations in Tau or excess of one or the other isoform changes the molecular parameters and control mechanisms, in as far that over a time-period of decades tau becomes phosphorylated to a degree that cannot be reversed. From that point the sequence of events is rather straightforward, and well recapitulated in transgenic mice: increased phosphorylation causes conformational collapse, dimerization, and further aggregation into fibrils (Terwel et al., 2008; Patterson et al., 2011). In sporadic AD, protein tau is not mutated, and the isoform ratio is normal, leading to the conclusion that out-of-balance kinase activity must explain the hyper-phosphorylation of protein tau in AD brain (**Figure 1**).

GSK3β was identified in this respect as the major kinase, denoted Tau Kinase I, first *in vitro* and established firmly *in vivo* in transgenic mouse brain by us (Ishiguro et al., 1993; Spittaels et al., 2000; Takashima, 2006; Terwel et al., 2008). To date over 30 distinct phosphorylation sites are known that can potentially be phosphorylated by GSK3β on protein Tau (Hanger et al., 2009). Although *in vivo*, GSK3β appears to be the most dominant Tau kinase, it is by no means the only kinase, indirectly observed *in vivo* in our different transgenic mouse strains. Increasing GSK3 activity transgenically in itself did not cause major pathological problems related to AD or tauopathy (Spittaels et al., 2000, 2002). Remarkably, increased GSK3β activity alleviated the axonopathy of our Tau4R mice but did not cause tauopathy (Spittaels et al., 2000), in contrast to the dramatic tauopathy evoked by the same GSK3β transgene in combination with Tau.P301L mice, denominated biGT mice (Terwel et al., 2008).

The former demonstrated for the first time *in vivo* that GSK3 effectively phosphorylates protein Tau4R in brain, and thereby decreased or prevented binding of Tau4R to MT to restore normal transport. Nevertheless, phosphorylation of wild-type human Tau4R by GSK3 was not sufficient to promote aggregation into tangles (Spittaels et al., 2000). Conversely, the biGT mice demonstrated that also Tau.P301L was effectively phosphorylated by GSK3 in brain and to a degree sufficient to form abundant tangles in forebrain neurons (Terwel et al., 2008). Obviously, while these data proved that the P301L mutation contributed additionally and essentially to the aggregation of Tau in conjunction with GSK3β, the nature of the P301L contribution to the process of Tau aggregation is not yet revealed.

We cannot but conclude that GSK3β is a most efficient Tau kinase, needed but not sufficient to force tau to aggregate into fibrils.

DE-REGULATED GSK3 ACTIVITY IN TRANSGENIC MOUSE BRAIN

We documented the increased tyrosine phosphorylation of both GSK isozymes in brain of relatively young APP.V717I mice, i.e., at age 4–8 months before their brain becomes littered with amyloid deposits (Moechars et al., 1999; Terwel et al., 2008). At that age most amyloid peptides are present as small aggregates, and inside or in close proximity of the neurons they are produced by (**Figure 2**).

In brain, the regulation of the activity of GSK3α/β by phosphorylation of S21/S9 (inhibition) and/or Y279/216 (activation) is a matter of controversy – not only in AD but in normal physiology. In this respect a fundamental question refers to the structure-function relation of both GSK3 isozymes: they are known as S/T kinases that act on primed and non-primed substrates and modulated by tyrosine phosphorylation (Y279/Y216 in GSK3α/β, respectively; review Medina and Wandosell, 2011; references therein). Both kinases are inactivated by the well-known serine phosphorylation (S21/S9 in GSK3α/β, respectively) which evidently overrides activation by tyrosine phosphorylation. We cannot indulge here in extensive structural considerations on the tyrosine phosphorylation, i.e., whether it is autocatalytic or intra- or inter-molecular, which would promote GSK3 to the status of "dual activity kinases" (Cole et al., 2004; Medina and Wandosell, 2011; references therein). Nevertheless, the problem is most actual and of direct interest for AD, because of our observations in APP.V717I mice (Terwel et al., 2008) and observations in AD patients (Leroy et al., 2007; next section).

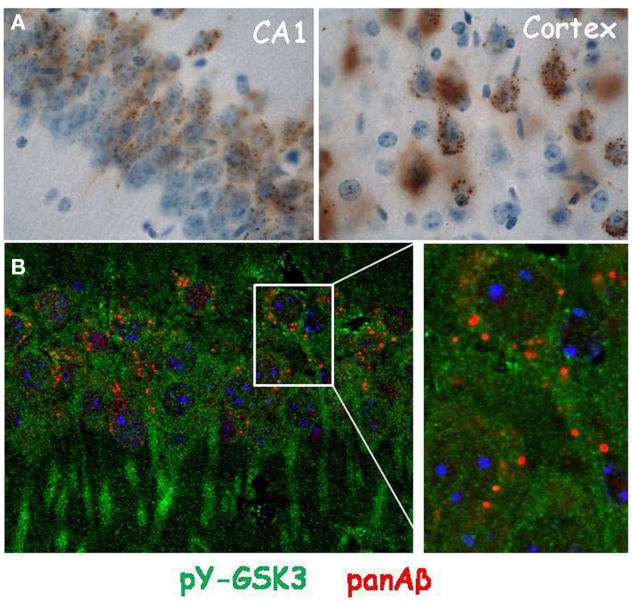

FIGURE 2 | Localization of amyloid peptides and pY-GSK3 in transgenic mouse brain. (A) Intracellular amyloid (panAβ) in granules in pyramidal neurons of CA1 (left) and cortical layers III/IV (right) in brain of APP.V717I mice (age 6 months); **(B)** localization of activated pY-GSK3 (green) and panAβ (red) in the cytoplasm of CA1 neurons in the brain of APP.V717I mice (age 6 months), at right higher magnification of boxed area.

The implications are profound: amyloid peptides, which are located in the secretory pathway or are secreted, apparently are able to act on GSK3 to increase its tyrosine (auto-)phosphorylation, and thereby increase the kinase activity toward protein Tau (**Figure 1**). This hypothesis explains the observations in our transgenic and viral models for AD while raising important mechanistic questions (reviews Jaworski et al., 2010a,b).

GSK3 IN HUMAN BRAIN, NOT ENOUGH DATA. . .

Solid data on the physiological and pathological aspects of GSK3 in healthy human brain and in neurological diseases and dementia are rare and far apart.

In the frontal cortex of AD patients a non-significant trend of increased total levels of GSK3β was noted, with no difference in pS9-GSK3β. That contrasted to the significant increase in pY216-GSK3β (Leroy et al., 2007) recapitulated in our APP.V717I mice, discussed in the previous section (Terwel et al., 2008). In AD-frontal cortex pY216-GSK3 co-localized in somatodendritic compartments with highly phosphorylated protein Tau, a finding that is not too surprising, because enzymes and substrates must eventually meet. Neurons containing pY216-GSK3β were evident and widespread in brain of AD patients of all Braak-stages, from pre-tangle to stage VI, while biochemically no differences in pS9-GSK3 were noted (Leroy et al., 2007).

This implicates pY216-GSK3β as a pathological marker for tangled neurons, prevailing from early to late stages, and therefore a likely candidate to cause, or at least contribute to the tauopathy. Nevertheless, the mechanism behind the activating tyrosine phosphorylation of pY216-GSK3β in neurons destined or forced to form tangles still eludes us, as discussed in the previous section.

In contrast, co-localization of pS9-GSK3β and Tau was evident in tangles, dystrophic neurites and neuropil threads in AD brain, while biochemical fractionation revealed considerable pS9-GSK3 in the sarkosyl insoluble Tau fraction (Ferrer et al., 2002). Granulovascular degeneration, a neuronal lesion often observed in AD, is immunoreactive for GSK3α/β and for pY216-GSK3β. The co-localization with phosphorylated Tau (pS262, AT100) in these granules could imply that activated pY216-GSK3 becomes preferentially localized in special cellular compartments, e.g., autophagosomes, contributing to a potentially protective mechanism for neurons (Taelman et al., 2010; references therein).

To be complete, GSK3β can also become inactive after phosphorylation of S389 by p38MAPK, at least in mouse brain (Thornton et al., 2008) but no data are available for human brain. We conclude that we lack sufficient data in healthy and diseased human brain, to answer or counteract the questions that are raised by the studies in transgenic models.

GSK3, APP PROCESSING, AND LI$^+$

A further problem in this sub-field is the eventual contribution of GSK3 to the pathogenesis of AD in terms of increased production of amyloid peptides. The question mainly originated from the finding that the cytoplasmic region of APP can become phosphorylated, with GSK3 one of the potential kinases (Aplin et al., 1996). Subsequently, and most amazingly, the less well-known GSK3α isozyme was proposed to regulate amyloidogenic processing of

APP (Phiel et al., 2003). The reported evidence was largely based on cellular systems, using non-specific inhibitors and Li^+, which evidently do not discriminate between the GSK3 isozymes. Additionally, Li^+ produces diverse pleiotropic effects in biological systems, unrelated to GSK3, even involving NMDA-R and nitric oxide signaling as most recently demonstrated (reviews Agam et al., 2009; Ghasemi and Dehpour, 2011).

We have analyzed this issue more extensively *in vivo* in mouse brain, to help define physiological functions and pathological roles of GSK3α in normal brain and in AD-related neuronal processes *in vivo*. We generated and analyzed GSK3α deficient mice and observed normal processing of endogenous murine APP, as well as human APP produced by intracerebrally injected AAV–APP.SLA constructs (Jaworski et al., 2009; data not shown). Moreover, we analyzed bigenic GSK3α.KO × APP.V717I mice, yielding essentially the same negative outcome. We conclude that at least the GSK3α isozyme does not contribute significantly to the processing of APP in mouse brain *in vivo*. We are extending these studies to the GSK3β isozyme, by generating mice with neuron-specific deficiency of the GSK3β isozyme.

Finally, we advocate strongly against the use of lithium salts in cell-biological and *in vivo* studies and even more strongly against the generalized claim that Li^+ is "a specific inhibitor" of GSK3 isozymes. Three points need to be brought to the attention of those engaging in this direction: Li^+ ions are easy to use or administer, but by no means specific inhibitors of the GSK3 isozymes, nor does Li^+ discriminate between isoforms, and lastly, Li^+ is not even a potent kinase inhibitor. We consistently find that at least 5 mM Li^+ is needed to inhibit GSK3 activity effectively in human retinoblastoma cell-based assays, while *in vivo* this concentration is highly toxic for mice on regimes that last for more than a day. Moreover, while LiCl incorporated in mouse food-pellets (2 g/kg) is not toxic for wild-type mice for up to 3 months, this diet effectively worsened the axonopathy in Tau4R mice and the tauopathy in Tau.P301L mice, without much effect on the phosphorylation status of protein Tau (Muyllaert et al., 2006, 2008; data not shown). Obviously, any effect noted using too low concentrations of lithium ions in any system, but particularly *in vivo*, cannot be attributed to inhibition of GSK3 but must be caused by other enzymes affected by Li^+, of which more and more being discovered (reviews Agam et al., 2009; Ghasemi and Dehpour, 2011).

CONCLUDING REMARKS: GSK3 AND PROTEIN TAU

In contrast to APP processing, the relation of GSK3 to phosphorylation of Tau and tauopathy became stronger than ever. Most informative were GSK3β mice crossed with Tau4R and Tau.P301L mice expressing wild-type or mutant human Tau, respectively (Spittaels et al., 2000; Terwel et al., 2008), proving that GSK3β effectively phosphorylates protein Tau in brain *in vivo*.

Tau.P301L mice develop tauopathy first in their brainstem and progressively suffer severe motor defects, eventually dying from breathing defects and asphyxia around an average age of 9.4 months, with none surviving beyond 12 months of age (Dutschmann et al., 2010; data not shown). Interestingly, the combination with GSK3β alleviates tauopathy in the brainstem of Tau.P301L mice, rescuing their early mortality despite the dramatically increased tangle-load in forebrain (Terwel et al., 2008).

A comparable effect on Tau pathology, albeit less strong, was apparent in the second bigenic model that combines APP.V717I and Tau.P301L (biAT), capturing both AD pathologies in one model (Terwel et al., 2008). A considerable fraction of biAT mice survive longer than the parental Tau.P301L mice, although with a strong gender effect: survivors are mostly females, but again with more severe and aggravated Tau pathology than Tau.P301L mice. Remarkably, the amyloid pathology in female biAT mice is comparable to that in the parental single transgenic APP.V717I mice (Moechars et al., 1999; Terwel et al., 2008).

These mouse models convincingly proved that insoluble Tau is not the neurotoxic lethal factor – at least not in mouse brain. Because in the parental APP.V717I mice both GSK3 isozymes are activated by increased phosphorylation of Y279/216, the similarity in effects on tauopathy in both bigenic models is explained by this increased GSK3 activity, which posts GSK3 even more firmly as the major link between the two hallmark pathologies in AD (**Figure 1**).

Overall, our studies in transgenic mouse models add considerable weight to the hypothesis of direct involvement of GSK3 in AD pathogenesis, although the lack of any effect on APP processing *in vivo* focuses their involvement completely onto the increased phosphorylation of protein Tau. This outcome does, however, not change the general sequence of events, starting with aberrant production of amyloid peptides that activate GSK3 kinases to increase phosphorylation of protein Tau, as proposed by us (Muyllaert et al., 2008; Terwel et al., 2008; Jaworski et al., 2010a,b). Moreover, the bypass of amyloid via the activation of GSK3 to increased phosphorylation of Tau considerably strengthens the importance of tauopathy in the overall pathological process in AD. The obvious condition is that we define tauopathy no longer as the classical pathological fibrillar deposits in neuronal somata and processes, but as the phosphorylated molecular species, yet to be identified, which we denoted as Tau-P* (**Figure 1**; Jaworski et al., 2010a,b).

These toxic tau-species are likely dimers or small aggregates of protein Tau, and balance between further aggregation into the well-known large deposits that are obviously inert, or to remain soluble and be the toxic species that cause clinical dysfunctions known in AD patients, and in other tauopathies.

Remaining challenges are to define molecular identity and exact mechanisms, as well as find the "X-factors" (**Figure 1**), which are likely a combination of genetic, epigenetic, and environmental influences, besides disease and all sorts of stress. Besides the elusive Tau-species, also the amyloid peptides and aggregates, as well as the activated GSK3 isozymes can, or rather will affect synaptic functions. Eventually, the final neurodegeneration will turn out to be a combination of these different molecular players in different combinations, affected by different X-factors, which then explains the large variation in clinical picture that characterizes sporadic AD.

A final important consideration refers to the potential of GSK3, either or both isozymes, as therapeutic targets in dementia, AD, or related tauopathies. Judging from available data, originating from studies on physiology of CNS and peripheral systems, from studies

on brain pathology, from pharmacological studies, including Li^+, from epidemiological studies, from experimental mice (transgenic and knock-out) the prospect looks dim. GSK3 is involved in many essential pathways in many if not all physiological systems. Pharmacological inhibitors will have to hit the real target, i.e., GSK3 in neurons in entorhinal cortex, hippocampus, and cortex, while GSK3 in all other cells of the human body must not be affected. Potential alternatives, e.g., local application, or aiming at neuron-specific interactions of GSK3 with its controlling partners, or others that aim more specifically.

To make this happen, we need to collect more facts. . .

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Modulation of GSK-3 as a therapeutic strategy on tau pathologies

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Glycogen synthase kinase-3 (GSK-3) is ubiquitously expressed and unusually active in resting, non-stimulated cells. In mammals, at least three proteins (α, β1, and β2), generated from two different genes, *gsk-3*α and *gsk-3*β, are widely expressed at both the RNA and protein levels although some tissues show preferential expression of some of the three proteins. Control of GSK-3 activity occurs by complex mechanisms that depend on specific signaling pathways, often controlling the inhibition of the kinase activity. GSK-3 appears to integrate different signaling pathways from a wide selection of cellular stimuli. The unique position of GSK-3 in modulating the function of a diverse series of proteins and its association with a wide variety of human disorders has attracted significant attention as a therapeutic target and as a means to understand the molecular basis of brain disorders. Different neurodegenerative diseases including frontotemporal dementia, progressive supranuclear palsy, and Alzheimer's disease, present prominent tau pathology such as tau hyperphosphorylation and aggregation and are collectively referred to as tauopathies. GSK-3 has also been associated to different neuropsychiatric disorders, like schizophrenia and bipolar disorder. GSK-3β is the major kinase to phosphorylate tau both *in vitro* and *in vivo* and has been proposed as a target for therapeutic intervention. The first therapeutic strategy to modulate GSK-3 activity was the direct inhibition of its kinase activity. This review will focus on the signaling pathways involved in the control of GSK-3 activity and its pathological deregulation. We will highlight different alternatives of GSK-3 modulation including the direct pharmacological inhibition as compared to the modulation by upstream regulators.

Keywords: GSK-3, kinases modulation, tau pathologies, Alzheimer

TAU HYPERPHOSPHORYLATION AS A COMMON MARKER IN TAU PATHOLOGIES

A number of neurodegenerative disorders present intraneuronal proteinaceous aggregates, denoted as neurofibrillary tangles (NFT), as common markers of disease. NFT are made up of hyperphosphorylated, aggregated tau. Thus, they are collectively referred as tauopathies, and include disorders such as frontotemporal dementia (FTP), progressive supranuclear palsy (PSP), and Alzheimer's disease (AD), among others.

The mechanisms responsible for tau aggregation are still unknown. Therefore, understanding how tau is regulated may be essential to identify the origin of tau aggregate formation. The regulation of tau takes place predominantly through post-translational modifications where tau phosphorylation has been suggested to play a central role in tau aggregation. It is generally accepted that an increase in tau phosphorylation reduces its affinity for microtubules, resulting in neuronal cytoskeleton instability. Even though abnormal tau phosphorylation occurs in many of the tau pathologies mentioned above, tau phosphorylation appears not to be sufficient to induce tau aggregation (Buee et al., 2000; Hernandez et al., 2009). There are at least 85 putative phosphorylation sites in tau, 45 serines, and 35 threonines. It has been shown that tau phosphorylation is regulated by many different kinases, including GSK-3β, CDK5, MAPKs, PKA, PKB/Akt, PKC, PKN, and CaMKII (Hanger et al., 1992; Harr et al., 1996; Yu et al., 2009). The precise role of some of these kinases is still under investigation with particular interest on glycogen synthase kinase-3 (GSK-3). However, we do not discard the important role of other kinases such as CDK5, p38, etc. In fact, the role of CDK5 in tau phosphorylation and NFT formation has been clearly established in transgenic mice (Noble et al., 2003; Piedrahita et al., 2010).

Tau hyperphosphorylation could result from the inhibition of phosphatases. Indeed, earlier work indicates that phosphatase activity is decreased in AD brains (Gong et al., 1993; Rahman et al., 2005). In summary, a combination of kinases and phosphatases may be postulated to play a central role in tauopathies.

ROLE AND REGULATION OF GSK-3 ACTIVITY

Glycogen synthase kinase-3 is a serine/threonine protein kinase initially described to phosphorylate and inhibit glycogen synthase (Rylatt et al., 1980). GSK-3 has been evolutionary conserved and homolog genes have been identified in practically every eukaryotic genome examined including *Xenopus laevis*, *Drosophila*

melanogaster, and *Dictyostelium discoideum* (Ruel et al., 1993; Itoh et al., 1995; Kim et al., 1999).

In mammals, GSK-3 is encoded by two genes known as *gsk-3α* and *gsk-3β* (Woodgett, 1990; Frame and Cohen, 2001; Grimes and Jope, 2001) that encode three different proteins, GSK-3α and two GSK-3β proteins (β1 and β2; Mukai et al., 2002; Schaffer et al., 2003). These isoforms are almost identical (98%) but differ in their N- and C-terminal domains (Woodgett, 1990). It has been extensively established that GSK-3 plays an important role in various essential physiological processes, including cell cycle or survival-apoptosis (Pap and Cooper, 1998; Lucas et al., 2001a; Ferkey and Kimelman, 2002). It is well known that GSK-3 has a plethora of substrates, identified in many cell types and in all cellular compartments; i.e., metabolic proteins (Alessi et al., 1996), cytoskeletal proteins (Hanger et al., 1992), as well as transduction (Cohen, 1999) and transcription factors (Hart et al., 1998).

Glycogen synthase kinase-3 is a kinase differentially regulated by tyrosine and serine/threonine phosphorylation (Wang et al., 1994). One of the main characteristics of GSK-3 is that its activity is relatively high in resting, non-stimulated cells. For many years, it was believed to be a constitutively active kinase. However, it has become apparent that the activity of GSK-3 may be regulated by a variety of signals (Sayas et al., 2002) and many extracellular signals may induce a rapid and reversible decrease in enzymatic activity. Moreover, the regulation of GSK-3 activity has turned out to be much more complex than originally thought (Medina and Wandosell, 2011). The regulatory mechanisms of GSK-3 activity may be classified as follows.

REGULATION BY PHOSPHORYLATION

Two general mechanism are involved in the regulation of GSK-3 activity, Ser/Thr phosphorylation of GSK-3 specific residues by other kinases, and Tyr phosphorylation, through auto-phosphorylation (Frame and Cohen, 2001; Harwood, 2001). Different regions can be identified in the GSK-3 structure that can be modified by phosphorylation: the first one corresponds to the amino-terminal domain and contains a serine residue at positions 21 in GSK-3α and 9 in GSK-3β. It has been undoubtedly demonstrated that phosphorylation of serine 21 or 9, correlates with inhibition of the kinase activity (Sutherland et al., 1993; Stambolic and Woodgett, 1994; Frame et al., 2001). Many protein kinases including Akt, ILK, PKA, p90Rsk are competent at phosphorylating GSK-3 at residues 21 and 9 *in vitro* and *in vivo* (Cross et al., 1995; Delcommenne et al., 1998; Fang et al., 2000).

Two other regulatory sites have been described: threonine 43, only present in the GSK-3β isoform, may be phosphorylated by Erk (Ding et al., 2005) whereas p38 MAPK phosphorylates serine 389 and threonine 390 present in GSK-3β (Thornton et al., 2008). In both cases, the data suggests that these phosphorylations may facilitate the competence of serine 9 to be phosphorylated rather than support a direct GSK-3 inhibition.

Conversely, an increase in tyrosine phosphorylation at residues 216 (GSK-3β) or 279 (GSK-3α) is in clear correlation with an increase on GSK-3 activity in neuronal cells, following exposure to lysophosphatidic acid (LPA; Sayas et al., 1999) or after neurotoxic insults such as β-amyloid or PrP (Munoz-Montano et al., 1997; Takashima et al., 1998; Perez et al., 2003b). Different candidates have been reported to phosphorylate GSK-3 on tyrosine 216/279 including Pyk-2 and Fyn kinases *in vitro* or MEK1/2 in fibroblasts (Lesort et al., 1999; Hartigan et al., 2001). These data are in contrast with those reported in *D. discoideum* where there is convincing evidence demonstrating that ZAK 1 (Kim et al., 1999, 2002) is the kinase responsible for this tyrosine phosphorylation of GSK-3. However, no homolog of ZAK1 has been found in mammals.

More recently, an alternative hypothesis has been proposed for the regulation of GSK-3 tyrosine phosphorylation. This hypothesis suggests that phosphotyrosine 279/216 in GSK-3 corresponds to an intra-molecular auto-phosphorylation event (Cole et al., 2004). However, this hypothesis still lacks a cellular demonstration. Data generated in our laboratory indicated that not all pharmacological inhibitors of GSK-3 decrease the level of phosphotyrosine (Simon et al., 2008). Thus, in view of the tantalizing autoregulatory system proposed and taken all data together, we hypothesize that some as-yet-unidentified tyrosine kinases and phosphatases may also regulate GSK-3 activity.

REGULATION BY PROTEIN COMPLEX ASSOCIATION

It is well known that GSK-3 forms part of a multiprotein complex formed by axin and adenomatous polyposis coli (APC) among others. This protein complex is the core of the canonical Wnt signaling (for review see Moon et al., 2004). In the absence of ligand, β-catenin is phosphorylated by GSK-3 in this multiprotein complex and targeted for proteasome degradation (Aberle et al., 1997). In addition, different GSK-3-binding proteins have been described.

The first GSK-3-binding protein was denoted as FRAT (Li et al., 1999; Fraser et al., 2002) and three different FRATs have been characterized since then. Surprisingly, FRAT1 appears to act as an inhibitory system, whereas FRAT2 appears to increase GSK-3 activity (Yost et al., 1998; Stoothoff et al., 2005). More recently, a GSK-3 interacting protein symbolized by GSKIP, has been cloned and characterized. GSKIP can block phosphorylation of different substrates and functions as a negative regulator of GSK-3β (Chou et al., 2006).

REGULATION BY PRIMING/SUBSTRATE SPECIFICITY

As a general rule, the specificity of many kinases is governed by a consensus sequence of amino acids present in their substrates. However, the crystal structure of human GSK-3β has provided a model for the binding of pre-phosphorylated substrates to the kinase (Dajani et al., 2001; ter Haar et al., 2001; Noble et al., 2005). It is now evident that some GSK-3 substrates require a previous (*primed)* phosphorylation by a *priming* kinase. This primed residue (Ser or Thr) is usually located four amino acids, C-terminal to the Ser or Thr residue to be modified by GSK-3 (Dajani et al., 2001; ter Haar et al., 2001). Several priming kinases have been identified, such as cdk-5, PAR-1, casein kinase I, PKC, or PKA (Sengupta et al., 1997; Amit et al., 2002; Liu et al., 2003; Noble et al., 2003; Nishimura et al., 2004; Alonso Adel et al., 2006). However, is not completely established whether a second set of "*non-primed*" substrates may define a different group of functions (Twomey and McCarthy, 2006). In addition, different GSK-3 isoforms appear to exhibit distinct substrate preferences in the brain (Soutar et al., 2010).

REGULATION BY SUBCELLULAR LOCALIZATION

The presence of GSK-3 appears to be developmentally regulated in the brain (Takahashi et al., 1994) and, in fact, the developmental profile of GSK-3α and GSK-3β is different and the putative differential role of each isoform has been explored (Garrido et al., 2007). It is important to indicate that a portion of GSK-3, mostly β, has been associated with the growth cone. This GSK-3 pool appears to respond rapidly, being modified by phosphorylation and/or relocated in the growth cone, by external signals such as semaphorins (Eickholt et al., 2002) or NGF (Zhou et al., 2004). The presence of GSK-3α and β in primary neurons have been reported in different neuronal compartments, like in axon and dendrites, as well as in the cytoplasm, endoplasmic reticulum (ER; Meares et al., 2011), nucleus, and the mitochondria (Grimes and Jope, 2001; Watcharasit et al., 2002; Bijur and Jope, 2003). With respect to the nuclear localization, GSK-3 may be involved in phosphorylation of many transcription factors such as: Cyclin D1, β-catenin, HSF-1, NFAT, and cAMP-response element-binding protein, among others (for review see Cohen and Frame, 2001; Harwood, 2001; Xu et al., 2009). Moreover, the nuclear presence of GSK-3 in the nucleus may also play a role in alternative splicing (Avila et al., 2004).

REGULATION BY PROTEOLYTIC CLEAVAGE

A new mechanism of GSK-3 regulation has been recently proposed. This regulation involves the removal by calpain of a fragment from the N-terminal region of GSK-3, including the regulatory serines 9/21 (Goni-Oliver et al., 2007). Interestingly, GSK-3β has also been recently shown to be cleaved at the N-terminus (and subsequently activated) by matrix metallo-proteinase 2 (MMP-2) in cardiomyoblasts (Kandasamy and Schulz, 2009).

PHYSIOLOGICAL AND PATHOLOGICAL REGULATION OF GSK-3

GSK-3 PHYSIOLOGY

In neuronal development, GSK-3 has been reported to control many different aspects of neuronal and glial physiology, like morphogenesis and axonal polarity (Shi et al., 2004; Garrido et al., 2007), synaptogenesis (Lucas and Salinas, 1997), and cell survival (Pap and Cooper, 1998; Lucas et al., 2001a). Some of these cellular activities of GSK-3 are regulated by signals orchestrated by a wide set of extracellular ligands including growth factors, neurotrophic factors, neurotransmitters, and hormones but also by cell–cell interactions. Most of these extracellular factors trigger signaling pathways which correlate with the inhibition, or transient inhibition, of GSK-3 activity. In the case of insulin/IGF1 (Cross et al., 1995; Delcommenne et al., 1998; Fang et al., 2000) and NGF (Zhou et al., 2004) the regulatory role of Akt and/or ILK has been reported to be responsible for the increased inhibitory serine phosphorylation. More recently, we and others have shown that estradiol may also inhibit GSK-3 by a pathway modulated by PI3K-Akt (Cardona-Gomez et al., 2004; Mendez et al., 2005; Varea et al., 2009).

An alternative system of GSK-3 regulation is represented by the Wnt signaling pathway in which, in the absence of ligand, GSK-3β phosphorylates β-catenin, as a part of a degradation signal (for review see, i.e., Moon et al., 2004; Rosso et al., 2005). When Wnt (at least some Wnt's) binds the Wnt receptor frizzled (*fz*), disheveled (*Dsh*) inhibits the activity of GSK-3β in a manner that is not fully understood. This system appears to be specific to GSK-3β as no counterpart has been described for GSK-3α to date. Some recent data suggests that this complex may be specific for the GSK-3β2 isoform (Castaño et al., 2010).

In addition, Reelin and Netrin signaling have been shown to regulate GSK-3 activity even though the exact mechanism is far from being fully elucidated. The binding of Reelin appears to induce signals which may activate GSK-3 (Gonzalez-Billault et al., 2005). In this pathway, the function of GSK-3 appears to be associated with CDK5, however, the specific system that controls GSK-3 activity has yet to be defined (Gonzalez-Billault et al., 2005). Similarly, Netrin 1 regulates, both *in vivo* and *in vitro*, the phosphorylation of neuronal MAP's, in a signaling pathway that depends essentially on the kinases GSK-3 and CDK5 (Del Rio et al., 2004).

As mentioned, GSK-3 is regulated by a plethora of membrane receptors, some of which show a specific subcellular localization in neurons. Among these receptors, some purinergic ATP/ADP receptors, such as P2 × 7, P2Y1, or P2Y13, show a localized subcellular expression in neurons (Diaz-Hernandez et al., 2008; del Puerto et al., 2011). Purinergic receptors are expressed in glial and neuronal cells in the central and peripheral nervous system and are activated by purines and pyrimidines. The ATP-gated P2 × 7 ionotropic receptor is expressed in the distal region of the axon of hippocampal neurons during development (Diaz-Hernandez et al., 2008) and has been identified in presynaptic terminals in adult brain. Inhibition of P2 × 7 with brilliant blue G (BBG) increases GSK-3 phosphorylation, reducing its activity, and favors axonal elongation and branching in cortical and hippocampal neurons (Diaz-Hernandez et al., 2008), similarly to activation of P2Y1-ADP receptors (del Puerto et al., 2011). Inhibition of GSK-3 decreases PHF-I levels (phosphorylated Tau) and increases Tau-1 levels (dephosphorylated Tau). Moreover, this effect is also produced by GSK-3 inhibition with AR-A014418 (Garrido et al., 2007), which can counteract the action of ATP activation of the P2 × 7 receptor (Diaz-Hernandez et al., 2008).

Conversely, some ligands such as LPA may trigger GSK-3 kinase activity. LPA is a bioactive lipid that can act as a growth factor and binds to specific G-protein-coupled seven transmembrane domain receptors (GPCR; Chun et al., 2002). In neurons, LPA has been shown to promote growth cone collapse and neurite retraction (Tigyi et al., 1996; Sayas et al., 1999). We have demonstrated this LPA-induced GSK-3 activation event, not only inferred from hyperphosphorylation of tau, but also directly measured from neuronal cell extracts (Sayas et al., 2002). Similarly, semaphorin 3A (Sema 3A), a molecule that inhibits axonal growth or neurite retraction, activates GSK-3 at the leading edge of neuronal growth cones. The inhibition of GSK-3 activity can prevent the growth cone collapse response induced by Sema 3A, suggesting that GSK-3 activity might play a role in coupling Sema 3A signaling to changes in cell motility, axonal growth and/or maintenance of axonal extension (Eickholt et al., 2002).

Other membrane receptors that regulate GSK-3 function are only expressed in brain regions associated to specific brain diseases. For example, D2 dopamine receptors are mainly expressed in striatum and can also regulate the activity of GSK-3. Activation

of D2-like receptors inhibits the phosphorylation of Akt, leading to activation (dephosphorylation) of GSK-3 through a G protein-independent mechanism involving a complex of Akt:PP2A scaffolded by β-arrestin-2. Lithium inhibits the behavioral actions of dopamine via disruption of the D2 receptor-mediated complex of Akt:βArr2:PP2A (Pawlak and Kerr, 2008). Similarly, other neuropeptide receptors, such as those of serotonin, regulate GSK-3 activity. Two serotonin receptors, 5-HT1A and 5-HT2A, appear to play antagonistic roles in regulating GSK-3β activity. Administration of a 5-HT1A agonist or a 5-HT2A antagonist to mice results in increased brain GSK-3β phosphorylation (Beaulieu et al., 2009). Moreover, drugs acting on 5-HT neurotransmission such as the selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase (MAO) inhibitors, and tricyclic antidepressants have been shown to inhibit GSK-3β in many brain regions, including the frontal cortex, hippocampus, and striatum of normal mice.

The acetylcholinesterase inhibitor physostigmine, the muscarinic agonist pilocarpine, and the *N*-methyl-D-aspartate (NMDA) receptors antagonist memantine, also modulate the phosphorylation state of the two isoforms of GSK-3 in mouse hippocampus, cerebral cortex, and striatum (De Sarno et al., 2006). Metabotropic glutamate receptor 5 (mGluR5) can modulate the PI3K/Akt/GSK-3 pathway in the hippocampus, and that modulation of this signaling pathway can reverse β-amyloid-induced neuronal toxicity (Liu et al., 2005).

Glycogen synthase kinase-3 or GSK-3-upstream elements can be selectively regulated in specific neuronal domains, such as the pre- or post-synaptic domains or the axon initial segment (place of neuronal action potential generation), in order to maintain or generate changes in neuronal function. For example, prolonged stimulation of neurons by depolarization results in GSK-3 phosphorylation and inactivation. Thus, GSK-3 activity could be acutely regulated by action potential stimulation (Smillie and Cousin, 2011). In conclusion, it is of great interest to identify proteins specifically located in domains that can regulate GSK-3 activity and the generation of selective pharmacological inhibitors to control GSK-3 activity at the subcellular level. Dendritic spines express GSK-3 regulated through NMDA receptors by two different stimuli (LTP stimulus or LTD stimulus), that, in turn, can activate or inhibit GSK-3 activity (Peineau et al., 2007).

IS DEREGULATION OR DYSFUNCTION OF GSK-3 THE CAUSE NEURODEGENERATIVE DISEASES?

Aberrantly phosphorylated tau is a common factor in tauopathies but the pathological mechanisms responsible may be diverse (Trojanowski and Lee, 2005). The presence of a FTDP-17 mutation in tau may result in altered splicing or may correspond with missense mutations that modify the microtubule binding capacity and subsequently the phosphorylation/dephosphorylation kinetics (Ballatore et al., 2007; Hernandez and Avila, 2008). In fact, early tau-related deficits could be the result of synaptic abnormalities caused by the accumulation of hyperphosphorylated tau within intact dendritic spines (Hoover et al., 2010). Indeed, alterations in the expression and/or the activity of tau kinases (i.e., GSK-3β; Pei et al., 1997; Swatton et al., 2004; Hye et al., 2005) have been reported in affected brains, suggesting that one or several kinases could be involved in tau hyperphosphorylation (for review see, i.e., Hernandez and Avila, 2008). In addition, in transgenic mice the over-expression of some kinases such as GSK-3 increase tau phosphorylation and neurodegeneration (Lucas et al., 2001b). Conversely, inhibition of GSK-3β reduces tau phosphorylation and neurodegeneration and may block NFT formation (Nakashima et al., 2005; Noble et al., 2005; Engel et al., 2006b). Without excluding that more than one kinase may be important in the pathological phosphorylation of tau, these data suggests that inhibition of GSK-3 is probably an excellent therapeutic strategy for the treatment of tauopathies.

Interestingly, the P2 × 7 receptor has also been related to Alzheimer's disease and β-amyloid production or involved in the regulation of a β-amyloid effect (Sanz et al., 2009). The purinergic receptor P2 × 7 triggers α-secretase-dependent processing of the amyloid precursor protein (APP; Delarasse et al., 2010). BBG treatment is also able to reduce inflammation after spinal cord injury in rats, reduce neuronal death, and it is a promising drug for axonal regeneration (Wang et al., 2004).

As mentioned, blockade of the mGluR5-mediated pathways inhibits GSK-3. The NMDA receptor antagonist memantine confers neuroprotection to Aβ peptides (Liu et al., 2005). Moreover, memantine, physostigmine, or pilocarpine, have been used as therapeutic agents in AD due to their capacity to regulate GSK-3 activity (De Sarno et al., 2006). When using GSK-3 inhibitors, one may also need to consider the different cell types involved in GSK-3 associated diseases. In some tauopathies glial cells and/or neuronal cells are an essential component. Thus, inhibition of GSK-3 through cell specific receptors may help avoid alteration of GSK-3 in other cell types.

Besides its association with AD (Ishiguro et al., 1993; Lovestone et al., 1994) GSK-3 dysfunction has also been associated with different pathological conditions or brain damage; such as Ischemia (Bhat et al., 2000), prion neurotoxicity (Perez et al., 2003b), or with some psychiatric disorders such as schizophrenia (Beaulieu et al., 2009). In fact in Disrupted in schizophrenia 1 mutant (DISC1) neuronal progenitor proliferation is altered and the inhibition of GSK-3 restore this proliferation (Mao et al., 2009). Thus, GSK-3 is emerging as a promising therapeutic target in several neurologic disorders.

THERAPEUTIC APPROACHES: DIRECT INHIBITION VERSUS PHARMACOLOGICAL DOWN-REGULATION

As mentioned above, GSK-3 unique position in modulating the function of a diverse series of proteins in combination with its association with a wide variety of human disorders has attracted significant attention to the protein both as a therapeutic target and as a tool to understand the molecular bases of these disorders. Furthermore, GSK-3 appears to be a cellular nexus, integrating several signaling systems, including numerous second messengers and a wide selection of cellular stimulants.

If we consider GSK-3 as an example of a kinase associated with tauopathies, two different approaches would be considered as therapeutic strategies: direct inhibition of GSK-3 activity or a more generic modulation of GSK-3 acting upstream of the kinase. The first strategy, has been widely considered and indeed, some general inhibitors such as lithium have been tested in several AD animal models (Engel et al., 2008). A second approach

is the search for candidates taking into account that neurodegeneration may reflect "pleiotropic" neuronal cell dysfunction. Accordingly, we have considered here some of the pathways that may control neural morphogenesis and survival including the PI3K-Akt, PKA, and Wnt pathways and their implication in neurodegeneration. Also, we propose alternative and complementary "non-target-based" approaches, that may also be useful for the study of neurodegeneration (Simon et al., 2011). Using cellular based assays we may identify molecules that regardless of their ability to cross the cellular membrane may be identified by a cellular/neuronal modification downstream of a selected kinase, i.e., the tau phosphorylation level. Activation of serotonergic 5-HT4 receptors have been shown to improve memory processes in preclinical cognition models, mainly through the stimulation of cholinergic neurotransmission (Lezoualc'h, 2007).

For many years, the mood stabilizing drug lithium has been considered as the reference GSK-3 inhibitor. Lithium ions directly inhibit GSK-3 (Klein and Melton, 1996), most likely by competing with magnesium but it also inhibits at least four phosphomonoesterases (including inositol monophosphatase; York et al., 1995), and phosphoglucomutase (Ray et al., 1978; Stambolic and Woodgett, 1994). Although the mechanism of action by which lithium exerts its therapeutic effects is unknown, GSK-3 is significantly inhibited at therapeutic lithium concentrations (Shaldubina et al., 2001; Gould and Manji, 2002; Phiel et al., 2003). It has been generally assumed that a significant proportion of the therapeutic action of lithium in bipolar disorders results from the inhibition of GSK-3 (Li et al., 2002; Rowe et al., 2007).

Inhibition of GSK-3 by lithium prevents Aβ-induced neurodegeneration of cortical and hippocampal primary cultures (Alvarez et al., 1999). With regards to tau metabolism, lithium is able to prevent tau phosphorylation in several mouse models of tauopathies (Perez et al., 2003a; Nakashima et al., 2005; Noble et al., 2005; Engel et al., 2006a; Caccamo et al., 2007). In AD models, lithium has been shown to block the accumulation of Aβ peptides in mice that overproduce human APP (Phiel et al., 2003). Furthermore, it has been suggested that lithium could also be effective in a mouse model of FTDP-17 (Perez et al., 2003a).

Only a few observational studies have attempted to address the clinical effect of lithium in patients with AD. A retrospective study with a large sample of patients with dementia resulted in an increased risk of AD in patients who had been treated with lithium within 4 years prior to diagnosis (Ayuso-Mateos et al., 2001). This effect might be partially accounted for by the increased occurrence of depression associated with AD. Moreover, a single case study reported in a dementia patient showed that lithium treatment alleviated symptoms of aggression and agitation, while cognition persisted after 1.5 years of treatment (Havens and Cole, 1982). Furthermore, a significantly increased global cognitive ability, as measured by MMSE in non-demented patients, appears to be associated with lithium intake (Terao et al., 2006). However, the study design and low sample size precludes drawing any causative conclusion from these studies.

In addition, some pilot studies have been carried out to directly address the effect of lithium treatment in AD patients. An open label feasibility and tolerability study with a small cohort of 22 subjects receiving a low dose of lithium was carried out in the UK and reported a high discontinuation rate despite few, relatively mild, and reversible side effects (Macdonald et al., 2008). A second randomized, single-blind, placebo-controlled, parallel group, multicenter 10-week study was carried out in Germany as a proof-of-principle (Hampel et al., 2009). A total of 71 patients with mild AD (MMSE scores between 21 and 26) were treated with lithium or placebo for 10 weeks after which neuropsychological and neuropsychiatric assessment was performed together with some biomarker determinations in plasma (Aβ1-42), lymphocytes (GSK-3 activity), and CSF (total tau, phospho-tau, and Aβ1-42). In spite of the fact that lithium plasma levels were within the therapeutic range, no treatment effect was observed in any of the cognition assessment scales or the selected biomarkers used. Given the short time of treatment of this study, the possibility that lithium has long-term effects on cognition or any other biomarker in AD remains to be tested.

Very recently, a randomized, double-blind, placebo-controlled study on 45 people with amnestic mild cognitive impairment (aMCI) treated with lithium for 12 months was reported (Forleza et al., 2011). Lithium treatment was associated with a significant decrease in CSF concentrations of pTau and with a better performance in cognitive tasks, strongly suggesting that lithium (most likely through GSK-3 inhibition) might have disease-modifying properties in AD and perhaps other tauopathies.

Use of lithium in clinical practice is problematic since it has a narrow therapeutic window (blood serum levels 0.6–1.2 mM) above which side effects are intolerable requiring titration. An overdose can lead to severe neurological dysfunction and in some cases death. Non-CNS side effects of lithium (common within therapeutic levels) include tremor, polyuria, polydipsia, nausea, and weight gain. Moreover, lithium can have adverse reactions with other drug classes including diuretics, NSAIDS, and other drugs that alter kidney function (Gould and Manji, 2006).

The unique position of GSK-3 as a pivotal and central player in the pathogenesis of both sporadic and familial forms of AD has attracted significant attention to this enzyme as a therapeutic target and has led to the synthesis of a high number of GSK-3 inhibitors, some of which are currently being tested in phase II proof-of-concept clinical trials (Mangialasche et al., 2010; Medina and Avila, 2010). Inhibition of GSK-3 with small-molecules would be expected to slow down progression of neurodegeneration in AD and perhaps other tauopathies as well.

A number of novel potent and relatively selective small-molecule inhibitors of GSK-3 activity from different chemical families have been recently described, including hymenialdisine, indirubins, paullones, maleimides, amino pyrazoles, thiazoles, and 2,4-disubstituted thiadiazolidinones (TDZD; reviewed in Medina and Castro, 2008). Most of them are ATP-competitive inhibitors, although more recently, new small-molecule derivatives that exhibit substrate competitive inhibition activity toward GSK-3 have been shown. Since different GSK-3 isoforms display a high degree of homology within the ATP-binding site, inhibitors are unable to exhibit isoform selectivity, as they all show similar potencies toward purified GSK-3α and GSK-3β. Thus, the development of isoform-specific inhibitors or the inhibition of

Table 1 | Selected GSK-3 kinase inhibitor drugs in clinical development for the treatment of tauopathies.

Drug	Company	Mechanism of action	Development phase	Indication
Tideglusib (NP-12)	Noscira	GSK-3 inhibitor	Phase II	Alzheimer's disease, PSP
Lithium	University of Sao Paulo	GSK-3 inhibitor	Phase II	Alzheimer's disease
Valproic acid	Nantes University Hospital	GSK-3 inhibitor	Phase II	PSP
AZD-1080	AstraZeneca	GSK-3 inhibitor	Discontinued	Alzheimer's disease

signaling pathways specific for one of the isoforms is necessary to selectively target the different substrates involved in different neurodegenerative diseases. Although ATP-competitive inhibitors occupy the general area of the highly conserved ATP-binding site, they also explore other available nearby space, depending on their structure. Thus, it may be possible to identify selective inhibitors by taking advantage of the small differences in primary sequence that exist between the different kinases. The crystal structures of GSK-3β complexed with a variety of ligands (ter Haar, 2006), together with molecular modeling approaches (Gadakar et al., 2007), provide the necessary clues for enhancing selectivity toward GSK-3 (ter Haar et al., 2001; Patel et al., 2007).

Besides small-molecule inhibitors, some physiological peptides act as GSK-3 inhibitors, including GBP, a maternal *Xenopus* GSK-3-binding protein homologous to a mammalian T cell proto-oncogene (Yost et al., 1998) and p24, a heat resistant GSK-3-binding protein (Martin et al., 2002). That finding led to a synthetic strategy to develop new inhibitors, such as L803-mts, a peptidic inhibitor that binds to the substrate site (Plotkin et al., 2003). L803-mts has been more recently used to examine the impact of long-term *in vivo* inhibition of GSK-3 and its effects in specific tissues (Kaidanovich-Beilin and Eldar-Finkelman, 2006).

The last few years have seen the synthesis of quite a number of relatively selective, potent GSK-3 inhibitors with relative efficacy *in vivo* in a diverse array of animal models of human diseases, including AD. Despite the challenges faced by this approach with respect to safety and specificity, a number of efforts are underway to develop kinase inhibitors and, in fact, Noscira's tideglusib (NP-12; see **Table 1**), is already in phase II clinical trials for the treatment of both AD and PSP, a tauopathy (Medina and Castro, 2008).

In summary, there is a significant effort being made to validate a number of tau-related targets and to develop novel alternatives for the treatment of tauopathies such as AD or PSP (reviewed in Medina and Wandosell, 2011) including GSK-3 inhibition. We hope that future clinical trials will reveal the safety and efficacy of potential treatment strategies that are being tested in translational laboratories across the world.

CONCLUSION

Three decades after its discovery as a protein kinase involved in glycogen metabolism, GSK-3 has revealed itself as a common link, integrating several signaling systems, including second messengers and a wide selection of cellular stimulants. Modulation of its activity has also turned out to be much more complex than originally thought, as GSK-3 regulation occurs by complex mechanisms that are dependent on specific signaling pathways, including post-translational modifications, protein complex formation, and subcellular localization. Although there seems to be a good degree of functional overlapping between different isoforms, some tissue- and isoform-specific functions and substrates are beginning to emerge. Thus, new data will open the possibility to design better and more specific inhibitors.

Deregulation or abnormal GSK-3 activity appears to be associated with various relevant pathologies, including AD and other tauopathies, as the enzyme is uniquely positioned as central player in AD pathogenesis. GSK-3 plays a critical role in key events such as tau phosphorylation, Aβ formation, and neurotoxicity, microtubule dynamics, synaptic plasticity, neuritic dystrophy, cognition, neuronal survival, and neurodegeneration.

In the last two decades, drug discovery and development efforts for AD have primarily focused on targets defined by the amyloid cascade hypothesis with disappointing results, underscoring the need for novel therapeutic targets. In contrast, tau-based approaches have received little attention until recently, despite the fact that tau pathology is paramount in all tauopathies including AD (Medina and Wandosell, 2011). A significant effort has being made in the last few years to synthesize a high number of relatively selective and potent GSK-3 inhibitors, while some of them have shown *in vivo* efficacy in various animal models of AD. Some of the known drug discovery and development challenges should be met, including: lack of good predictive animal models, the need for validated biomarkers of disease progression, appropriate clinical trial design, early diagnosis, and treatment, definition of target populations, difficulties in demonstrating disease-modifying effects, etc. Despite the challenges faced by this approach with respect to safety and specificity, a number of efforts are underway to develop GSK-3 inhibitors as potential drugs for the treatment of AD. Some agents have already reached phase II clinical trials and some proof-of-concept studies are currently ongoing or planned.

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Emerging roles of glycogen synthase kinase 3 in the treatment of brain tumors

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The constitutively active protein glycogen synthase kinase 3 (GSK3), a serine/threonine kinase, acts paradoxically as a tumor suppressor in some cancers while potentiates growth in others. Deciphering what governs its actions is vital for understanding many pathological conditions, including brain cancer. What are seemingly disparate roles of GSK3 stems from the complex regulation of many cellular functions by GSK3. This review focuses on the regulation of GSK3, its role in survival, apoptosis and DNA damage, and finally its potential therapeutic impact in brain cancer. A thorough understanding of this versatile protein is critical for improving the outcome of various diseases, especially cancer.

Keywords: glycogen synthase kinase 3, neuroprotection, lithium, brain cancer, DNA damage, epidermal growth factor receptor, NFκB, apoptosis and autophagy

INTRODUCTION

Glycogen synthase kinase 3 (GSK3) has been shown over the past three decades to regulate a myriad of cellular functions including cell polarity, cell fate, metabolic homeostasis, development, apoptosis, microtubule function, and neuronal growth and differentiation. Elegant cellular checkpoints and highly regulated equilibrium of protein levels are responsible for the precise control of GSK3 activity. GSK3, while involved in many critical functions, still remains a nearly untapped source of modulation therapeutically. The emergence of GSK3 as an attractive therapeutic agent could stem from its involvement in several of the critical signaling pathways. Multiple genes involved in these pathways are often susceptible to activating mutations which lead to multiple tumor types and/or tumor resistance (Brugge et al., 2007). By targeting GSK3, one could potentially attenuate these dysregulated pathways to improve tumor response in patients.

More importantly, GSK3 inhibition may also impact quality of life. The standard curative treatment regimen for primary and metastatic brain tumors typically involves cranial irradiation (IR), which often results in long-term neurological side effects, especially in the pediatric population (Roman and Sperduto, 1995; Anderson et al., 2000; Boehme et al., 2008). There have been reports of intellectual impairment, reduction in performance IQ, memory loss, and dementia after exposure of the brain to radiation. This cognitive decline observed may be due to IR-induced damage to the hippocampus, a critical area of the brain responsible for learning and memory (Yazlovitskaya et al., 2006). Consistent with these findings, radiation to the hippocampus is associated with more pronounced cognitive deficits compared with radiation to other areas of the brain (Abayomi, 2002). As GSK3 inhibition has been shown to protect neurocognitive function following cranial irradiation, the therapeutic value of GSK3 in cancer may also be related, in part, to its effects on DNA repair and its emerging role in neuroprotection of normal neuronal tissues from radiation induced apoptosis (Thotala et al., 2008; Yang et al., 2009, 2011). These combined effects of GSK3 inhibition, namely inducing tumor cell death while protecting normal cells, again make GSK3 an attractive therapeutic target for brain tumors.

In this review, we will discuss the rationale for therapeutic targeting of GSK3 in oncology, due to the involvement of GSK3 in apoptosis, DNA damage/repair, and autophagy. Obstacles to targeting GSK3 include its varied and highly regulated role in cellular homeostasis in addition to the existence of highly homologous GSK3 isoforms, which will be discussed. Despite these hurdles, GSK3 nevertheless has been tagged as a potential target in human cancers, and more importantly, as a protector of normal tissues at high risk for treatment-related side effects. These characteristics may ultimately improve the therapeutic ratio and enhance patient quality of life.

GSK3 MODULATION OF TUMORIGENIC SIGNALS

Glycogen synthase kinase 3 is essentially expressed ubiquitously in all mammalian tissues and is almost always in an *active* state in cells, even when not stimulated by mitogenic or hormonal signals. There are two mammalian GSK3 isoforms encoded by distinct genes: GSK3α and GSK3β. GSK3α and GSK3β, although structurally similar, are functionally diverse. GSK3α and GSK3β are highly conserved and widely expressed kinases that share 98%

sequence homology within their catalytic domains. Interestingly, loss of GSK3β isoform is embryonically lethal due to liver degeneration caused by extensive hepatocyte apoptosis (Hoeflich et al., 2000). Moreover, GSK3α is unable to rescue this phenotype. It is also well established that GSK3 activity is regulated primarily at the posttranslational level, chiefly by protein–protein interactions or posttranslational modifications (Cohen and Frame, 2001). Phosphorylation of GSK3 N-terminally at S9 lowers its activity toward its substrates (Cohen and Frame, 2001). We refer the readers to some excellent reviews in this special issue for further details on GSK3, including its isoforms, regulation of these isoforms, and their distinct functions.

Dysregulation in GSK3 activity has been linked to multiple cancers. However, the direction by which GSK3 is dysregulated, i.e., suppressed vs. activated, is heterogeneous among tumor types as discussed below. In general, GSK3 primarily functions by inactivating its substrates via phosphorylation, thus altering their conformation, localization, and/or degradation (**Figures 1** and **2**). This, in turn, can affect the subsequent ability of these substrates to interact and trigger downstream signaling events. Usually, the substrates of GSK3 need to be "primed" by a separate kinase to allow GSK3 to bind and subsequently phosphorylate the target molecule. Here we look at the direct and indirect roles of GSK3 in cancer.

DIRECT ROLES OF GSK3 IN CANCER

Though various roles of GSK3 in cancer have been proposed, the direct vs. indirect roles of GSK3 in this disease are difficult to tease out due to the embryonic lethality of GSK3 loss. In addition, the significant cross talk between different signaling pathways and varied role of GSK3 in these pathways makes it all the more difficult to pinpoint one player. However, we will detail in the next section the direct roles of GSK3 in cancer as reported in the literature.

Expression of GSK3β is drastically diminished in multiple cancers as listed in **Table 1**. Ma et al. (2007) have demonstrated that normal patient skin tissue specimens express higher GSK3β and pGSK3β expression when compared to cancer. Moreover, utilizing different constructs, they show that modulation of GSK3β activity negatively regulates epidermal cell transformation. In the complex web underlying skin tumorigenesis and involving interactions among multiple signaling cascades and various transcription factors, GSK3β appears to be an important component in the signaling cascade since modulation of GSK3β expression/activity is sufficient to alter the transformation potential of epidermal cells. Thus, GSK3β is a target for skin cancer prevention and treatment strategies.

Interestingly, in pancreatic cancer, pharmacological inhibition of GSK3 decreased proliferation and survival of these cancer cells (Ougolkov et al., 2005). The mechanism was shown to be related to blockade of GSK3β-mediated upregulation of NF-κB-mediated gene transcription. Similarly, brain, bladder, colorectal, myeloma, leukemia, and stomach cancers were also responsive to inhibition of GSK3 (Mishra, 2010; Naito et al., 2010). For example, pharmacological inhibition of GSK3 downregulated BCL-2 and XIAP protein expression levels leading to decreased survival of bladder cancer cells. Furthermore, aberrant nuclear accumulation of GSK3β in urothelial and bladder cancer was observed. Nuclear expression of GSK3β has been associated with high grade tumors, metastasis and worse survival in bladder cancer patients and thus may be a prognostic marker for this cancer (Naito et al., 2010).

In addition, GSK3 inhibition has been shown to promote apoptosis in leukemic cells via modulation of chromatin structure through histone modifications that abrogate the transcriptional activity of NFκB (Ougolkov et al., 2007). GSK3 can also promote mixed lineage leukemia (MLL) cell proliferation and transformation via destabilization of the cyclin-dependent kinase inhibitor p27Kip1 (Wang et al., 2008). Similarly, inhibition of GSK3β has been shown to promote apoptosis in neuroblastoma by promoting G2/M cell cycle arrest, accumulation of β-catenin and subsequent inhibition of NFκB activity (Dickey et al., 2011). Furthermore, recent studies in prostate, pancreatic, and colorectal cancer cell lines indicate that GSK3 inhibitors significantly decrease cell growth and proliferation (Martinez et al., 2006). Thus, taken

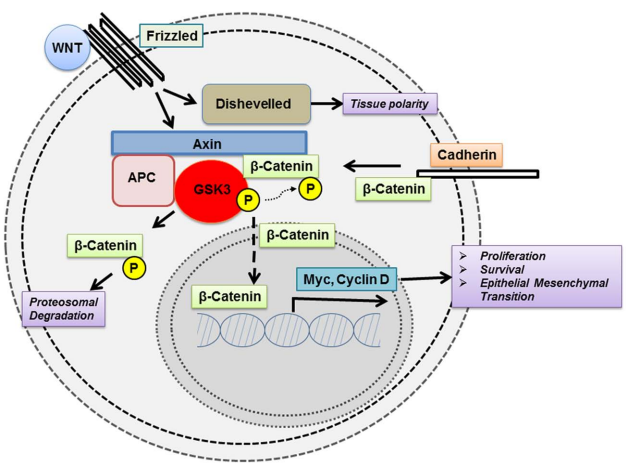

FIGURE 1 | Glycogen synthase kinase 3 modulates the function of key signaling proteins in the wnt pathway.

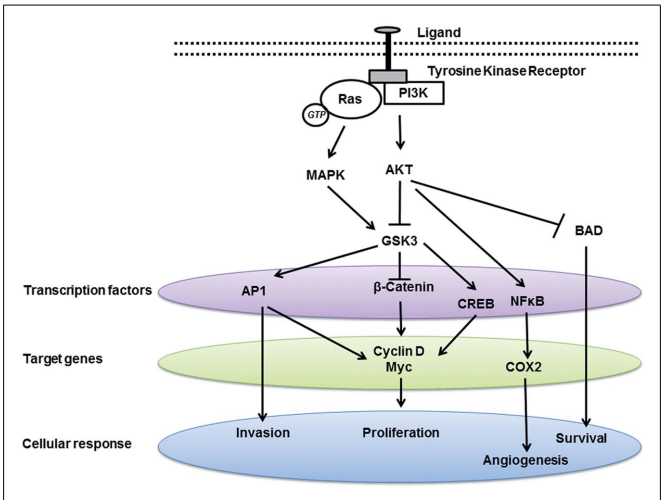

FIGURE 2 | Glycogen synthase kinase 3 modulates multiple signaling pathways involved in carcinogenesis.

Table 1 | Evidence for GSK3 involvement in distinct cancers.

Evidence	Cancers	References
Active GSK3β promotes growth and inhibition of GSK3 decreases cell growth and proliferation of cancer cells	Colorectal Leukemia Liver Pancreas Prostate Renal Stomach Thyroid	Mishra (2010) Ougolkov et al. (2005) Aijing Sun et al. (2007) Tiebang Kang et al. (2008) Mai et al. (2006) Adler et al. (2009)
High level of expression and nuclear accumulation of GSK3 is associated with kinase activity and tumor dedifferentiation	Pancreas	Ougolkov et al. (2005)
Pharmacological inhibition of GSK3 has been shown to promote cell death (apoptosis)	Brain Colorectal Myeloma Pancreas Stomach	Zhou et al. (2008) Mishra (2010) Thotala et al. (2008) Mai et al. (2006)
Higher pGSK3β (Ser9) expression is observed, indicative of inactivated GSK3β	Colorectal Lung Oral Skin	Leis et al. (2002) Tiebang Kang et al. (2008) Tian et al. (2006)

together, these results suggest GSK3 as a potential new therapeutic target in human cancer.

In the brain specifically, cancers result from abnormal cell growth and may arise from different cell lineages: neuronal or glial progenitor cells. Medulloblastomas originate from neuronal progenitors which ultimately gives rise to neurons. On the other hand, glial progenitors are far more plastic and may give rise to astrocytoma or glioblastoma (from astrocyte progenitor) or oligodendroglioma (from oligodendrocyte progenitor). We refer the readers to an excellent review on brain cancer by Huse and Holland (2010).

Medulloblastoma, one of the more common malignant brain tumors in children which originates in the area between the brainstem and the cerebellum, remains responsible for a significant level of morbidity and mortality among cancer patients. A proposed pathway involved in the development of medulloblastoma is the sonic hedgehog (SHH) pathway (Weiner et al., 2002). Approximately 15% of sporadic medulloblastomas present with genomic alterations in components of the SHH signaling pathway (Taylor et al., 2002). Normally, the SHH mitogen enables the cerebellar granule neuron precursors (CGNPs) to exhibit rapid cellular growth that eventually progresses to terminal differentiation into glutamatergic neurons. Dysregulation of SHH activation is implicated in medulloblastoma and its proliferative effects are enabled by its transcription factor target, N-myc (Knoepfler and Kenney, 2006). N-myc has been shown to be critical for normal brain growth as well as being a key player in medulloblastoma. Importantly, GSK3 is reported to be a regulator of N-myc phosphorylation (Knoepfler and Kenney, 2006). Phosphorylation of N-myc leads to its destabilization and degradation. Thus, treatment of CGNPs with LiCl or GSK3 chemical inhibitor leads to a rapid decrease in N-myc phosphorylation. Furthermore, inhibition of PI3K leads to an increase in GSK3 activity resulting in a rapid turnover of N-myc (Kenney et al., 2004). Thus, dysregulation of GSK3 is a key player in medulloblastoma and targeting this protein may be a novel therapeutic approach.

Inhibition of GSK3β activity also induces tumor cell differentiation and enhances apoptosis in glioblastoma. Targeted inhibition of GSK3β results in a decrease in progenitor markers while inducing expression of GFAP, β-tubulin III, CNPase, all being cellular differentiation markers. In addition, GSK3 inhibition has a negative effect on the subpopulation of cancer stem cell-like cells, depleting them and pushing into differentiation. These observations strengthen the claim that GSK3 inhibition in glioma cells results in decreased proficiency in anchorage-independent growth compared to wild-type. Impairment in the formation of neurospheres and decrease in stem cell markers are also observed following GSK3β inhibition (Kotliarova et al., 2008; Korur et al., 2009).

INDIRECT ROLES OF GSK3 IN CANCER

In addition to the aforementioned direct roles of GSK3 in cancer, it may also indirectly be involved in cancer due to its participation in dysregulated signaling pathways. For example, one of the major upstream protein kinases known to phosphorylate and inactivate GSK3 is protein kinase B (PKB)/AKT, which is often dysregulated in tumors (Robinson et al., 2011). AKT is able to enhance growth-factor mediated survival by multiple mechanisms. One method is the phosphorylation of the pro-apoptotic protein, BCL2-associated agonist of cell death (BAD), leading to inhibition of complex formation between BAD and BCL-2. This dissociation causes a shift from apoptosis to cellular survival (Downward, 2004). Another possibility is the ability of AKT to activate NF-κB by the non-canonical pathway involving activation of IKKα which increases p53 production. This increase in NF-κB activity is implicated in transformation of murine fibroblasts as well as involvement in breast and skin cancers (Gustin et al., 2006). Additionally, AKT promotes nuclear translocation of mouse double minute 2 (MDM2), an E3 ubiquitin ligase, thereby downregulating p53-mediated apoptosis (Mayo and Donner, 2001). The tumor suppressor phosphatase and tensin homolog (PTEN) antagonizes this PI3K/Akt pathway. For example, in certain brain cancers, PTEN dysfunction may contribute to the formation of gliomas in multiple ways including having effects on stem cell self-renewal, migration, invasion as well as by enhanced degradation of p53 via increased expression of MDM2 (Endersby and Baker, 2008).

Glycogen synthase kinase 3 can also phosphorylate the pro-oncogenic molecules β-catenin, c-myc, and c-Jun, targeting them for degradation or inactivation. This results in inhibition of cell proliferation and self-renewal. Dysregulation in these pathways and gain-of-function mutations in these three proteins interfere with the function of GSK3 and have been linked to cancers of the skin, colon, prostate, and liver (Polakis, 2007).

Dysregulation in the GSK3 pathway has also been implicated in oral cancer. Tumor recurrence and metastasis pose a challenge to

controlling this malignancy. Emerging research suggests a critical role of GSK3β as a tumor suppressor in oral cancer. Furthermore, GSK3β may be vital in regulating key players that control transcription, accelerated cell cycle progression, activation of invasion/metastasis, and anti-apoptosis (Goto et al., 2002; Chien et al., 2010; Iwai et al., 2010). P-cadherin triggers GSK3β mediated inactivation and subsequent cytoplasmic translocation of Snail in oral squamous cell carcinoma, thus conferring mesenchymal cells with epithelial features (Bauer et al., 2009). These results contradict the idea of GSK3 as a therapeutic target. However, as discussed below, in a subset of cancers, GSK3 inhibition can induce death or halt tumor progression.

Specifically, in brain tumors, GSK3β may have an indirect role in glioblastomas which is one of the most lethal brain tumors. Glioblastomas are notoriously difficult to treat and have a median survival time of ∼14 months. As mentioned above cancers are driven by cells with stem cell properties. Expression of GSK3β, which is consistently expressed in primary glioblastoma, is functionally linked to Bmi1, a protein involved in neural stem cell self-renewal and anti-oxidant defense in neurons (Kotliarova et al., 2008; Korur et al., 2009). Research has also shown that inhibition of Bmi1, a member of the polycomb family of proteins, results in reduced GSK3β as well as increased differentiation of glioma (Korur et al., 2009). While fully differentiated normal brain tissue has no Bmi1 expression, it is marked in 84% of primary glioblastomas and 71% of primary oligodendroglioma. Downregulation of Bmi1 in glioma cells results in an increase in cell differentiation toward an astrocytic fate and a decrease in expression of Nestin, Sox2, and GSK3β.

Furthermore, inactivation of GSK3α and GSK3β has been reported to block differentiation in brain progenitor cells, and instead lead to substantial hyperproliferation of the neural progenitor cells, thus, expanding the population (Kim et al., 2009). These observations were attributed to dysregulation of β-catenin, SHH, Notch, and fibroblast growth factor signaling. This critical GSK3-mediated homeostatic control was only removed by a major reduction of GSK3 signaling. Since brain development requires a balance of progenitor proliferation and differentiation this might, in a more sporadic background, lead to amplification of stem cells. This may, at least theoretically, lead to subsequent tumor formation. Thus, GSK3 signaling is integral to homeostatic controls and further dissection of these intricate processes will elucidate the therapeutic role of GSK3 in cancer.

Another potential mechanism of GSK3 activation in cancer may be via the epidermal growth factor receptor (EGFR) pathways. The EGFR family of proteins (EGFR/HER/ErbB2) plays an essential role in modulating proliferation, differentiation, and survival. EGFR has become a heavily targeted pathway as a novel cancer therapeutic strategy since its overexpression confers resistance to therapy. It has also been recently reported that lapatinib, a small molecule tyrosine kinase inhibitor targeting EGFR and HER2, is associated with changes in Akt and GSK3 (Li et al., 2011). Accordingly, EGFR and phosphorylated GSK3β have been validated as good prognostic markers for EGFR overexpressing lung carcinoma (Zheng et al., 2007). Thus, it is intriguing to hypothesize that there may be significant cross-talk between GSK3 and EGFR signaling pathways. Interestingly, dysregulation in the EGFR family of proteins is observed in glioblastoma (Furnari et al., 2007; Chin, 2008; Parsons et al., 2008; Bredel et al., 2011). The EGFR signaling pathway may be activated alternatively via NF-κB and can be repressed by the nuclear factor of κ-light polypeptide gene enhancer in B-cells inhibitor-α (*NFκBIA*). It has been suggested that *NFκBIA* may function as a tumor suppressor in multiple cancers (Osborne et al., 2005; Sjöblom et al., 2006; Bu et al., 2007). Not surprisingly, *NFκBIA* is often deleted in glioblastomas and confers resistance to chemotherapy (Bredel et al., 2011). Since NF-κB is also known to be an important player in the survival of glioma cells, it is not surprising that attenuation of GSK3 inhibits NF-κB leading to decreased glioma cell growth (Kasuga et al., 2004; Robe et al., 2004). TRAIL, DR4/5, and c-myc are induced upon GSK3 inhibition in a dose-dependent manner. Furthermore, GSK3 inhibition has been reported to have synergistic effects in combination with the chemotherapeutic drug, carboplatin, on glioma cytotoxicity.

Further evidence of this link lies in the signal transducer and activator of transcription (STAT) family of transcription factors, which is downstream of EGFR and plays a central role in cancer. STAT3 is constitutively activated in many human cancers where it functions as a critical mediator of oncogenic signaling through transcriptional activation of genes encoding apoptosis inhibitors [e.g., BCL-x(L) and survivin], cell-cycle regulators (e.g., cyclin D1 and c-myc) and inducers of angiogenesis (e.g., vascular endothelial growth factor). The STAT3 gene has been reported to regulate cancer stem cells in brain cancer. Importantly, GSK3 inhibitors have been demonstrated to block STAT3 DNA binding activity and reduce the expression of STAT3-induced Glial fibrillary acidic protein (GFAP) and B-cell CLL/lymphoma 3 (BCL-3; Beurel and Jope, 2008).

The above findings suggest that GSK3 inhibition indeed may be beneficial in brain tumors. However, one must be cautioned and consider the actions of GSK3 in normal neurons. Dysregulation of GSK3 in a variety of brain abnormalities supports its function as the switch between basic mechanisms of neuronal functions. It plays a pivotal role in brain bioenergetics, establishment of neuronal circuits, modulation of neuronal polarity, migration, neuronal proliferation, and survival (Frame and Cohen, 2001). In particular, the role of GSK3 in phosphorylation of cytoskeletal proteins impacts neuronal plasticity. Since cytoskeletal constituents are involved in the development and maintenance of neurites, alterations in the rate of stabilization/destabilization of microtubules influences major cellular compartments of neurons, such as dendrites, spines, axons, and synapses. This modulates synaptic plasticity in the adult brain (Peineau et al., 2008). Due to the effects of GSK3 on synaptic plasticity, the use of GSK3 inhibitors for cancer therapy must be approached with caution. However, a subset of cancer patients may benefit from GSK3 inhibition, due to the paradoxical effect of GSK3 activity on tumor growth. Further investigation to determine the full extent of GSK3 dysregulation in cancer is thus warranted.

GSK3 INHIBITION AND PROTECTION OF IRRADIATED NEURONS

Nevertheless, the role of GSK3 inhibition in brain tumor therapy may extend beyond tumoricidal effects and provide neuroprotection following irradiation, which increases the therapeutic index

and improves patient quality of life (**Figure 3**). Cranial irradiation therapy is a standard method for treatment of brain cancer. Since radiation cannot be selectively delivered to cancer cells, it leads to many destructive cellular processes including apoptosis, genomic instability, and autophagy. Cranial irradiation is known to cause long-term cognitive defects especially in the developing brains of children (Meadows et al., 1981). The subgranular zone of the hippocampus, housing the actively proliferating neuronal progenitor cells, is thought to be the area most affected by cranial irradiation leading to neurocognitive deficits (Nagai et al., 2000).

Interestingly, it has been known for some time that lithium treatment has neuroprotective effects against various insults to neuronal tissues (Nonaka et al., 1998; Cimarosti et al., 2001; Hongisto et al., 2003). As previously mentioned, lithium is known to modulate GSK3 activity by attenuating GSK3 activity at therapeutic concentrations via the PI3K/AKT pathway (∼1 mM; De Sarno et al., 2002; Jope, 2003). These neuroprotective effects of GSK3 inhibition have led to the hypothesis that GSK3 can potentially be targeted to attenuate the side effects of cranial irradiation (Inouye et al., 1995; Yazlovitskaya et al., 2006; Thotala et al., 2008). Treatment with LiCl increases phosphorylation of GSK3β at S9 and concomitantly decreases the activating phosphorylation of GSK3β, GSK3β-T216. The major kinase responsible for the inhibitory phosphorylation of GSK3 at S9 is Akt which is activated upon LiCl treatment as well as treatment with ionizing radiation. A two-fold increase in protein levels of the downstream targets of GSK3, β-catenin, and cyclin D1, has been reported following treatment with LiCl. Together these show that LiCl treatment has a direct effect on GSK3 activity, which may be explained by an increase in Akt activity. Importantly, both lithium prophylaxis and prophylaxes with small molecule GSK3β inhibitors prior to cranial irradiation maintained neurocognitive function in irradiated mice (Thotala et al., 2008). Thus lithium can potentially improve neurocognitive function following irradiation and the mechanism of action may be attributed to reduction of radiation-induced apoptosis of hippocampal neurons.

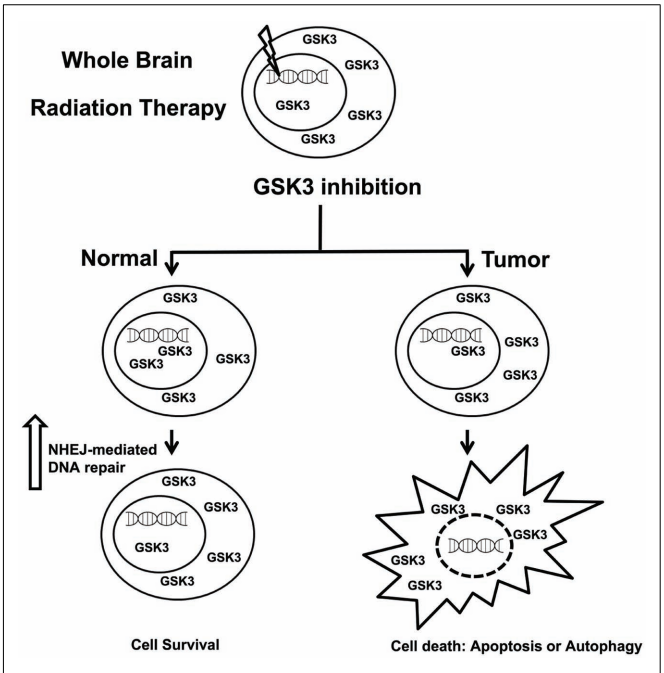

FIGURE 3 | Inhibitors of GSK3 can selectively protect normal tissues from radiation induced toxicities.

While apoptosis is one consequence of this type of treatment, radiation induced DNA damage is certainly another potential trigger leading to neuronal death and subsequent neurocognitive decline. There are multiple types of DNA damage that can occur in a given cell, the most lethal and critical being the double-strand break (DSB; Rich et al., 2000). There are two major DSB repair pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ; van Gent et al., 2001). Cells with radiation-induced DSBs utilize the NHEJ pathway predominantly. During NHEJ-mediated repair, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) undergoes autophosphorylation upon exposure to ionizing radiation. Thus, another mechanism of neuroprotection by GSK3 inhibition may involve augmenting DNA repair via one of these major repair pathways to attenuate DNA damage.

Supporting this notion, GSK3 inhibition using LiCl selectively augments DSB repair following IR in normal tissues but not cancer (Yang et al., 2009). Moreover, the accelerated repair of DSB in normal tissues but not tumor involves the NHEJ-mediated repair pathway. These findings support the rationale for the use of lithium as a neuroprotector for normal cells exposed to ionizing radiation.

Since LiCl has multiple targets, neuroprotection via specific inhibition of GSK3β was subsequently investigated. Thotala et al. (2008) used specific GSK3β inhibitors or a kinase-inactive GSK3β to show that GSK3β is indeed required for apoptosis and neurocognitive decline in hippocampal neurons as a result of radiation treatment. Additionally, it has been shown that GSK3 inhibition using these selective inhibitors augment DSB repair following IR in normal tissues but not cancer (Yang et al., 2011). Moreover, the accelerated repair of DSB in normal tissues but not tumor involves the NHEJ-mediated repair pathway. These results were validated using genetic models as well and support the rationale for the use of GSK3 inhibitors as neuroprotectors for normal cells exposed to ionizing radiation.

The role of GSK3 in genomic instability is only recently gaining attention. It follows that since LiCl and other inhibitors of GSK3 exhibits neuroprotective effects manifesting as a decrease in apoptosis resulting from radiation treatment, and DNA damage is another known characteristic of radiation exposure, that GSK3 could potentially modulate DNA damage or repair. It should be noted that lithium has its drawbacks as a neuroprotector. Besides being non-specific, lithium requires a long 7-day prophylaxis, has a narrow therapeutic window, and lacks specificity. Thus, it is not feasible for the majority of brain tumors which require immediate treatment. This necessitates efforts to discover novel GSK3 inhibitors that have greater therapeutic potential. Nevertheless, several clinical trials have been initiated to test the efficacy of lithium in neuroprotection during the treatment of brain tumors (**Table 2**). It should be noted that the trials are still in their early stage. It remains to be seen if, indeed, lithium can improve the lives of cancer patients.

Table 2 | Lithium in clinical trials as a potential neuroprotector.

Institution	Phase	Trial	Study population	Treatment
New York University School of Medicine	II	NCT01105702	Newly diagnosed high grade glioma (WHO Grade III and IV)	Temodar (Temozolomide), Bevacizumab, lithium, and radiation for high grade glioma
Vanderbilt-Ingram Cancer Center	I	NCT00469937	–Histopathologically confirmed extracranial primary malignancy –Multiple (i.e., >3) brain metastases OR < 3 metastases with at least 1 metastasis >4.0 cm in diameter –Not eligible for radiosurgery –No requirement for immediate whole-brain radiotherapy –No metastases to the midbrain or brainstem	Lithium during whole brain radiotherapy for patients with brain metastases

Information from www.Clinicaltrials.gov accessed on August 1, 2011.
∗Italicized = completed trials.

It is perplexing that inhibition of GSK3 selectively upregulates DNA repair in normal cells. One explanation may be that GSK3 is already maximally inhibited in the majority of cancer. It is also intriguing to postulate that, perhaps, the status of p53 determines cellular response to GSK3 inhibition. Perhaps, there are different roles of GSK3 in p53 wild-type vs. mutated tumors and the p53-independent component also mediates repair that is only apparent when p53 is mutated as is the case in the majority of cancer. Further research is warranted in this avenue.

The mechanisms by which GSK3 is involved in DNA repair are still forthcoming. Since GSK3 is implicated in several of the pathways that contribute to successful DNA repair, it is likely GSK3 could act as a direct regulator. One method could involve GSK3 interaction with key repair proteins. Our lab is currently exploring this and other avenues to determine the full extent by which GSK3 is involved DNA damage/repair and how this relates to GSK3-induced neuroprotection. This has the potential to improve the quality of life of cancer patients, especially in the pediatric population.

SUMMARY AND CONCLUSION

Glycogen synthase kinase 3 is proving to be an even more complex regulator of cellular function than was previously thought. Its role in programmed cellular death pathways including apoptosis and autophagy as well as its effects on DNA damage/repair and subsequent neuroprotection provide rationale for novel and specific methods of targeting GSK3, especially for brain tumors. This would be the ideal therapeutic strategy which would maximize the therapeutic index by promoting tumor cell kill while protecting normal tissues. Nevertheless, the utility of GSK3 as a therapeutic target must be approached with caution. Firstly, one must verify the direction of GSK3 dysregulation as the impetus for tumor development to ensure that benefit can be realized with GSK3 inhibition. Second, due to the effects of GSK3 on synaptic plasticity in the adult brain, the use of GSK3 inhibitors for cancer therapy must be approached with caution. Third, effects on both peripheral tissue and tumor from using GSK3 inhibitor as neuroprotector need to be teased out. Expression profile and subcellular localization of GSK3 in different cancers may provide a lead in this aspect. Furthermore, since GSK3 has multiple critical roles in cellular metabolism, inhibition of its activity may have unwanted side effects. Further thorough evaluation is necessitated in this field before GSK3 can be embraced as a target for cancer therapy.

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GSK-3 inhibitors: preclinical and clinical focus on CNS

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Inhibiting glycogen synthase kinase-3 (GSK-3) activity via pharmacological intervention has become an important strategy for treating neurodegenerative and psychiatric disorders. The known GSK-3 inhibitors are of diverse chemotypes and mechanisms of action and include compounds isolated from natural sources, cations, synthetic small-molecule ATP-competitive inhibitors, non-ATP-competitive inhibitors, and substrate–competitive inhibitors. Here we describe the variety of GSK-3 inhibitors with a specific emphasis on their biological activities in neurons and neurological disorders. We further highlight our current progress in the development of non-ATP-competitive inhibitors of GSK-3. The available data raise the hope that one or more of these drug design approaches will prove successful at stabilizing or even reversing the aberrant neuropathology and cognitive deficits of certain central nervous system disorders.

Keywords: protein kinases, GSK-3, GSK-3 inhibitors, CNS

INTRODUCTION

The serine/threonine kinase GSK-3 is a conserved signaling molecule with essential roles in diverse biological processes. Aberrant GSK-3 activity has been linked with several human diseases including diabetes, inflammation, and neurodegenerative and psychiatric disorders (Eldar-Finkelman, 2002; Doble and Woodgett, 2003; Gould et al., 2004b; Jope et al., 2007; Hernandez and Avila, 2008; Hooper et al., 2008; Hur and Zhou, 2010). This supported the hypothesis that inhibition of GSK-3 will have therapeutic benefit and intensive efforts have been made in the search for and design of selective GSK-3 inhibitors. The reported GSK-3 inhibitors are of diverse chemotypes and mechanisms of action. These include inhibitors isolated from natural sources, cations, and synthetic small molecules. Regarding the mechanism of inhibition, we can find ATP-competitive inhibitors, non-ATP-competitive inhibitors, and substrate–competitive inhibitors. A major challenge in the field is achieving specificity, and advanced structure-based computational studies are conducted to improve GSK-3 inhibitor specificity and possibly to ensure targeting of specific GSK-3 isozymes. Here we review the state of the art of GSK-3 inhibitors with focus on their biological activities in neurons and neurological disorders. We further highlight our current progress in the development of non-ATP-competitive inhibitors of GSK-3 and their implications in CNS disorders.

EVOLUTIONARY, STRUCTURAL, AND REGULATORY FEATURES OF GSK-3-LEADINGS IN DRUG DESIGN

The importance of GSK-3 as a therapeutic target highlighted the need for in depth understanding of different features of GSK-3 with respect to sequence, structure, and regulation. GSK-3 is highly conserved in the animal kingdom. In mammals, GSK-3 is expressed as two isozymes: GSK-3α and GSK-3β. An alternative splice variant of GSK-3β, GSK-3β2, has also been reported (Mukai et al., 2002). GSK-3α and β share extensive similarities in their catalytic domains, but differ in their N- and C-terminal regions (Woodgett, 1990). In lower organisms such as choanoflagellates, sea squirts, and nematodes, a single gene encodes GSK-3 (Alon et al., 2011), whereas in vertebrates such as fish, amphibians, reptiles, and lizards the two genes coding for GSK-3α and β are identified; interestingly birds lack a copy of the GSK-3α gene (Alon et al., 2011). The existence of two GSK-3 isozymes suggested that at least one of the isozymes took on unique functions tied to the emergence of vertebrates, likely related to the development of highly ordered systems such as the central nervous system (CNS). Recent studies had found certain physiological differences between GSK-3 isozymes in functions related to embryonic development, brain structure, and behavior; although other studies clearly demonstrated redundant function for the two isozymes (Hoeflich et al., 2000; Hernandez et al., 2002; Prickaerts et al., 2006; Terwel et al., 2008; Kaidanovich-Beilin et al., 2009; Kim et al., 2009; Mines et al., 2010; Alon et al., 2011; Soutar et al., 2011). Our understanding of the distinct functions of GSK-3 isozymes in neuronal systems and, in particular, their relative contributions to neuropathologies is far from clear. This is of particular importance as we seek to determine the worthiness of development of isozyme-specific inhibitors.

Like other protein kinases, GSK-3 is composed of a conserved catalytic domain folded into a bi-lobal architecture with a smaller N-terminal lobe responsible for ATP binding and a larger, globular C-terminal domain that contains the conserved "activation loop" important for the kinase activity (Hanks and Hunter, 1995; Taylor et al., 1995). Tyrosine residue located within the activation loop is essential for full activation of GSK-3, and this process is a chaperone-dependent auto-phosphorylation event (Hughes et al.,

1993; Cole et al., 2004; Lochhead et al., 2006). GSK-3 activity is further regulated by regions outside the catalytic domain. Its N-terminal end contains a highly conserved RPRTTSF motif that acts as an auto-inhibitory pseudosubstrate when phosphorylated at serine (Frame and Cohen, 2001; Ilouz et al., 2008). Another site located within the C-terminal region of GSK-3β (Thr 390) was recently identified as an inhibitory site (Thornton et al., 2008). In some instances, GSK-3 activity is also regulated via interactions with regulatory proteins. For example, GSK-3 interacts with presenilin proteins that, in turn, may regulate the production of the Alzheimer's amyloid beta peptide (Aβ; Phiel et al., 2003). Interaction of GSK-3 with the scaffold protein Axin regulates the stability of the Wnt signaling effector β-catenin (Ikeda et al., 1998; Wu and Pan, 2011). FRAT/GBP competes with Axin and inhibits GSK-3 activity toward β-catenin (Yost et al., 1998). Axin and FRAT bind to GSK-3 via a similar hydrophobic patch located within the C-terminal region of GSK-3 (Fraser et al., 2002), this interaction has been exploited for inhibitor design (Hedgepeth et al., 1999; Thomas et al., 1999). Finally, the intracellular distribution of GSK-3 isozymes is differentially regulated (Diehl et al., 1998; Bijur and Jope, 2003; Meares and Jope, 2007; Caspi et al., 2008; Adachi et al., 2011; Azoulay-Alfaguter et al., 2011; Wu and Pan, 2011). Hence, GSK-3 function can be regulated at many levels, which in turn, may be exploited in development of selective GSK-3 inhibitors.

Unlike other protein kinases, GSK-3 is constitutively active in resting conditions and is inhibited in response to upstream signals. It can be inhibited or over-activated by diverse post-transductional modifications such as phosphorylation in response to upstream signals (Eldar-Finkelman, 2002). In addition, GSK-3 shows a unique preference in substrate recognition as it requires pre-phosphorylation of its substrates in the context of SXXXS(p) (Woodgett and Cohen, 1984; Fiol et al., 1994). Crystallographic studies of GSK-3β identified a phosphate binding pocket comprised of three basic residues, Arg 96, Lys 205, and Arg 189, that presumably binds the phosphorylated substrate (Dajani et al., 2001; ter Haar et al., 2001). Phosphorylation of GSK-3-downstream targets typically results in attenuation of the signaling pathway and/or inhibition of the substrate's activity. In neurons, GSK-3 is intimately involved with control of apoptosis, synaptic plasticity, axon formation, and neurogenesis (Crowder and Freeman, 2000; Jiang et al., 2005; Yoshimura et al., 2005; Kim et al., 2006; Zhao et al., 2007; Muyllaert et al., 2008; Hur and Zhou, 2010). *In vivo* studies indicate that over-activity of GSK-3 results in adverse effects. This over-activity should be produced by an increase in GSK-3 expression or by an imbalance of its phosphorylation state leading to a super-active enzymatic state. Transgenic animals that overexpress GSK-3 display alterations in brain size, impaired long-term potentiation (LTP), and deficits in learning and memory (Lucas et al., 2001; Hernandez et al., 2002; Spittaels et al., 2002; Hooper et al., 2008). These animals also have characteristics typical of Alzheimer's disease such as hyperphosphorylation of tau and enhanced production of Aβ peptide (Lucas et al., 2001; Phiel et al., 2003; Engel et al., 2006; Rockenstein et al., 2007). In addition, data from pharmacological and genetic models implicate GSK-3 activity in mood behavior and indicate that elevated GSK-3 activity is associated with manic and depressive behavior (Gould et al., 2004a; Kaidanovich-Beilin et al., 2004; O'Brien et al., 2004; Prickaerts et al., 2006; Beaulieu et al., 2008; Mines et al., 2010; Polter et al., 2010). Finally, abnormal regulation of GSK-3 activity was reported in patients with Alzheimer's disease, amyotrophic lateral sclerosis (ALS), major depression, schizophrenia, and bipolar disorder (Kozlovsky et al., 2002; Hu et al., 2003b; Hye et al., 2005; Karege et al., 2007; Lovestone et al., 2007; Pandey et al., 2010; Saus et al., 2010; Forlenza et al., 2011). Hence, increasing efforts are focused on development of selective GSK-3 inhibitors able to modulate this abnormal over-activity.

SMALL METAL CATIONS AS GSK-3 INHIBITORS

The cation lithium was the first "natural" GSK-3 inhibitor discovered (Klein and Melton, 1996; Stambolic et al., 1996). Lithium (meaning lithium salts) is a mood stabilizer long used in treatment of bipolar disorders. Lithium inhibits GSK-3 directly by competition with magnesium ions (Klein and Melton, 1996; Ryves and Harwood, 2001) and indirectly via enhanced serine phosphorylation and autoregulation (De Sarno et al., 2001; Zhang et al., 2003; Kirshennboim et al., 2004). Lithium has been widely used in many studies as a pharmacological inhibitor of GSK-3; these demonstrated that lithium produces similar biological consequences as inhibition of GSK-3 via other means. For example, treatment with lithium increases cellular β catenin levels (Stambolic et al., 1996; O'Brien and Klein, 2009), reduces tau phosphorylation at GSK-3 epitopes in neurons (Noble et al., 2005), activates glycogen synthase (Cheng et al., 1983), and promotes embryonic axis duplication (Klein and Melton, 1996). Lithium has striking morphological effects on neurons including a reduction in axon length, increase in growth cone area, and an increase in synapse formation (Burstein et al., 1985; Takahashi et al., 1999; Owens et al., 2003; Kim and Thayer, 2009). The therapeutic range of lithium is 0.5–1.5 mM, and its IC_{50} toward GSK-3 is 1–2 mM (Klein and Melton, 1996), suggested that lithium may clinically inhibit GSK-3. Indeed, numerous studies have evaluated the therapeutic activity of lithium in various neuronal systems, and verified a profound effect of lithium in neuroprotection against variety of insults in apoptotic and brain injury paradigms (Bijur et al., 2000; Hongisto et al., 2003; Perez et al., 2003; Williams et al., 2004; Jin et al., 2005; Wada et al., 2005; Brewster et al., 2006; Chuang and Manji, 2007; Mathew et al., 2008).

Lithium has been then tested in Alzheimer's and related neurodegenerative models. These studies demonstrated that lithium blocks amyloid precursor protein (APP) deposits and reduces Aβ secretion in cells and transgenic mice overexpressing APP (Sun et al., 2002; Phiel et al., 2003; Rockenstein et al., 2007). Treatment with lithium also prevented Aβ neurotoxicity in rat brain (De Ferrari et al., 2003) and reduced tauopathy in transgenic mice overexpressing human mutant tau (Noble et al., 2005; Caccamo et al., 2007). Lithium was shown to provide therapeutic benefit in models of epileptic neurodegeneration (Busceti et al., 2007), motor performance in Huntington's disease (Wood and Morton, 2003), and hippocampal neuropathology and neurological functions in spinocerebellar ataxia type 1 (SCA1; Watase et al., 2007). However, some studies reported that lithium had no effect on tau phosphorylation, Aβ loads, and neuroprotection (Ghribi et al., 2003; Song et al., 2004; Caccamo et al.,

2007). These could be due to differences in the experimental sets (e.g., age, dose, time of treatment etc.) used in the different studies. Several clinical trials with lithium in AD and elderly patients have been conducted but results are not conclusive. Chronic treatment with lithium yielded positive results in dementia patients (Havens and Cole, 1982) and improved cognition and memory scores (MMSE) in patients receiving the drug as compared to non-treated patients (Terao et al., 2006). In AD patients, lithium reversed the reduction in brain-derived neurotrophic factor (BDNF) serum concentrations (Leyhe et al., 2009) and reduced the prevalence of AD in elderly patients with bipolar disorder (Nunes et al., 2007). A different clinical study found that treatment with lithium slowed the progression in ALS (Bedlack et al., 2008) via autophagy-induced degradation of aggregate-prone proteins (Bedlack et al., 2008). Other clinical trials, however, did not confirm the ability of lithium to prevent dementia or improve cognition or to reduce tau phosphorylation or Aβ levels in AD patients (Pachet and Wisniewski, 2003; Dunn et al., 2005; Hampel et al., 2009). In addition, lithium had toxic side effects in some elderly patients (Macdonald et al., 2008), and studies with lithium were thus discontinued (Tariot and Aisen, 2009).

Other metal ions such as beryllium, zinc, mercury, and copper are potent GSK-3 inhibitors (Ilouz et al., 2002; Ryves et al., 2002). Interestingly, these cations are more potent inhibitors of GSK-3 than lithium (IC_{50} in the micromolar concentration range as compared to the IC_{50} of lithium which is within the millimolar concentration range). Of particular interest is the trace element zinc, that unlike lithium, and other metal ions is naturally found in the body tissues. Zinc inhibits GSK-3 in the low micromolar range ($IC_{50} = 15\,\mu M$) and elevates cellular β-catenin levels (Ilouz et al., 2002). It is noteworthy that zinc levels are linked with major depression and mental functions. In animal models, zinc deficiency results in increased depressive- and anxiety-like behaviors (Kroczka et al., 2001; Tassabehji et al., 2008), and treatment with zinc produces anti-depressive like activity in the mouse forced swimming test (FST) model (Kroczka et al., 2001). Hypozincemia is often detected in patients with major depression, and dietary supplementation of zinc improves symptoms of depression (Bodnar and Wisner, 2005; Nowak et al., 2005). Hence, it is tempting to speculate that the therapeutic activity of zinc in mood behavior and other cognitive symptoms is mediated by its ability to inhibit GSK-3. However, zinc is a co-factor of many enzymes and, like lithium, may initiate many cellular effects independently of GSK-3. Still, research with lithium and zinc may further our understanding of the biological functions of GSK-3 in man. Worth mentioning is also the indirect GSK-3 inhibition produced by the inorganic salt sodium tungstate (Gómez-Ramos et al., 2006). As a consequence of the GSK-3 inactivation, the phosphorylation of several GSK-3 dependent sites of the microtubule tau protein decreases (Gómez-Ramos et al., 2006). This fact points to a new potential drug for treatment of AD. Remarkably, this compound has a low toxicity profile and is currently in phase I of clinical trials as an antiobesity agent. Altogether, although mechanisms of action is not fully clear, these cations may serve as a stepping stone for development of new GSK-3 inhibitors that mimic their inhibitory paradigms.

ORGANIC MOLECULES AS GSK-3 INHIBITORS

Much effort is done in the discovery and development of GSK-3 inhibitors in the last years being a very active field of research for academic centers and pharmaceutical companies. Nowadays, several chemical families have emerged as GSK-3 inhibitors, including great chemical diversity. Some of these GSK-3 inhibitors have synthetic origin but others have been derived directly or indirectly from small molecules of natural origin. Worth mentioning is the fact that the marine environment has been shown recently to provide a source of chemical structures with promising biological activities for CNS diseases. In fact, marine invertebrates have played a prominent role in the generation of novel GSK-3 inhibitors.

The number of small molecule GSK-3 inhibitors is continuously increasing with most in the early discovery phase. Here, we mainly focus on the GSK-3 inhibitors that have been tested in biological systems. Collectively, these studies provided compelling evidence of the specific roles of GSK-3 in neuronal functions under both normal and pathological conditions. Generally speaking, inhibition of GSK-3 has profound effects on neuroprotection, self renewal and pluripotency in embryonic stem (ES) cells, axonal morphogenesis, and mood behavior. As kinase selectivity is one of the main hazards in the development of this class of therapeutic agents, we will discuss GSK-3 inhibitors based on their ability to compete or not with ATP.

GSK-3 ATP-COMPETITIVE INHIBITORS FROM NATURAL RESOURCES

Many of this kind of GSK-3 inhibitors isolated from marine organisms were identified during the search for inhibitors for cyclin-dependent protein kinases (CDKs) with anti-tumor activity. The dual activity of these inhibitors (and others) toward GSK-3 and CDKs is a direct result of their structural similarity within the ATP-binding domain (about 86% sequence similarity).

The bis-indole indirubin isolated from the traditional Chinese medicine for treatment of myelocyte leukemia was initially characterized as a CDK inhibitor and then found to be a potent GSK-3 inhibitor (Hoessel et al., 1999; Leclerc et al., 2001). It inhibits both protein kinases within the nanomolar concentration range. The indirubin analogs that were synthesized and tested (collectively termed here "indirubins") showed inhibitory activity toward both GSK-3 and CDKs (Leclerc et al., 2001; Meijer et al., 2003). The indirubin analog 6-bromoindirubin, isolated from a marine invertebrate, the mollusk known as "Tyrian purple," showed a certain selectivity toward GSK-3 over CDKs (Meijer et al., 2003). Accordingly, a synthetic cell-permeable derivative, 6-bromoindirubin-3′-oxime (6BIO; **Figure 1**), was developed and was about 16-fold more selective for GSK-3 relative to CDKs (Meijer et al., 2003; Polychronopoulos et al., 2004). The biological activity of 6BIO has been evaluated in several neuronal systems. It reduced tau phosphorylation in cultured cortical neurons (Martin et al., 2009), and inhibited neurite outgrowth in cerebellar and embryonic or postnatal dorsal root ganglion (DRG) neurons (Kim et al., 2006; Alabed et al., 2011). This effect appeared to be dependent on the degree of GSK-3 inhibition, as a weak inhibition of GSK-3 promoted axon branching (Kim et al., 2006). 6BIO enhanced self renewal and pluripotency in human ES cells (Sato et al., 2004), via its ability to act as a Wnt mimetic (Sato

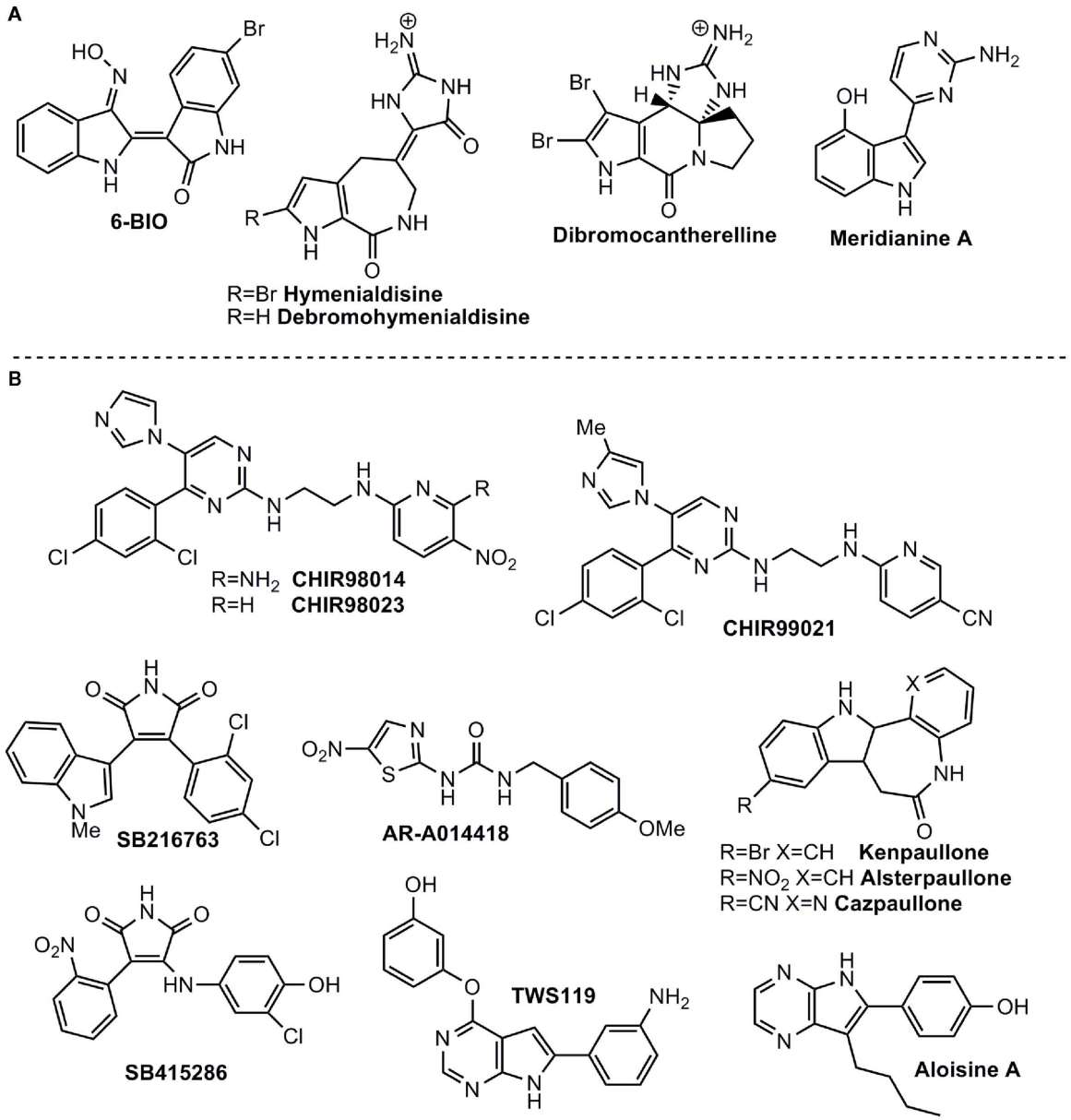

FIGURE 1 | Some ATP-competitive GSK-3 inhibitors with potential for CNS disorders. (A) Isolated from marine organisms. **(B)** Small molecules from organic synthesis programs.

et al., 2004). Additional studies with indirubins demonstrated their ability to provide neuroprotection against kainic acid, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and trophic deprivation (Hongisto et al., 2003; Jin et al., 2005; Wang et al., 2007; Magiatis et al., 2010). *In vivo* systemic administration of indirubin-3′-oxime to APP/Presenilin-1 transgenic mice, a established AD model, attenuated many AD symptoms including tau hyperphosphorylation, Aβ accumulation, inflammation, and spatial memory deficits (Ding et al., 2010). In contrast, treatment with indirubin-3′-oxime did not reduce tau phosphorylation in cultured neurons or in the rat brain (Selenica et al., 2007). These discrepancies could be due to the limited bioavailability of indirubin-3′-oxime, which, like other indirubins, has limited water solubility (Leclerc et al., 2001). Recent work reported the synthesis of additional indirubin derivatives with improved water solubility (Vougogiannopoulou et al., 2008). Their biological activity has not yet been reported.

Sponges (Porifera), as the best known source of bioactive marine natural products in metazoans, play a significant role in marine drug discovery and development. The alkaloids debromohymenialdisine (DBH) and hymenialdisine (HD), which contain both bromopyrrole and guanidine groups (Williams and Faulkner, 1996; **Figure 1**) were originally isolated from sponges belonging to the *Agelasidae, Axinellidae*, and *Halichondridae.* HD structure was established using X-ray crystallography (Cimino et al., 1982)

and it is a potent protein kinase inhibitor targeting GSK-3β, CDKs, MEK1, CK1, and Chk1 (Tasdemir et al., 2002; Sharma and Tepe, 2004) among others. In addition, HD has the ability to inhibit GSK-3β *in vivo* and also blocks the *in vivo* phosphorylation of the microtubule-binding protein tau at sites which are hyperphosphorylated by GSK-3 in AD (Meijer et al., 2000). As observed with other GSK-3 inhibitors, HD, and derivatives act in competition with ATP (Wan et al., 2004). Recently, it has been shown that HD and DBH are stored in spherulous cells from *Axinella* sp. (Song et al., 2011), which can help to define the bioactive production strategy in terms of sponge aquaculture.

The potent activity of HD as a competitive kinase inhibitor aroused interest in synthesizing a pyrrole-azepin-8-one ring system bonded to a glycocyamidine ring scaffold (Nguyen and Tepe, 2009). Crystallographic data of the HD complex with CDK2, led to the rational development of new analogs with increased potency and selectivity over GSK-3, CDK5, and CDK1. Moreover, using different docking methods and molecular dynamics simulation, the structural determinants that govern target selectivity on HD derivatives has been studied, explaining the significant inhibitory effect on GSK-3 of the related HD metabolite dibromocantharelline (Zhang et al., 2011). The specific residue Cys199 near the binding site of GSK-3 provides new clues for the design of potent and selective inhibitors.

Meridianins are brominated 3-(2-aminopyrimidine)-indoles, which are isolated from the tunicate *Aplidium meridianum,* an ascidian collected near the South Georgia Islands. These compounds inhibit various protein kinases such as CDKs, GSK-3, PKA, and CK1 (Gompel et al., 2004; **Figure 1**). The ability of Meridianins to prevent cell proliferation and to induce apoptosis, demonstrated their ability to enter cells and to interfere with the activity of kinases important for cell division and cell death (Gompel et al., 2004). Different medicinal chemistry approaches were employed to prepare more selective GSK-3 inhibitors (Akue-Gedu et al., 2009). However, in all cases, they were most potent toward CDKs, and inhibition of GSK-3 was marginal (Echalier et al., 2008).

SYNTHETIC, ATP-COMPETITIVE GSK-3 INHIBITORS

Among the first synthetic small molecule GSK-3 inhibitors reported were the purine analogs, the aminopyrimidines, developed by Chiron. The potent inhibitors CHIR98014 (CT98014), CHIR98023 (CT98023), CHIR99021 (CT99021) (collectively termed here the CHIRs) inhibit GSK-3 within the nanomolar concentration range (Ring et al., 2003; **Figure 1**). Systemic analysis that profiled protein kinase inhibitors also confirmed the high selectivity of CHIRs toward GSK-3 (Bain et al., 2003, 2007). A limited number of studies have tested CHIRs in neuronal systems (these compounds had been chiefly tested in diabetic models). They showed that CHIRs potently reduced tau phosphorylation in cultured neurons and the rat brain (Selenica et al., 2007). In addition, treatment with CHIRs inhibited neurite outgrowth in cerebellar and DRG neurons (Alabed et al., 2011) and blocked NMDA-mediated long-term depression (LTD) in hippocampus slices (Peineau et al., 2009), indicating an unexpected role of GSK-3 in LTD maintenance (Peineau et al., 2009). In agreement with the 6BIO studies, CHIRs enhanced self renewal and pluripotency in mouse ES cells mimicking the activation of Wnt signaling pathway (Ying et al., 2008; Li et al., 2011). Finally, CHIRs reduced neuronal death in a cerebral ischemia rat model (Kelly et al., 2004), and enhanced the levels of the survival motor neuron protein (SMN) in spinal muscular atrophy (SMA; Makhortova et al., 2011).

The arylindolemaleimides SB-216763 and SB-415286 are highly selective GSK-3 inhibitors developed by GlaxoSmithKline that inhibit GSK-3 within the low nanomolar concentration range (collectively termed here SBs; Coghlan et al., 2000; **Figure 1**). A Number of studies demonstrated the neuroprotective effects of SBs against variety of pro-apoptotic conditions including inhibition of the PI3 kinase/Akt survival pathway, trophic deprivation, Aβ toxicity, heat shock, ethanol, NMDA excitotoxicity, and polyglutamine toxicity caused by the Huntington's disease protein (Bijur et al., 2000; Cross et al., 2001; Culbert et al., 2001; Carmichael et al., 2002; Facci et al., 2003; Hongisto et al., 2003, 2008; Takadera and Ohyashiki, 2004; Hu et al., 2009). In addition, SB-216763 was shown to inhibit axon growth in postnatal and embryonic DRG neurons (Owens et al., 2003; Alabed et al., 2011). On the other hand, different studies reported that SB-216763 induced the formation of multiple long axons in hippocampal, cerebellar granular (CG), and DRG neurons (Padilla et al., 1997; Jiang et al., 2005; Yoshimura et al., 2005; Seira et al., 2011). Furthermore, it improved axon regeneration in lesioned neurons (Seira et al., 2011). Apparently it appeared that axon fate is dependent on the degree of inhibition of GSK-3, namely, strong inhibition of GSK-3 with high concentration of inhibitor inhibited axon growth, while weak inhibition promoted axon branching (Kim et al., 2006). An alternative explanation for these discrepancies relies on different culture length (Jiang et al., 2005). In any event, it has been clearly demonstrated that GSK-3 regulates neurite polarity and neurite outgrowth. The therapeutic activity of SBs has been further tested in several *in vivo* models. SB-216763 reduced ischemic cerebral damage in mice subjected to middle cerebral artery occlusion (Valerio et al., 2011), and enhanced locomotor recovery after spinal cord injury (Padilla et al., 1997). Like CHIRs, treatment with SBs inhibited NMDA-induced LTD (Peineau et al., 2009). In an AD model of mice injected with Aβ peptide, SB-216763 reduced Aβ neurotoxic effects including reduction in tau phosphorylation, caspase-3, and the activity of the stress activated kinase JNK (c-Jun N-terminal kinase; Hu et al., 2009). Administration of SB-216763 to disrupted-in-schizophrenia-1 (DISC1) knockdown mice ameliorated schizophrenic symptoms such as depressive behavior and hyper-locomotion (Mao et al., 2009). In the postnatal rat model, administration of SB-216763 reduced tau phosphorylation in the hippocampus (Selenica et al., 2007). In one of these studies, however, SB-216763 produced neurodegenerative-like effects and behavior deficits in healthy mice (Hu et al., 2009). This demonstrates that over-inhibition of GSK-3 may result in conditions that prevent neurons from operating normally. Thus, GSK-3 inhibitors should be used preferentially in pathologies associated with elevated GSK-3 activity. Finally, additional maleimide derivatives were recently identified by phenotypic screen for defects in zebrafish embryogenesis (Zhang et al., 2011). These compounds were potent GSK-3 inhibitors (Zhang et al., 2011), but their biological activity remains to be tested in relevant systems.

The amino thiazole AR-A014418 developed by AstraZeneca was shown to be a selective inhibitor toward GSK-3 when compared to other protein kinases including CDKs (Bhat et al., 2003; **Figure 1**). In their initial study, Bhat et al. (2003) showed that AR-A014418 was neuroprotective in apoptotic conditions induced by inhibition of PI3 kinase and prevented neurodegeneration in hippocampal slices exposed to Aβ peptide. Consistent with studies using SBs and 6BIO (Owens et al., 2003; Kim et al., 2006; Alabed et al., 2011), treatment with AR-A014418 prevented axon elongation in hippocampal neuronal culture (Shi et al., 2004). AR-A014418 has also been examined in behavior models. It was shown to produce an anti-depressive like behavior in the mouse FST (Gould et al., 2004a; Silva et al., 2008) and, in a manic behavior model of amphetamine-induced hyperactivity, it reduced manic activity (Kalinichev and Dawson, 2011). These results suggest that inhibition of GSK-3 may be beneficial in both depressive and manic episodes. Finally, in JNPL3 transgenic mice overexpressing mutant human tau, AR-A014418 reduced levels of aggregated insoluble tau in the brainstem (Noble et al., 2005), and, in an ALS transgenic mouse model, AR-A014418 attenuated motor neuron death and improved cognition (Koh et al., 2007). Yet, unexpectedly, AR-A014418 showed no effect on tau phosphorylation in the cortex or hippocampus in the postnatal rat model (Selenica et al., 2007). A structurally closed related compound AZD-1080 entered into clinical trials phase I for AD in 2006, but unfortunately the development has been discontinued.[1]

The group of paullone compounds, in particular kenpaullone and alsterpaullone, are widely used in various experimental settings as GSK-3 inhibitors (**Figure 1**). Paullones are fused tetracyclic compounds that inhibit both GSK-3 and CDKs within the nanomolar concentration range (Schultz et al., 1999; Leost et al., 2000). A structurally similar compound, 1-azakenpaullone, is a more selective GSK-3 inhibitor (Kunick et al., 2004). Its derivative cazpaullone (9-cyano-1-azapaullone; **Figure 1**) has been recently characterized as a selective GSK-3 inhibitor (Stukenbrock et al., 2008). Both alsterpaullone and kenpaullone prevented neuron cell death in response to variety of insults including trophic deprivation, thapsigargin treatment, and mitochondrial stress (Takadera and Ohyashiki, 2004; Mishra et al., 2007; Petit-Paitel et al., 2009; Skardelly et al., 2011). Alsterpaullone was shown to reduce tau phosphorylation in cultured neurons (Leost et al., 2000; Selenica et al., 2007), and to block NMDA-induced LTD in hippocampal slices (Peineau et al., 2008). Kenpaullone decreased Aβ production in cells overexpressing APP (Phiel et al., 2003) and promoted differentiation of precursor cells into dopamine neurons (Castelo-Branco et al., 2004). This last supported the use of GSK-3 inhibition in treatment of Parkinson's disease (Castelo-Branco et al., 2004). Recent work implicated a role for GSK-3 in SMA (Makhortova et al., 2011). Apparently, a search for small molecules that elevate the expression levels of SMN identified GSK-3 inhibitors as potential agents. Treatment with alsterpaullone verified this observation and showed that alsterpaullone slowed down the degradation of SMN in SMA human fibroblasts (Makhortova et al., 2011). Furthermore, the death of motor neurons induced by depletion of SMN was rescued by alsterpaullone (Makhortova et al., 2011). This study is a first indication of a therapeutic potential of inhibition of GSK-3 in SMA (Makhortova et al., 2011). Reports of alsterpaullone or kenpaullone in *in vivo* systems are limited. One study demonstrated the ability of alsterpaullone to reduce tau phosphorylation in the rat brain (Selenica et al., 2007).

Additional compounds were recently described as dual CDK/GSK-3 inhibitors. These include the purine derivatives pyrazolo [3,4-*b*] quinoxalines (Ortega et al., 2002), the pyrazolo[3,4-*b*]pyridine ring system (Chioua et al., 2009), the 9-oxo-thiazolo [5,4-*f*] quinazoline-2-carbonitrile derivatives (Loge et al., 2008), and the thiazolo[5,4-*f*]quinazolin-9-ones (Testard et al., 2006). Aloisines (6-phenyl[5*H*]pyrrolo[2,3-*b*]pyrazines) are a different class of dual CDK/GSK-3 inhibitors that inhibit the two kinases in the sub-micromolar range (Mettey et al., 2003). Aloisine A (**Figure 1**) is the most potent of those analogs tested and showed anti-proliferative effects in differentiated postmitotic neurons (Mettey et al., 2003). A series of bisindolylmaleimides were identified as potent GSK-3 inhibitors and were shown to enhance mouse ES cells self renewal in the presence of the leukemia inhibitory factor (LIF; Bone et al., 2009). Recent work identified TWS119, a 4,6-disubstituted pyrrolopyrimidine (**Figure 1**), from a phenotypic cellular screen for compounds with the ability to induce neuronal differentiation of pluripotent mouse ES cells; the compound proved to be a GSK-3 inhibitor (Ding et al., 2003). This result contrasts with earlier studies that demonstrated maintenance of pluripotency by other GSK-3 inhibitors (Sato et al., 2004; Ying et al., 2008). These differences could be due to differences between mouse and human ES cells, culture conditions, or differences in the developmental stages derivation (Welham et al., 2007). Finally, a novel series of macrocyclic polyoxygenated bis-7-azaindolylmaleimides were shown to be highly selective toward GSK-3 (Kuo et al., 2003; Shen et al., 2004). Their biological activity remains to be further elucidated.

Collectively, studies with ATP-competitive inhibitors verified that GSK-3 is essential for maintenance of normal neuronal activities, but also demonstrated an important role for GSK-3 in the etiological mechanisms of neurodegeneration and psychiatric disorders. Crystallographic data identified specific interactions within the ATP-binding pocket and with additional residues located at the surface of the C-lobe. These include Asp 133, Arg 141 Gln185, Asp200, and Arg220 and the conserved salt bridge of Lys 85/Glu 97 (**Figure 3A**). However, the limited specificity of ATP-competitive inhibitors is a serious drawback as demonstrated in series of studies that profiled many protein kinase inhibitors (Davies et al., 2000; Bain et al., 2003, 2007). It is possible that the high toxicity of this type of drugs prevented entering into the clinical phase or resulted in a failure in clinical treatments. It may be possible to improve specificity by exploiting unique features within the ATP-binding fold of GSK-3, but this strategy requires further validation. As a last caveat, the dual inhibition of GSK-3 and CDKs may be not disadvantageous. CDK5 which is largely restricted to neurons, is essential for regulation of many neuronal functions (Jessberger et al., 2009), and its aberrant activity has been implicated in variety of neurodegenerative conditions (Cruz and Tsai, 2004). CDK5 also serves as the "priming kinase" that phosphorylates prior its phosphorylation by GSK-3 (Li et al., 2006; Plattner

[1] http://www.astrazeneca.com/article/511390.aspx, Accessed on April 29, 2008

et al., 2006). Thus, inhibition of both GSK-3 and CDK5 may be of therapeutic benefit, and the use of such dual activity inhibitors should not be discouraged until further evaluation in preclinical models.

SYNTHETIC, NON-ATP-COMPETITIVE GSK-3 INHIBITORS

As we have highlighted above, there are many compounds with different scaffolds able to inhibit GSK-3 in an ATP-competitive manner; these compounds may have adverse secondary effects if used in chronic treatment. The human kinome has more than 500 protein kinases that share a high degree of homology in the catalytic site, and in particular, within the ATP-binding pocket. Achieving kinase selectivity is one of the main challenges in the search and design of protein kinases inhibitors (Eglen and Reisine, 2009). ATP non-competitive GSK-3 inhibitors are likely to be more selective than those that inhibit ATP binding, since they should bind to unique regions within the kinase providing a more subtle modulation of kinase activity than simply blocking ATP entrance. This point is of utmost importance for GSK-3 modulation as a therapeutic approach, because only the aberrant GSK-3 activity should be inhibited.

There are different chemical families of organic compounds reported in the literature that do not compete with ATP in their GSK-3 inhibition and different binding modes to the enzyme have been described. The first reported family was the small heterocyclic thiadiazolidinones (TDZD) family (Martinez et al., 2002; **Figure 2A**). Although their mechanism of action has not yet been experimentally confirmed, a possible role has been postulated for an interaction with cysteine 199, a key residue located in the active site of GSK-3 (Mazanetz and Fischer, 2007). TDZD did not show inhibition over various kinases including PKA, PKC, CK-2, and CDK1/cyclin B. Treatment of primary culture neuronal cells with TDZD reduced tau phosphorylation, and treatment with diverse TDZDs such as NP00111 (**Figure 2A**), NP031112, NP03115,

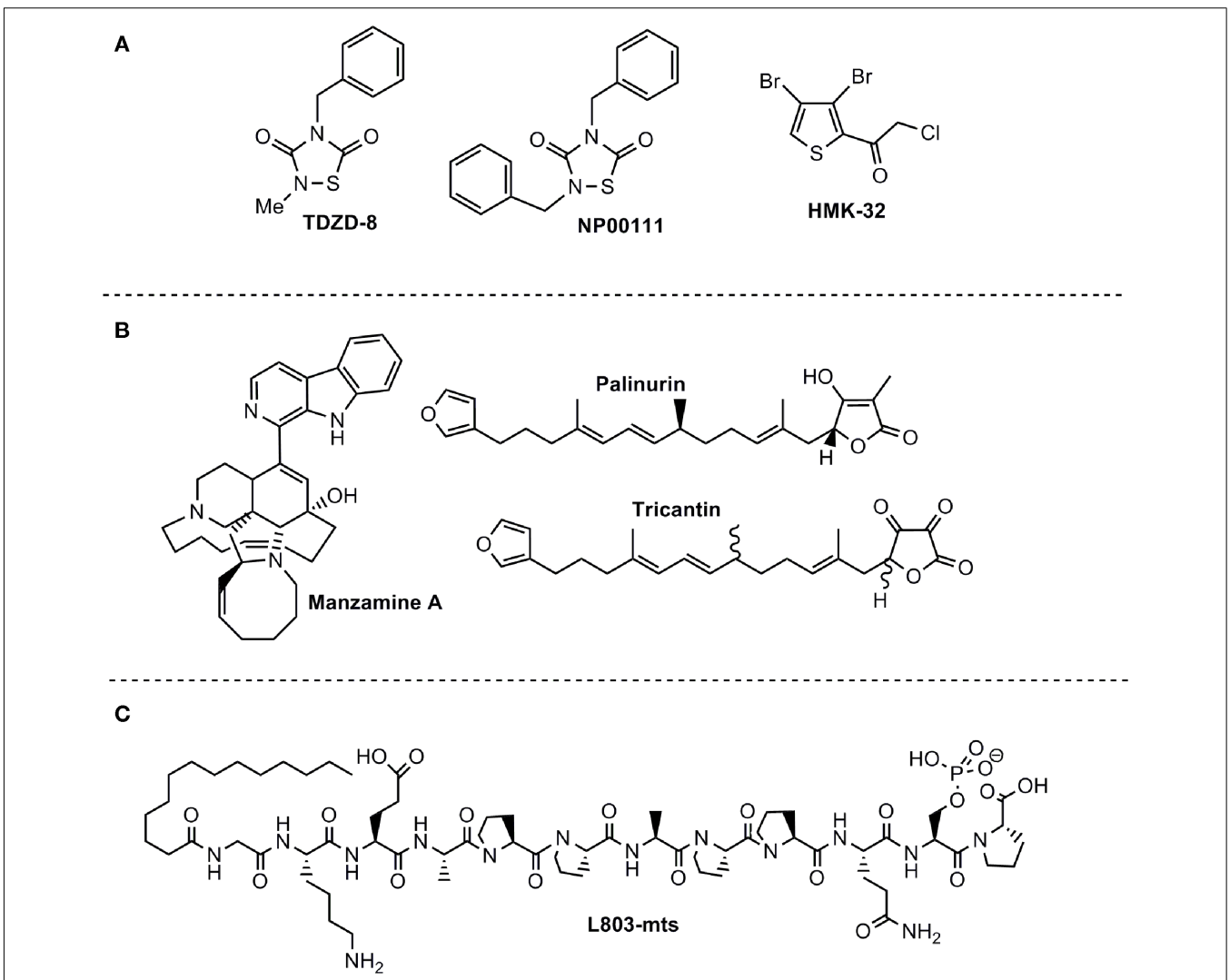

FIGURE 2 | ATP non-competitive GSK-3 inhibitors with potential for CNS disorders. (A) Small molecules from organic synthesis programs. **(B)** Natural compounds isolated from marine organisms. **(C)** Peptide competitive with substrate.

produced neuroprotective and antidepressant activities in several animals models (Luna-Medina et al., 2007; Rosa et al., 2008a,b).

With these others positive data, safety regulatory studies of one TDZD candidate was done with the aim to determinate the human dose. Very relevant for the translation from the bench to the treatment of AD patients are the results obtained in the chronic oral treatment for 3 months performed with TDZD compound named NP-12, to the double transgenic APP × tau rodent model (Ribe et al., 2005). Treatment with NP-12 improved cognitive performance in the Morris water maze test, while all histopathological features related to AD pathology such as beta-amyloid plaque load, tau hyperphosphorylation, gliosis, and neurons death were significantly reduced (Sereno et al., 2009). Of interest is the observation regarding the increase in insulin growth factor-1 (IGF-1) in mice brains of both on wild type and APP × PS1 mice, after an acute oral treatment with NP-12 for 5 days (Bolos et al., 2011). IGF-1 is a potent neurotrophic peptide with therapeutic value for many neurodegenerative diseases including AD pathology (Torres-Aleman, 2007). These increased IGF-1 levels in mice would mean that there is not only a direct action of GSK-3 inhibitors on the brain, but, there is also an effect on some peripheral signaling pathways (as activation of the IGF-1 transporter megalin), that could be modified with this innovative treatment improving the AD pathology.

Much of the work done in the hit-to-lead process, and lead optimization of TDZD heterocyclic family has been reported recently (Martinez et al., 2008). Two molecules have been well characterized. The first is TDZD-8, which is one of the pharmacological tools frequently used to study the biological and pathological roles of GSK-3 in cellular and animal models (Beaulieu et al., 2004; Cuzzocrea et al., 2006; Lipina et al., 2011). The second molecule is NP031112, also termed NP-12 or tideglusib. It is a brain permeable small molecule currently used in clinical trials phase II for AD and progressive supranuclear palsy (PSP).

Data from the phase IIa trial of tideglusib were recently reported and indicated a trend in improved cognitive abilities of the mild to moderate AD patients treated for 24 weeks (del Ser, 2010). The phase IIb trial for AD[2] is ongoing with more than 20 centers involved. FDA and EMEA have approved the orphan drug status for the development of tideglusib in the rare tauopathy PSP[3]. The TAUROS study is ongoing and results are expected to be finalized by the end of 2011.

The second family of compounds known as ATP non-competitive GSK-3 inhibitors is the halomethylketone (HMK) derivatives (Conde et al., 2003) which have been recently described as the first irreversible inhibitors of this enzyme (Perez et al., 2009a; **Figure 2A**). In this case, inactivation of the enzyme is due to the formation of an irreversible covalent sulfur–carbon bond between the key cysteine 199 located at the entrance to the ATP site of GSK-3 and the HMK moiety (Perez et al., 2011).

HMKs are cell-permeable compounds. They are able to decrease tau hyperphosphorylation on primary neurons cell culture after 2 h of treatment (Perez et al., 2009a). They are rather selective in a wide protein kinase panel and its off-target activity were determined on different CNS receptors binding assays without any significant positive data (Perez et al., 2009a). There are a couple of HMKs commercially available from different commercial sources that confirms the importance of this series of compounds as pharmacological tools for the study of GSK-3 physiology and pathology in different cell models (Yasuda et al., 2009).

NON-ATP-COMPETITIVE GSK-3 INHIBITORS FROM NATURAL RESOURCES

Manzamines are complex β-carboline alkaloids isolated from Indo-Pacific sponges and characterized as having an intricate and novel polycyclic system (Hu et al., 2003a). Following a discovery program of GSK-3 inhibitors from marine sources, it was found that manzamine A (**Figure 2B**) inhibits human GSK-3β *in vitro* more than 70% at 25 μM (Rao et al., 2006). In order to identify the pharmacophore responsible for this new enzymatic inhibition, the potential GSK-3 inhibition of carboline and ircinal A, which can be considered the chemical precursors of manzamine A, were tested. Both moieties are inactive in their ability to bind to GSK-3, indicating the entire manzamine molecule is responsible for this activity. To further assess the potential of manzamine A in the treatment of AD, its ability to inhibit several different kinases (GSK-3β, GSK-3α, CDK1, PKA, MAPK, and CDK5) and decrease the hyperphosphorylation of tau protein mediated by GSK-3 in human neuroblastoma cell cultures was investigated (Hamann et al., 2007). Manzamine A specifically inhibits GSK-3β and CDK5 (the two key players in the hyperphosphorylation of tau protein in AD) with IC_{50}s of 10 and 1.5 μM respectively; it is ineffective toward others kinases tested (Hamann et al., 2007). Kinetic studies indicated an ATP non-competitive inhibition regarding GSK-3 (Hamann et al., 2007) while susbstrate competitive inhibition has been recently proved experimentally (Palomo et al., 2011).

Treatment of SH-SY5Y cells in culture with manzamine A at different concentrations (5, 15, and 50 μM) resulted in a decrease in tau phosphorylation at the GSK-3 epitope Ser 396 (Hamann et al., 2007). as quantified by a specific ELISA sandwich methodology. Cell survival was determined in parallel by measuring LDH release. Manzamine A constitutes a promising scaffold from which more potent and selective GSK-3 inhibitors could be designed as potential therapeutic agents for the treatment of diseases mediated by GSK-3 such as the AD (Wahba and Hamann, 2011). Recently a potential binding site of manzamine A with GSK-3 was identified, and this will provide new directions in substrate competitive drug design (Peng et al., 2011).

Very recently extracts and compounds obtained from the marine organism *Ircinia* sp., and, more particularly, the furanoterpenoids isolated from the Mediterranean sponges *Ircinia dendroides, Ircinia variabilis*, and *Ircinia oros,* have been claimed as inhibitors of GSK-3 (Alonso et al., 2005). Fractionation and purification of active components from these extracts, guided by a GSK-3 inhibition assay, resulted in the isolation of furanosesquiterpenoids as new GSK-3 inhibitors with potential use as therapeutic agents. Palinurin and one unknown metabolite called tricantin were mainly isolated (**Figure 2B**). Kinetic analyses of isolated compounds were performed and showed that

[2]http://clinicaltrials.gov/show/NCT01350362

[3]http://clinicaltrials.gov/ct2/show/NCT01049399?term=tideglusib&rank=1

tricantin inhibits recombinant human GSK-3β with an IC_{50} value of 7.5 μM, whereas palinurin exhibited an IC_{50} value of 4.5 μM. They are cell-permeable inhibitors and described as ATP non-competitive GSK-3 inhibitor able to reduce tau phosphorylation in cell cultures (Alonso and Martinez, 2006). Total synthesis of palinurin has been recently described providing a new source for this lead compound (Perez et al., 2009b). Different studies have been performed to determine structure activity relationships among a series of congeneric molecules and the bioactive conformation has been proposed (Ermondi et al., 2011). However, the binding mode to GSK-3 remains unknown.

PEPTIDES AS SUBSTRATE–COMPETITIVE INHIBITORS

Peptides have also been described as potential protein kinase inhibitors (Eldar-Finkelman and Eisenstein, 2009). The use of peptides, which copy natural motifs that specifically influence kinase activity and/or its intracellular interactions with associated partners, may be a promising approach for selective inhibition of protein kinases. Although substrate–competitive inhibitors have often been overlooked due their relative weak inhibition, they provide numerous advantages over the ATP-competitive inhibitors, mainly in selectivity. This is due to the fact that substrate recognition and types of interactions vary considerably among kinases, whereas ATP-binding domains are structurally conserved. In the case of GSK-3, the relatively weak affinities of substrate–competitive inhibitors, and, in general, ATP non-competitive inhibitors, may be advantageous. GSK-3 is essential for many aspects of neuronal function; hence, drastic inhibition may result in deleterious effects (as has been observed with some of GSK-3 inhibitors). Furthermore, pathological GSK-3 over-expression does not exceed two to threefold over normal levels. Thus, moderate-to-weak inhibition of GSK-3 (about 50% inhibition) is actually a desired approach for treatment of disorders associated with elevated activity of GSK-3. Finally because substrate–competitive inhibitors are more selective than ATP-competitive molecules, substrate–competitive inhibitors may be a favorable choice for clinical use.

The specific requirement of GSK-3 for pre-phosphorylated substrates supported the rational for the use of synthetic phosphorylated peptides as substrate–competitive inhibitors (Plotkin et al., 2003). The peptide L803-mts (11 residues) is a cell-permeable phosphorylated peptide derived from the GSK-3 substrate heat shock factor-1 (HSF-1; **Figure 2C**) that is very selective and inhibits cellular activity of GSK-3 within the low micromolar concentration range (Plotkin et al., 2003). L803-mts showed biological activity in diabetic models consisting with the paradigm of GSK-3 acting as a negative regulator of insulin signaling (Kaidanovich-Beilin and Eldar-Finkelman, 2006; Rao et al., 2007). Recent work further demonstrated the therapeutic activity of L803-mts in CNS models. Like other GSK-3 inhibitors, L803-mts promoted axon formation and elongation in hippocampal neurons (Kim et al., 2006). It was also shown to provide neuroprotection effects in neuron cultured cells exposed to 6-hydroxydopamine-induced cell death (Chen et al., 2004). *In vivo* treatment with L803-mts increased β-catenin levels in the mouse hippocampus and produced anti-depressive like activity in the FST (Kaidanovich-Beilin et al., 2004). Administration of L803-mts in a traumatic brain injury (TBI) model reversed depressive behavior in the injured animals (Shapira et al., 2007). Finally, L803-mts conferred low toxicity in neurons as compared with other GSK-3 inhibitors (Kim and Thayer, 2009).

Understanding of the mode of interaction of GSK-3 with its substrates is necessary for effective design and development of substrate–competitive inhibitors. A combined approach of mutagenesis and computational protein–protein docking analyses identified a novel substrate-binding site within the catalytic core of GSK-3β formed by Phe 67, Gln 89, Phe93, and Asn 95 (Ilouz et al., 2006; Licht-Murava et al., 2011), and Asp 181 as an additional binding site for the N-terminal pseudosubstrate (Ilouz et al., 2008; **Figure 3B**). These residues are spatially located near the ATP-binding site and the phosphate binding pocket that interacts with the phospho-serine moiety of the substrate (Dajani et al., 2001; ter Haar et al., 2001; **Figure 3B**). From studies of the binding mode of L803-mts with GSK-3 it was found that the inhibitor- and substrate-binding sites are not identical. Both substrate and L803-mts interact with the phosphate binding pocket, but the substrate interacts with the cavity bordered by Gln 89 and Asn 95, whereas L803-mts mainly interacts with Phe 93 and with a hydrophobic surface located away from the ATP-binding site (Licht-Murava et al., 2011; **Figure 3B**). This clarified our understanding of the different binding modes of substrates and inhibitors. Whereas the substrate requires appropriate alignment with the catalytic domain to allow catalysis, the inhibitor does not require an exact positioning within the catalytic cleft. Contacts other than those used by the substrate may be crucial for converting a substrate to an inhibitor. Based on this idea, new L803-mts variants were synthesized, and some were 3- to 10-fold better inhibitors than L803-mts (Licht-Murava et al., 2011). Taken together, substrate–competitive inhibitors of GSK-3 hold tremendous promise as potential therapeutics. An extensive understanding of molecular recognition of GSK-3 with its substrates and inhibitors should provide the basis for rational design and optimization of efficient and high affinity substrate–competitive inhibitors.

A different example for peptide inhibitors are two peptides derived from Frat (FRATide-39 residues) or Axin (Axin GID-25 residues) that compete with Axin binding to GSK-3, which in turn, leads to activation of Wnt signaling pathway (Hedgepeth et al., 1999; Thomas et al., 1999). Treatment with Axin GID induced multiple axon formation in hippocampal neurons (Jiang et al., 2005), and its exogenous expression in cerebellar granule neurons protected against trophic deprivation-induced cell death (Hongisto et al., 2003). These results could suggest that GSK-3 inhibition-mediated neuroprotection or axon formation involves activation of Wnt signaling and or an increase in Axin non-bound GSK-3 pool (Hongisto et al., 2003). However, from the therapeutic point of view, more relevant are small peptides.

PROSPECTIVE AND CHALLENGES

Cumulative data now suggest a promising future for GSK-3 inhibitors (**Table 1**). However, some concerns had been raised regarding the potential toxicity of these compounds ranging from hypoglycemia to tumorigenesis and neuron deregulation. Furthermore, GSK-3 is essential for life, and there is a concern that its inhibition could prevent cells from operating normally. Nevertheless, it is worth mentioning that GSK-3 activity is elevated

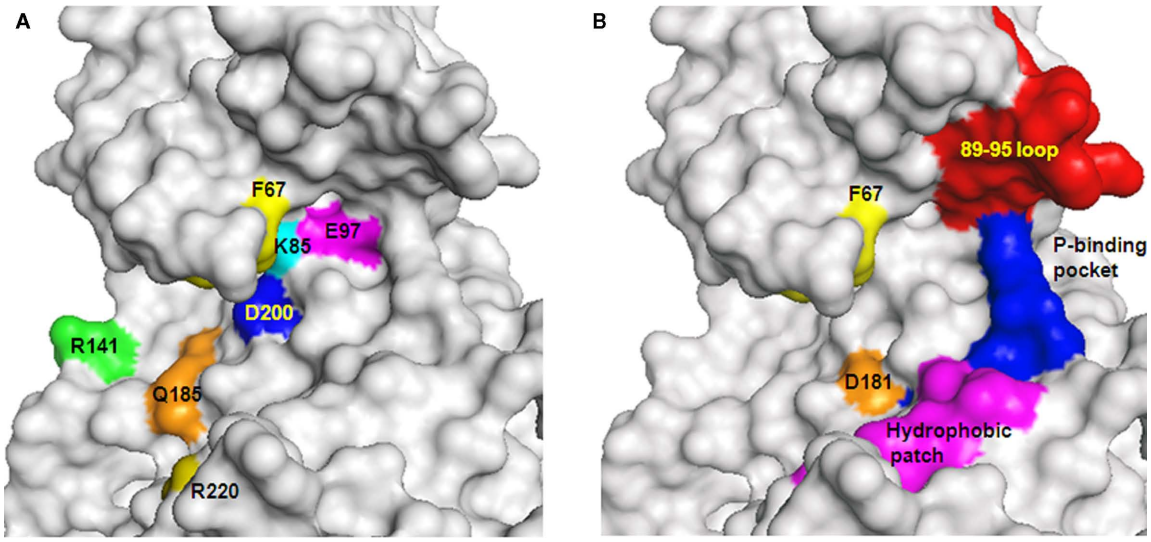

FIGURE 3 | GSK-3-interacting sites with ATP-competitive inhibitors, substrates, and L803-mts. (A) Sites interacting with ATP or ATP-competitive inhibitors are indicated. These interacting sites are located at the interface within the N- and C-lobes of the catalytic domain. F67 (yellow) locates the P-loop interacting with ATP **(B)** Distinct and overlapping element in GSK-3 interaction with substrates and inhibitors. Sites interacting with substrates: F67 (yellow), 89–95 loop (red), phosphate binding pocket (P-binding pocket, blue). D181 (orange) interacts with the pseudosubstrate. Sites interacting with L803-mts: F93 (within the 89–95 loop), hydrophobic patch (V214, I217 Y216 magenta), and the phosphate binding pocket (blue). GSK-3 structure is based on PDB code 1gng and images were processed by PyMol software.

in pathological conditions, thus, a smooth inhibition of GSK-3 able to restore down levels of activity to physiological ones would be enough to produce an important therapeutic effects in unmet diseases, being that point crucial for not produce adverse effects.

Evidently, treatment with GSK-3 inhibitors restored glucose homeostasis and did not provoke hypoglycemia or hyperinuslinemia in diabetic models. Activation of the proto-oncogenic molecule β-catenin by inhibition of GSK-3 is another major concern claiming that long-term inhibition of GSK-3 may promote cancer. However, no direct *in vivo* evidence has indicated tumorigenesis upon administration of GSK-3 inhibitors. On the contrary, in certain cancers GSK-3 inhibitors reduced cell proliferation and enhanced cell death upon irradiation treatment. Compelling evidence in this regard is the fact that treatment with the drug lithium which has been used as standard therapeutic for the treatment of bipolar disorder since the 1950s is not associated with increased levels of tumorigenesis or deaths from cancer. Certainly the degree of GSK-3 inhibition is a crucial element affecting toxicity, and a weak to moderate inhibition of GSK-3 is an optimal therapeutic approach.

GSK-3 inhibitors that are non-ATP-competitive provide important benefits in therapeutic use for several reasons. First of all because, better kinase selectivity may be expected from inhibitors that bind outside the ATP pocket, and secondly, because, this kind of kinase inhibitors should have lower values of IC_{50}, which in the case of GSK-3 is not only beneficial but also necessary to avoid toxicity. Thus, non-ATP-competitive GSK-3 inhibitors, which comprise covalent inhibitors, substrate–competitive inhibitors and allosteric modulators, arise as the unique real potential drugs for the treatment of at least chronic diseases as AD. An interesting question in this regard is the feasibility in the design of selective inhibitors toward GSK-3α or GSK-3β. The fact that the catalytic domains of the two isozymes share more than 90% homology suggests that inhibitors targeting this domain may not discriminate between the two isozymes (as indeed is deduced in the *in vitro* kinase assays). A different strategic approach exploiting unique properties of GSK-3 isozymes such as protein–protein interactions, distinct cellular localization etc. should be used in development of such inhibitors. Hence, a better understanding of the distinct structure–function properties of GSK-3 isozymes is required for future design of isozymes-selective inhibitors.

Another important challenge to overcome for a GSK-3 inhibitor to be converted in an effective drug for AD treatment is its specific brain distribution. The drug needs to cross the blood brain barrier to exert its action in the regulation of exacerbated GSK-3 brain levels. Usually this is not an easy task for organic compounds and/or peptides, moreover when oral bioavailability is the preferred administration route for chronic AD treatment. It is very difficult to balance the equilibrium between molecular lipophilicity to enter into the brain and molecular hydrophilicity to be orally administrated. That reason has ruled out several promising GSK-3 inhibitors from the race to the market. Determination of potential brain penetration should be incorporated in the first stages of GSK-3 inhibitors development.

Finally, our knowledge regarding human clinical side effects of GSK-3 inhibitors is rather scarce since a limited number of compounds have reached the clinical phase. Moreover, these compounds are of distinct chemical structures; and thus differ in their bioclinical and pharmacological properties (absorption, distribution, metabolism, etc.). It is thus difficult to determine at this point what adverse events will be commonly associated with inhibition of GSK-3. Lithium is the only GSK-3 inhibitor that has been in

Table 1 | GSK-3 inhibitors.

Type	Source	Inhibitor	Inhibition potency (IC_{50})	Biological activity in CNS	Drug development state
Cations	Inorganic atom	Lithium	2 mM	Mood stabilizer, neuroprotection, therapeutic benefits (tau and amyloid) in AD models (1-9).	Drug of reference for bipolar disorders. Conflicting results in the clinic for AD.
		Zinc	15 μM	Antidepressant (10, 11).	----
		Tungstate	Indirect inhibition	Decrease tau phosphorylation (12)	Phase I as antiobesity agent
ATP-competitives	Marine organism	**Indirubins** 6-BIO	5-50 nM 1.5 μM	Neuroprotection, axon formation, maintain pluripotency, reduced tau phosphorylation (13-16).	Pre-clinical
		Hymenialdisine Dibromocantharelline	10 nM 3 μM	Decrease tau phosphorylation, neuroprotection (17).	Pre-clinical
		Meridianins		Decrease tau phosphorylation (18).	-----
	Organic synthesis	**Aminopyrimidines** CT98014 CT98023 CT99021 TWS119	0.6-7 nM	Block LTD, maintain pluripotency, enhance neurite outgrowth, supports cell renewal of ESC, reduce tau phosphorylation (5, 19-22). Induces neuronal differentiation. Support self renewal of ESC (23).	----- -----
		Arylindolemaleimide SB-216763 SB-41528	34 nM 77 nM	Neuroprotection, induce axon formation, shows therapeutic benefits in AD and schizophrenic models(5, 9, 24-32).	Pre-clinical
		Thiazoles AR-A014418 AZD-1080	104 nM n.r.	Neuroprotection, shows antidepressive and anti-manic activity, shows therapeutic benefits in AD and ALS model (33-39).	----- Withdraw from clinical phase I
		Paullones Kenpaullone Alsterpaullone Cazpaullone	4-80 nM	Neuroprotection, blocks LTD, reduces Aβ production, reduces tau phosphorylation (19, 26, 40-45)	Pre-clinical
		Aloisines	0.5-1.5 μM	Blocks cell proliferation (46).	-----
Type	**Source**	**Inhibitor**	**Inhibition potency (IC_{50})**	**Biological activity in CNS**	**Drug development state**
Non-ATP competitives	Marine organism	**Manzamines** Manzamine A	1.5 μM	Decrease tau phosphorylation (47).	-----
		Furanosesquiterpenes Palinurine Tricantine	4.5 μM 7.5 μM	Decrease tau phosphorylation	-----
	Organic synthesis	**Thiadiazolidindiones** TDZD-8 NP00111 NP031115 NP031112(tideglusib)	2 μM 2 μM 4 μM n.r.	Neuroprotection, decrease tau phosphorylation, beta-amyloid and gliosis in vivo, antidepressants, affects locomotor activity and reverse schizophrenic like behavior, promotes tissue recovery after spinal cord injury (48-55).	----- ----- ----- Phase II as orphan drug for PSP Phase IIb for AD
		Halomethylketones HMK-32	1.5 μM	Neuroprotection, decrease tau phosphorylation (56).	-----
	Peptides	L803-mts	40 μM	Neuroprotection, antidepressive activity, axon morphogenesis (14, 57-59).	Pre-clinical

Inhibitors are sorted by mode of action (ATP competitive vs. non-ATP competitive) and source (natural vs. synthetic).

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clinical use for a significant time. However, lithium lacks target specificity, and its adverse side effects and high toxicity do not necessarily reflect events associated with inhibition of GSK-3 *per se*. AZD-1080 (AstraZeneca) and NP-12/Tideglusib (Noscria) reached the clinic in 2006. AZD-1080 was abandoned as a drug candidate due to nephrotoxicity observed in phase I clinical trials. NP-12 is currently in phase IIb trials for Alzheimer's disease and paralysis supranuclear palsy, and no side effects/off targets effects have been described at this time. Their distinct chemical structures and/or different inhibition mode are most likely responsible for the different clinical impacts observed with these two compounds. Results from TAURUS and ARGO studies will reveal the safety and efficacy of Tideglusib in humans. Meanwhile, an increasing number of GSK-3 inhibitors are being tested in preclinical models, and it is anticipated that some will enter clinical trials.

CONCLUSION

As GSK-3 plays an important role in AD and some others unmet diseases, more of them related to CNS, many inhibitors have been discovered in the last years. Some of them have proven to be effective in specific cellular and animal models of different CNS pathologies. However, due to different hazards previously considered when GSK-3 is targeted, some risks should be avoid in a GSK-3 inhibitor development. High values for IC_{50} and ATP-competition when the compound binds to the enzyme, should not be present in a GSK-3 inhibitor if we want that the molecule become an effective drug. A mild inhibition of GSK-3 is indispensable to treat pathological states because it will be able to decrease the exacerbated GSK-3 function in the tissue affected by the disease but the simultaneous decrease of activity in other healthy tissues, will be compensate by alternative cellular mechanisms present in the human being.

ATP non-competitive GSK-3 inhibitors such allosteric modulators, substrate competitive or covalent inhibitors are emerging as an alternative and promising approach for a safer use in the clinic.

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