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Research Article **Neuroscience**

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Glycogen synthase kinase-3 is essential for β-arrestin-2 complex formation and lithium-sensitive behaviors in mice

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Lithium is the first-line therapy for bipolar disorder. However, its therapeutic target remains controversial. Candidates include inositol monophosphatases, glycogen synthase kinase-3 (GSK-3), and a β-arrestin-2/AKT/protein phosphatase 2A (β-arrestin-2/AKT/PP2A) complex that is known to be required for lithium-sensitive behaviors. Defining the direct target(s) is critical for the development of new therapies and for elucidating the molecular pathogenesis of this major psychiatric disorder. Here, we show what we believe to be a new link between GSK-3 and the β-arrestin-2 complex in mice and propose an integrated mechanism that accounts for the effects of lithium on multiple behaviors. GSK-3β (*Gsk3b*) overexpression reversed behavioral defects observed in lithium-treated mice and similar behaviors observed in *Gsk3b*$^{+/-}$ mice. Furthermore, immunoprecipitation of striatial tissue from WT mice revealed that lithium disrupted the β-arrestin-2/Akt/PP2A complex by directly inhibiting GSK-3. GSK-3 inhibitors or loss of one copy of the *Gsk3b* gene reduced β-arrestin-2/Akt/PP2A complex formation in mice, while overexpression of *Gsk3b* restored complex formation in lithium-treated mice. Thus, GSK-3 regulates the stability of the β-arrestin-2/Akt/PP2A complex, and lithium disrupts the complex through direct inhibition of GSK-3. We believe these findings reveal a new role for GSK-3 within the β-arrestin complex and demonstrate that GSK-3 is a critical target of lithium in mammalian behaviors.

Introduction

Bipolar disorder (BPD) is a potentially devastating affective disorder affecting 1%–2% of the population worldwide (1). Lithium has remained the first line of therapy for decades (2–4), and yet the molecular target of lithium in BPD remains highly controversial (5). The leading candidates under active investigation as potential therapeutic targets of lithium include inositol monophosphatases and related phosphomonoesterases (6–11), glycogen synthase kinase-3 (GSK-3) (12), and the scaffolding function of β-arrestin-2 (13).

GSK-3 has emerged as a strong candidate for the relevant in vivo target of lithium. Lithium inhibits GSK-3 in vitro (12) and in vivo at therapeutically relevant concentrations (14–20). Inhibition of GSK-3 provides a compelling explanation for the effects of lithium on the development of diverse organisms (8), and these observations are supported by pharmacologic (21–23) and genetic loss-of-function approaches that closely mimic lithium effects on development (8, 24–29). Systemic lithium inhibits GSK-3 in the mouse brain (16, 20, 30, 31) and in peripheral blood cells of patients with BPD (32, 33). Genetic loss of function and inhibition of GSK-3 with structurally diverse inhibitors also parallel lithium effects in diverse behaviors in rodents (13, 20, 34–37). These correlations support GSK-3 as the relevant target of lithium in mammalian behavior.

However, the specificity of lithium action in these settings has not been assessed by rescue experiments to demonstrate a causal role for GSK-3. Furthermore, elegant recent work suggests that behavioral effects of lithium arise through inhibition of the scaffolding function of β-arrestin-2 that mediates interaction of Akt and protein phosphatase 2A (PP2A) to regulate Akt (acting upstream of GSK-3) (13, 38). To test whether lithium-mediated behavioral effects are specifically due to inhibition of GSK-3, we have generated transgenic mice that overexpress GSK-3β (*Gsk3b*) in the brain and show that *Gsk3b* overexpression reverses the effects of lithium in multiple behaviors. Using *Gsk3b* gain-of-function and loss-of-function approaches, we also show that GSK-3 is required for stability of the β-arrestin-2/Akt/PP2A complex and that direct inhibition of GSK-3 disrupts the β-arrestin-2 complex in vivo. Taken together, these findings reconcile 2 leading hypotheses for lithium action and provide strong support for GSK-3 as an essential direct target of lithium in mammalian behaviors.

Results

Overexpression of Gsk3b reverses lithium-sensitive behaviors. Rescue of a gene knockout or drug-induced phenotype by restoring activity of the putative target provides strong support for the hypothesis that the phenotype is due to specific inhibition of the target. This is considered an essential control in the genetic analysis of model organisms, such as yeast and fruit flies, although it is less often applied in mammalian systems. We therefore tested whether transgenic expression of *Gsk3b* in the brain would reverse lithium-sensitive phenotypes, including established lithium-sensitive behaviors (20) and assembly of the β-arrestin-2/Akt/PP2A complex (13). Initially, we tested whether increasing GSK-3 levels would reverse inhibition of substrate phosphorylation in the presence of lithium chloride (LiCl) in vitro. Raising the overall level of GSK-3 protein by 50% restored substrate phosphorylation in the

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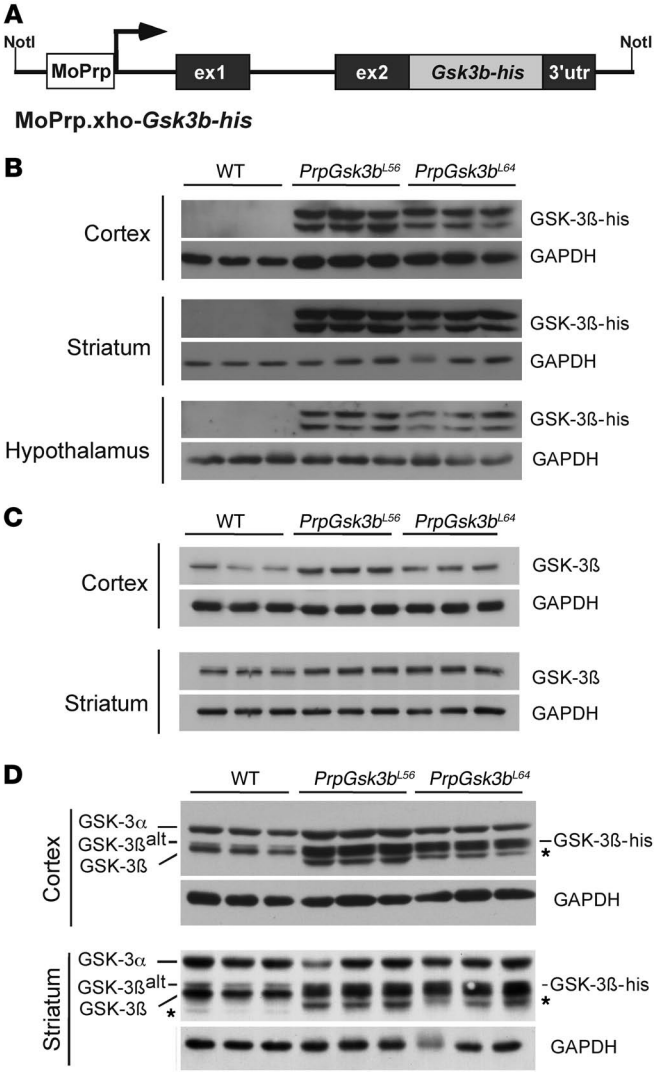

Figure 1
Expression of *Gsk3b*-his in mouse brain. (**A**) Schematic of the *Gsk3b*-his transgene inserted downstream of MoPrp (MoPrp.xho). ex1, exon 1; utr, untranslated region. (**B**) GSK-3β-his protein expression in cortex, striatum, and hypothalamus of WT, *PrpGsk3b*^{L56}, and *PrpGsk3b*^{L64} transgenic mice was assessed by immunoblotting for 6X-his tag. Partial N-terminal proteolysis yielded a second, smaller band that reacted with C-terminal GSK-3 antibodies and was also observed for endogenous GSK-3β (see **D**), as reported previously (40, 41). GAPDH was used as the loading control. (**C**) Total GSK-3β protein expression in cortex and striatum of WT, *PrpGsk3b*^{L56}, and *PrpGsk3b*^{L64} mice detected with an N-terminal, GSK-3β–specific antibody. (**D**) GSK-3α and GSK-3β protein expression in cortex and striatum, detected with an antibody that recognizes the C-termini of both GSK-3α and GSK-3β antibody, confirms partial proteolysis of endogenous GSK-3β and GSK-3β-his (asterisks). An alternatively spliced form of endogenous GSK-3β is indicated (GSK-3β^{alt}). GSK-3β-his migrates between the endogenous GSK-3β and GSK-3β^{alt} forms.

presence of LiCl to that of control levels in vitro (Supplemental Results and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI45194DS1). *Gsk3b* overexpression also restored lithium-inhibited phosphorylation of the endogenous GSK-3 substrate glycogen synthase in human embryonic kidney cells (Supplemental Results and Supplemental Figure 1B) and reversed activation of a Wnt-dependent transcription reporter (similar to TOPFLASH) that was robustly activated by lithium (Supplemental Results and Supplemental Figure 1C). Thus, increasing GSK-3 levels reversed lithium effects in vitro and in cultured cells. We next tested whether transgenic expression of *Gsk3b* in the brain rescues lithium-sensitive behaviors in mice.

Chronic lithium treatment reduces immobility in the forced swim test (FST), reduces exploratory behavior, and increases time in the open area of the elevated zero maze (EZM) (20). These parallels between lithium action and *Gsk3b* loss of function are consistent with the hypothesis that GSK-3 is the relevant target of lithium in these behaviors, but to establish a causal role for inhibition of GSK-3 by lithium, we tested whether overexpression of *Gsk3b* would reverse the behavioral effects of lithium. The mouse prion promoter (MoPrp) drives transgene expression in neurons and glia throughout the central nervous system of mice (39). This promoter was used to drive expression of *Gsk3b* with a C-terminal 6X-his tag (Figure 1A). Two transgenic founder lines were obtained (*PrpGsk3b*^{L56} and *PrpGsk3b*^{L64}) and were backcrossed into the C57BL/6 background. Expression of the transgene in cortex, hippocampus, amygdala, hypothalamus, striatum, nucleus accumbens, and cerebellum was confirmed by RT-PCR (data not shown) and by Western blot. GSK-3β-his was detected in all brain regions tested, with *PrpGsk3b*^{L56} mice showing higher GSK-3β-his expression compared with that of *PrpGsk3b*^{L64} mice (Figure 1B). Increased GSK-3β protein expression was confirmed by Western blotting with antibodies that recognize the GSK-3β N terminus (Figure 1C) or a C-terminal epitope conserved in GSK-3α and GSK-3β (Figure 1D); increased GSK-3β protein expression was also confirmed by LI-COR imaging, which showed a 30% increase in overall GSK-3β protein in the cortex of *PrpGsk3b*^{L56} mice. A higher-mobility his-tagged band observed in all transgenics (Figure 1B) also reacted with the C-terminal GSK-3 antibody (Figure 1D), and a similar band at lower intensity was also observed in WT brain extracts, consistent with partial N-terminal proteolysis, which may represent endogenous calpain-mediated cleavage, as reported previously (40, 41), although we cannot rule out partial proteolysis during sample preparation. The *PrpGsk3b* mice also demonstrated increased GSK-3 activity in lysates prepared from striatum and cortex (Supplemental Methods, Supplemental Results, and Supplemental Figure 2). Coordination, overall activity in the open field (Supplemental Results and Supplemental Figure 5), and the general state of *Gsk3b* transgenic mice were indistinguishable from those of their WT littermates, indicating no overt physiological or behavioral state changes in *Gsk3b*-overexpressing mice.

In the FST, mice are placed in a cylinder partially filled with water, and the time that the mouse remains immobile is measured in the last 4 minutes of a 6-minute test (42). To investigate whether the effects of lithium on the FST are reversed by overexpression of *Gsk3b*, we tested WT and *PrpGsk3b* mice, with and without lithium treatment. In the absence of lithium treatment, there was no effect of *Gsk3b* overexpression. A 1-way ANOVA on the FST data revealed a robust effect of lithium in WT mice ($P < 0.01$), as described previously (20, 43). In contrast, the effect of lithium was blocked in *PrpGsk3b*^{L56} mice, indicating that *Gsk3b* overexpression rescues this effect of lithium (Figure 2A). There was no significant effect of *Gsk3b* overexpression in *PrpGsk3b*^{L64} mice, which may reflect the relatively lower level of GSK-3β-his expression in these mice (Figure 1B).

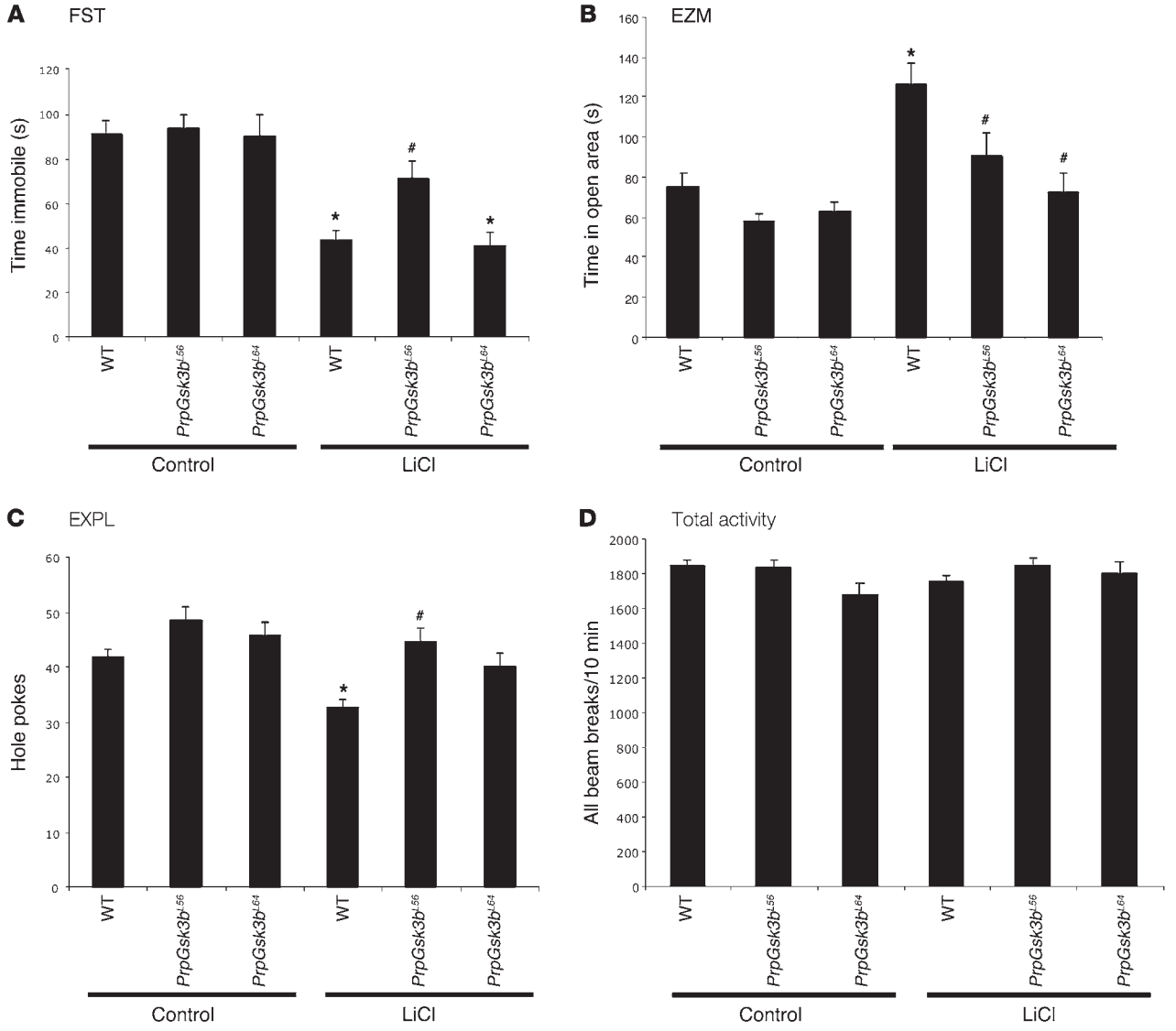

Figure 2

Overexpression of *Gsk3b* reverses the behavioral effects of lithium. WT, *PrpGsk3b*^{L56}, and *PrpGsk3b*^{L64} mice were treated with control or lithium diet for 1 week and were then tested with the (**A**) FST (n = 68, 73, 29, 48, 46, and 32 mice, respectively), (**B**) EZM (n = 53, 58, 15, 42, 36, and 19 mice, respectively), (**C**) exploratory behavior test (EXPL) (n = 22, 25, 29, 18, 14, and 32 mice, respectively), and (**D**) total activity test (n = 22, 25, 29, 18, 14, and 32 mice, respectively). Numbers in parentheses represent the number of mice for each bar of each bar graph, respectively. *P < 0.05 compared with WT; $^{\#}P$ < 0.05 compared with WT plus LiCl.

In the EZM, mice are placed on a circular track with 2 symmetrically opposed open and enclosed areas, and the time spent in the open versus the closed arms is measured. As shown previously (20), lithium treatment increased time in the open areas, and overexpression of *Gsk3b* reversed this effect in both transgenic lines (P < 0.05), restoring time spent in the open area to that of WT control levels (Figure 2B). These observations are consistent with and complementary to the recent observation that knockin of phosphorylation-defective forms of GSK-3α and GSK-3β, which prevents inhibitory phosphorylation at N-terminal serines, increases time in the open area of the elevated plus maze (33).

Hole poke exploratory behavior measures the frequency at which mice explore holes in the floor of an observation chamber. Chronic lithium reduces the number of hole pokes without affecting overall activity (20). Consistent with previous findings, lithium reduced hole pokes in WT mice (P < 0.05). Overexpression of *Gsk3b* restored the number of hole pokes in lithium-treated *PrpGsk3b*^{L56} mice to those of WT control levels (Figure 2C); lithium-treated *PrpGsk3b*^{L64} mice also showed an increase in the number of hole pokes that approached but did not achieve statistical significance, which may reflect the lower expression of GSK-3β-his in this line. To rule out an effect of overt changes in the state of the mice that could confound the exploratory behavior assessment, data for total activity were collected in the hole board arena. No significant differences were found among the WT, WT with lithium, *PrpGsk3b*, and *PrpGsk3b* with lithium groups (Figure 2D).

Heterozygous loss of *Gsk3b* causes behavioral defects that mimic lithium action, including reduced time immobile in the FST and

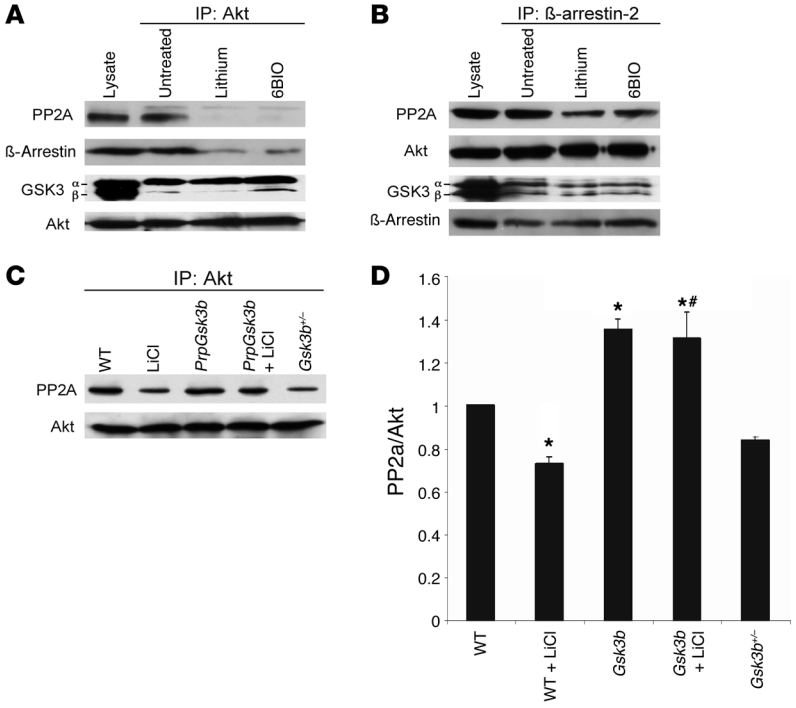

Figure 3

GSK-3 stabilizes the β-arrestin-2/Akt/ PP2A/GSK-3 complex. (**A**) Striatal homogenates from WT mice were exposed to the GSK-3 inhibitors LiCl and 6BIO (or AR-AO14418, Supplemental Figure 6) and subjected to immunoprecipitation with anti-Akt antibody, followed by immunoblotting for PP2A, β-arrestin-2, GSK-3α/β (using an antibody to an epitope in the C terminus; both GSK-3 isoforms were present in the immunoprecipitate, although more GSK-3α was detected than GSK-3β), and Akt. (**B**) Striatal homogenates were treated with GSK-3 inhibitors, as in **A**, and then subjected to immunoprecipitation with anti–β-arrestin-2 antibody, followed by immunoblotting for PP2A, Akt, GSK-3α/β, and β-arrestin-2. (**C**) Complex formation after in vivo inhibition of GSK-3. WT and *PrpGsk3b*^{L56} mice were treated with control or lithium for 1 week, while *Gsk3b*$^{+/-}$ mice received control diet only. Striatum was isolated, and Akt was immunoprecipitated, as in **A**, followed by immunoblotting for PP2A and Akt (n = 3 mice per group) The experiment was repeated 3 times with similar results. (**D**) Autoradiographs from 3 experiments were scanned and quantitated using ImageJ (http://rsbweb.nih.gov/ij/), and mean abundance of each band was normalized to Akt (which did not vary significantly among the samples). *P < 0.05 compared with WT; #P < 0.05 compared with lithium-treated animals. The PP2A/Akt interaction in *Gsk3b*$^{+/-}$ mice was reduced but did not achieve statistical significance based on the ANOVA with Dunn's post-hoc test, although a 1-tailed Student's t test comparing WT with *Gsk3b*$^{+/-}$ mice showed P = 0.006.

striata from WT mice, immunoprecipitated AKT, and blotted for components of the complex. Under basal conditions, Akt, PP2A, β-arrestin-2, GSK-3α, and GSK-3β interacted in the striatum, and association of Akt, PP2A, and β-arrestin-2 was disrupted by LiCl (Figure 3, A–C), as previously published (13, 44, 45). If GSK-3 stabilizes this complex, then structurally distinct GSK-3 inhibitors should mimic lithium and disrupt the complex. Indeed, addition of the GSK-3 inhibitors 6BIO (Figure 3A) or AR-A014418 (Supplemental Results and Supplemental Figure 6) to the immunoprecipitations disrupted interaction of Akt with β-arrestin-2 and PP2A (Figure 3A), similar to LiCl. We also note that the level of GSK-3α/β in the Akt immunoprecipitates is not reduced upon inhibition, perhaps reflecting additional Akt/GSK-3–containing complexes that are not sensitive to GSK-3 inhibition (46–50). To confirm that the β-arrestin-2 complex is sensitive to GSK-3 inhibitors, we performed immunoprecipitations from striatum using an anti–β-arrestin-2 antibody. Consistent with Figure 3A and prior reports, β-arrestin-2 interacted with PP2A (13, 44, 45), and addition of lithium or 6BIO disrupted this interaction (Figure 3B). The β-arrestin-2 immunoprecipitates also contained Akt and GSK-3α/β, but we did not observe dissociation of Akt or GSK-3 when GSK-3 was inhibited; while the reasons for this are unclear, each of these signaling molecules are known to associate in multiple and distinct complexes (46–50), and the β-arrestin antibody used here may identify additional complexes that are not sensitive to GSK-3 inhibition.

These observations suggest that GSK-3 contributes to β-arrestin-2 complex stability and that lithium disrupts the β-arrestin scaffold in vivo by inhibiting GSK-3, in addition to the reported direct effect on β-arrestin-2/Akt interaction. To investigate whether GSK-3 regulates the β-arrestin-2 complex in vivo, we also tested complex formation in the striatum of *Gsk3b*$^{+/-}$ mice and found reduced interaction of Akt and PP2A in mice lacking one copy of *Gsk3b* (Figure 3, C and D). WT and *PrpGsk3b*^{L56} mice were also treated for 12 days with LiCl, as above. Chronic LiCl reduced interaction between Akt and PP2A in WT mice, as reported previously (13), and this was restored in *PrpGsk-3b*^{L56} mice (Figure 3, C and D). Taken together, these observations show that the β-arrestin-2/Akt/PP2A/GSK-3 complex requires GSK-3, as both pharmacologic and genetic interference with *Gsk3b* disrupts complex formation and, importantly, overexpression of *Gsk3b* restores complex formation in parallel with the rescue of lithium-sensitive behaviors.

reduced exploratory behavior (20, 43); we found that transgenic expression of *Gsk3b* in *Gsk3b*$^{+/-}$ heterozygotes reversed these behavioral defects, demonstrating that the behavioral defects observed in *Gsk3b*$^{+/-}$ mice are also specifically due to *Gsk3b* loss of function (Supplemental Results and Supplemental Figure 4).

Gsk3b is required for the β*-arrestin-2/Akt/PP2A complex*. Previous work has defined a central role for β-arrestin-2 as a molecular scaffold that binds Akt and PP2A in neurons of the striatum to mediate dephosphorylation of Akt and, indirectly, reactivation of GSK-3. This complex is disrupted by lithium in striatal homogenates (13). Lithium also inhibits the in vitro interaction of recombinant β-arrestin-2 and Akt, although at a relatively high concentration of lithium (50 mM). Earlier work has shown that GSK-3 is also a component of the complex (44); we therefore tested whether GSK-3 contributes to the stability of the β-arrestin-2 complex. We isolated

Discussion

Defining the direct molecular target of lithium is critically important for the development of new drugs to treat BPD and to define the molecular pathogenesis of this common psychiatric disease. We have proposed a set of criteria to validate a putative target of lithium that can be applied in each lithium-sensitive context (5), including (a) in vivo inhibition, (b) pharmacologic

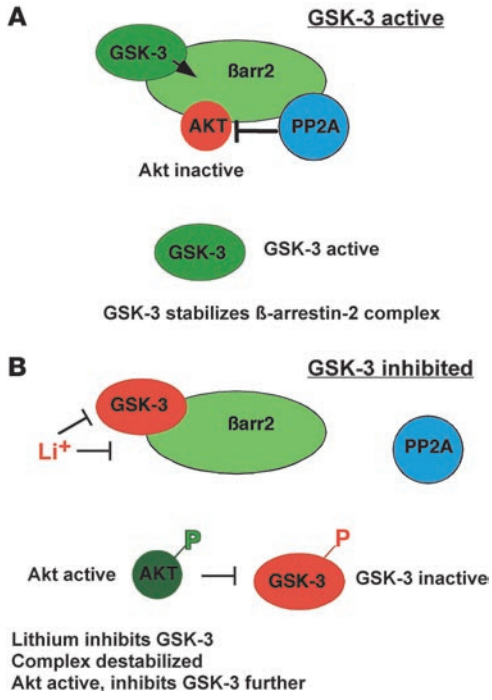

Figure 4
Model for lithium action and GSK-3 stabilization of the β-arrestin-2/Akt/ PP2A/GSK-3 complex. Neurotransmitter signaling in the striatum promotes interaction of β-arrestin-2 (βarr2), Akt, PP2A, and GSK-3 (45). (**A**) In the model presented here, GSK-3 stabilizes the complex, enhancing PP2A-mediated dephosphorylation of Akt, and hence preventing inhibitory phosphorylation of GSK-3. (**B**) Inhibition of GSK-3, for example, by lithium (Li+), destabilizes the complex, preventing dephosphorylation of Akt by PP2A. Akt remains active and phosphorylates GSK-3 at inhibitory sites, enhancing the inhibition of GSK-3 by lithium. As reported previously, lithium also interferes with interaction between β-arrestin-2 and Akt (13).

evidence that structurally diverse inhibitors mimic lithium, (c) genetic evidence through mutation of the target, and (d) reversal of lithium-sensitive behaviors by restoring activity of the target in the presence of lithium.

Each of these criteria is now fulfilled for GSK-3: GSK-3 is inhibited by lithium in the rodent brain (16, 20, 30, 31); diverse GSK-3 inhibitors mimic lithium action in multiple behaviors (13, 34–37); deletion of *Gsk3a* or *Gsk3b* mimics lithium action in mouse behaviors (13, 20, 34); and, as shown here, lithium-sensitive behaviors (and dissociation of the β-arrestin-2 complex) were reversed by raising *Gsk3* expression in the brain, demonstrating that these actions of lithium are specifically and causally related to GSK-3 inhibition.

Applying these criteria to validate GSK-3 in the therapy of BPD is more challenging, but early data are encouraging. GSK-3 N-terminal phosphorylation, a marker for GSK-3 inhibition, is increased in PBMCs from patients with BPD treated with lithium (51) and is reduced in PBMCs of untreated patients with BPD compared with that of healthy controls, implying increased baseline GSK-3 activity (33). Furthermore, olanzapine, an alternative medication for BPD, has also been reported to be a direct GSK-3 inhibitor (52). The observation that a structurally distinct GSK-3 inhibitor may have similar therapeutic actions as lithium supports the hypothesis that GSK-3 is the therapeutically relevant target of lithium in the treatment of BPD.

Other GSK-3 transgenic mouse lines have been reported, but these were not tested for reversal of lithium-sensitive behaviors (30, 53, 54). Interestingly, overexpression of the phosphorylation-defective GSK-3β^{S9A} increases locomotor activity, which was interpreted as a manic-like state (53). Similarly, knockin mice in which N-terminal phosphorylation sites in *Gsk3a* and *Gsk3b* were mutated to alanine, preventing inhibitory phosphorylation, show hyperlocomotion in a novel environment (33, 55), enhanced locomotor response to amphetamine, increased immobility in the FST and tail suspension test, and reduced time in the open arms of the elevated plus maze (33). All of these behaviors are opposite to the behaviors observed with lithium or *Gsk3b* haploinsufficiency and are therefore consistent with GSK-3 as the relevant target of lithium in these behaviors. It should be pointed out, however, that Ackermann et al. observed reduced immobility in the FST and increased time in the open arms of the EZM (55); although the hyperactivity of the mice in their study may make it difficult to interpret increased swimming activity in the FST, nevertheless the reason for the differences between these 2 studies is not clear, as both groups used mice on a similar, albeit mixed, genetic background.

Many lithium-sensitive behaviors in mice require β-arrestin-2, and assembly in the striatum of a complex that includes β-arrestin-2, Akt, and PP2A is sensitive to lithium. β-Arrestin-2 recruits PP2A to dephosphorylate and inactivate Akt (Figure 4). As Akt phosphorylates and inactivates GSK-3, assembly of this complex is predicted to enhance GSK-3 activity. Lithium interferes with stability of this β-arrestin complex, and, by preventing PP2A-dependent dephosphorylation of Akt, enhances Akt-mediated inhibition of GSK-3. This model is strongly supported by the observations that either β-arrestin-2 knockout or lithium enhances GSK-3 phosphorylation in vivo. However, while lithium can disrupt the direct interaction of recombinant β-arrestin-2 and Akt, this was done in the presence of 50 mM lithium, and whether interaction of the purified proteins is sensitive to more therapeutically relevant lithium concentrations has not been reported. Alternatively, we propose that GSK-3 plays a functional role in complex stability. In support of this hypothesis, structurally diverse GSK-3 inhibitors disrupted the β-arrestin-2 complex in striatal extracts, loss of one copy of *Gsk3b* also disrupted the complex in vivo, and overexpression of *Gsk3b* restored complex formation in the presence of lithium. These data show that GSK-3 is required in vivo and in vitro for the stability of this complex (Figure 4A) and support the hypothesis that direct inhibition of GSK-3 by lithium contributes substantially to disassembly of the β-arrestin-2/Akt/PP2A complex (Figure 4B).

The IC_{50} for lithium inhibition of GSK-3 is approximately 1 mM, at the high end of the therapeutic window for lithium therapy. However, lithium treatment enhances inhibitory phosphorylation of GSK-3, both in vivo and in cell culture, and this has been proposed to amplify the in vivo response to lithium in target tissues (31, 56). Thus, GSK-3 plays a positive role in its own activation by promoting activation of a phosphatase that removes N-terminal inhibitory phosphate groups on GSK-3 (57) and by promoting dephosphorylation and inhibition of Akt by stabilizing β-arrestin-2/Akt/PP2A interaction in the striatum. Lithium, through direct inhibition of GSK-3, blocks both mechanisms of autoactivation of GSK-3, providing at least two mechanisms to enhance lithium inhibition of GSK-3 in a tissue-dependent manner. Conversely, sensitivity

to lithium is markedly affected by the level of GSK-3, and small increases in GSK-3 expression can therefore attenuate sensitivity to lithium, as shown here and discussed previously (57).

While the behavioral effects of *Gsk3b* haploinsufficiency reported here and in prior work from this and other groups are highly concordant, one group reported that they were unable to observe behavioral phenotypes in *Gsk3b*$^{+/-}$ mice (58). However, these behaviors were tested in mice with a mixed genetic background that included the 129 strain, whereas the mice in this study and in the work of Beaulieu et al. were backcrossed into the C57BL/6 background (13). In this context, it should be noted that 129 mice are insensitive to lithium in amphetamine-induced hyperlocomotion, whereas C57BL/6 mice are highly sensitive (59), an intriguing observation that deserves further study, as it may help to explain the variable response to lithium in patients with BPD.

In summary, we have shown that overexpression of *Gsk3b* rescues the behavioral phenotypes observed in *Gsk3b* heterozygotes and lithium-treated mice and that direct inhibition of GSK-3 by lithium destabilizes the interaction of β-arrestin-2/Akt/PP2A in the striatum. Taken together with prior work using alternative GSK-3 inhibitors and genetic studies using both loss of function and knockin of constitutively active forms *Gsk3*, these findings provide strong support for GSK-3 as the relevant target of lithium in animal behaviors. Important questions to address in future work are how GSK-3 regulates these behaviors at a molecular level and, importantly, in which neuronal populations is GSK-3 function important for regulating these behaviors.

Methods

Generation of transgenic mice. The *Gsk3b* transgene was generated with *Xenopus Gsk3b* (93% identical to mouse and human *Gsk3b* and 97% similar to mouse and human *Gsk3b*; ref. 27), provided by David Kimelman (University of Washington, Seattle, Washington, USA), and driven by the mouse prion promoter (MoPrP.Xho; gift of David Borchelt, Johns Hopkins University, Baltimore, Maryland, USA) (39, 60). A 6X-his tag was added to the C terminus to distinguish endogenous from transgenic GSK-3β. Linearized plasmid was purified and injected into C57B6/129 heterozygous blastocysts by the University of Pennsylvania Transgenic and Chimeric Mouse Facility for generation of mice. Two founders were used to establish distinct lines, *PrpGsk3b*^{L56} and *PrpGsk3b*^{L64}, which were backcrossed into the C57/B6 strain for more than 10 generations. Both transgenic lines had normal litter sizes, and the transgene was transmitted in normal Mendelian fashion. Tissue from 3-month-old mice of each line was harvested for molecular analysis. Several brain regions were microdissected, and transgene expression was confirmed for both lines by RT-PCR. Transgenic protein expression was assessed by Western blot for the C-terminal his tag and for GSK-3β. Inositol levels were similar in the cortex, striatum, and hippocampus of WT, *PrpGsk3b*^{L56}, and *PrpGsk3b*^{L64} mice (Supplemental Methods, Supplemental Results, and Supplemental Figure 3).

Mice were housed 2 to 3 per cage, segregated by gender, in a 12-hour light/dark cycle. No overt changes in home cage behavior were observed, including overall activity, grooming, and social interactions. At 10 to 12 weeks, mice were randomly assigned to experimental and control groups. All animal experimentation was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and the University of Pennsylvania Laboratory Animal Resource Committee.

Behavioral testing. In regard to strain background, behavioral analysis was initially performed in mice backcrossed a minimum of 5 to 10 generations into C57/B6; all behavioral data were confirmed in a larger cohort backcrossed more than 10 generations into C57/B6. Porsolt FST, hole board exploratory behavior, and EZM were performed, as described previously (20), on days 7, 9, and 11 of lithium treatment. The order of the respective tests was varied to avoid an order effect.

Lithium dosing and testing schedule. Control and transgenic mice were allowed free access to water and standard mouse chow throughout the experiment. For lithium treatment, mice were fed 0.2% (w/w) LiCl mouse chow (Harlan Teklad) ad lib for 3 days, followed by 0.4% (w/w) LiCl mouse chow for the duration of the experiment (20). All mice had access to supplemental 450 mM NaCl drinking solution.

Immunoblotting and immunoprecipitations. Frontal cortex, hippocampus, hypothalamus, striatum, nucleus accumbens, amygdala, olfactory bulb, and cerebellum were harvested on day 12 of lithium treatment, frozen in liquid nitrogen, and stored at –80°C. All behavior experiments and tissue harvest took place between 8:00 AM and 12:30 PM. Antibodies to GSK-3β (N terminus) and β-catenin were from BD Transduction Laboratories. Antibodies to glycogen synthase, phosphorylated glycogen synthase (GS^{S641P}), and Akt were from Cell Signaling Technology. Anti–β-arrestin-2 antibodies were from Cell Signaling Technology. Anti–β-tubulin antibodies were from Promega. Frozen samples of dissected brain regions were homogenized with a Polytron PT10-35 Tissue Homogenizer in 200 μl 0.75% NP-40 lysis buffer (61). Protein content was determined by Bradford assay. Samples from individual animals (5–10 μg per lane) were analyzed by SDS-PAGE and immunoblotting. Immunoblots were visualized by ECL or ECL plus (Amersham). To measure band intensity in immunoblots, filters were probed with infrared-labeled secondary antibodies (Rockland Inc.), imaged with a LI-COR Odyssey Infrared Imager according to the manufacturer's instructions, and quantitated using LI-COR software (LI-COR Biosciences). For immunoprecipitations, striatal lysates were prepared as above and pooled from 3 mice per group. One mg of striatal lysate was used for immunoprecipitation with an antibody to Akt immobilized on sepharose beads (Cell Signaling Technology). Immunoprecipitation was conducted overnight at 4°C, followed by immunoblotting with an anti-PP2A antibody (Cell Signaling Technology).

Statistics. A 1-tailed Student's *t* test was performed on all behaviors to test for gender differences. No significant gender differences were found so the data were combined. For all other data, a 1-way ANOVA was used, followed by Dunn's post-hoc analysis when a significant difference was found among groups. The level of significance was set at $P < 0.05$. Histograms show mean values ± SEM throughout.

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1. Gould TD, Manji HK. The Wnt signaling pathway in bipolar disorder. *Neuroscientist*. 2002;8(5):497–511.
2. Cade J. Lithium salts in the treatment of psychotic excitement. *Med J Aust*. 1949;2(10):349–352.
3. Schou M. Lithium treatment at 52. *J Affect Disord*. 2001;67(1–3):21–32.
4. Pilcher HR. Drug research: the ups and downs of lithium. *Nature*. 2003;425(6954):118–120.
5. O'Brien WT, Klein PS. Validating GSK3 as an in vivo target of lithium action. *Biochem Soc Trans*. 2009;37(Pt 5):1133–1138.
6. Berridge MJ, Downes CP, Hanley MR. Neural and developmental actions of lithium: a unifying hypothesis. *Cell*. 1989;59(3):411–419.
7. York JD, Guo S, Odom AR, Spiegelberg BD, Stolz LE. An expanded view of inositol signaling. *Adv Enzyme Regul*. 2001;41:57–71.
8. Gurvich N, Klein PS. Lithium and valproic acid: parallels and contrasts in diverse signaling contexts. *Pharmacol Ther*. 2002;96(1):45–66.
9. Williams RS, Cheng L, Mudge AW, Harwood AJ. A common mechanism of action for three mood-stabilizing drugs. *Nature*. 2002;417(6886):292–295.
10. Tanizawa Y, Kuhara A, Inada H, Kodama E, Mizuno T, Mori I. Inositol monophosphatase regulates localization of synaptic components and behavior in the mature nervous system of C. elegans. *Genes Dev*. 2006;20(23):3296–3310.
11. Acharya S, Bussel JB. Hematologic toxicity of sodium valproate. *J Pediatr Hematol Oncol*. 2000;22(1):62–65.
12. Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci U S A*. 1996;93(16):8455–8459.
13. Beaulieu JM, et al. A beta-arrestin 2 signaling complex mediates lithium action on behavior. *Cell*. 2008;132(1):125–136.
14. Hedgepeth C, Conrad L, Zhang Z, Huang H, Lee V, Klein P. Activation of the Wnt signaling pathway: a molecular mechanism for lithium action. *Dev Biol*. 1997;185(1):82–91.
15. Stambolic V, Ruel L, Woodgett J. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr Biol*. 1996;6(12):1664–1668.
16. Munoz-Montano JR, Moreno FJ, Avila J, Diaz-Nido J. Lithium inhibits Alzheimer's disease-like tau protein phosphorylation in neurons. *FEBS Lett*. 1997;411(2–3):183–188.
17. Hong M, Chen DC, Klein PS, Lee VM-Y. Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3. *J Biol Chem*. 1997;272(40):25326–25332.
18. Lovestone S, Hartley CL, Pearce J, Anderton BH. Phosphorylation of tau by glycogen synthase kinase-3 beta in intact mammalian cells: the effects on the organization and stability of microtubules. *NeuroScience*. 1996;73(4):1145–1157.
19. Mudher A, et al. GSK-3beta inhibition reverses axonal transport defects and behavioural phenotypes in Drosophila. *Mol Psychiatry*. 2004;9(5):522–530.
20. O'Brien WT, et al. Glycogen synthase kinase-3beta haploinsufficiency mimics the behavioral and molecular effects of lithium. *J NeuroSci*. 2004; 24(30):6791–6798.
21. Meijer L, et al. GSK-3-selective inhibitors derived from Tyrian purple indirubins. *Chem Biol*. 2003; 10(12):1255–1266.
22. Hedgepeth CM, Deardorff MA, Rankin K, Klein PS. Regulation of glycogen synthase kinase 3beta and downstream Wnt signaling by axin. *Mol Cell Biol*. 1999;19(10):7147–7157.
23. Williams DS, Atilla GE, Bregman H, Arzoumanian A, Klein PS, Meggers E. Switching on a signaling pathway with an organoruthenium complex. *Angew Chem Int Ed Engl*. 2005;44(13):1984–1987.
24. Harwood AJ, Plyte SE, Woodgett J, Strutt H, Kay RR. Glycogen synthase kinase 3 regulates cell fate in Dictyostelium. *Cell*. 1995;80(1):139–148.
25. Cohen ED, et al. Wnt/beta-catenin signaling promotes expansion of Isl-1-positive cardiac progenitor cells through regulation of FGF signaling. *J Clin Invest*. 2007;117(7):1794–1804.
26. Doble BW, Patel S, Wood GA, Kockeritz LK, Woodgett JR. Functional redundancy of GSK-3alpha and GSK-3beta in Wnt/beta-catenin signaling shown by using an allelic series of embryonic stem cell lines. *Dev Cell*. 2007;12(6):957–971.
27. Pierce SB, Kimelman D. Regulation of Spemann organizer formation by the intracellular kinase Xgsk-3. *Development*. 1995;121(3):755–765.
28. He X, Saint-Jeannet J-P, Woodgett JR, Varmus HE, Dawid IB. Glycogen synthase kinase-3 and dorsoventral patterning in Xenopus embryos. *Nature*. 1995;374(6523):617–622.
29. Dominguez I, Itoh K, Sokol SY. Role of glycogen synthase kinase 3 beta as a negative regulator of dorsoventral axis formation in *Xenopus* embryos. *Proc Natl Acad Sci U S A*. 1995;92(18):8498–8502.
30. Noble W, et al. Inhibition of glycogen synthase kinase-3 by lithium correlates with reduced tauopathy and degeneration in vivo. *Proc Natl Acad Sci U S A*. 2005;102(19):6990–6995.
31. De Sarno P, Li X, Jope RS. Regulation of Akt and glycogen synthase kinase-3beta phosphorylation by sodium valproate and lithium. *Neuropharmacology*. 2002;43(7):1158–1164.
32. Li X, et al. Lithium regulates glycogen synthase kinase-3beta in human peripheral blood mononuclear cells: implication in the treatment of bipolar disorder. *Biol Psychiatry*. 2007;61(2):216–222.
33. Polter A, et al. Deficiency in the inhibitory serine-phosphorylation of glycogen synthase kinase-3 increases sensitivity to mood disturbances. *Neuropsychopharmacology*. 2010;35(8):1761–1774.
34. Beaulieu JM, et al. Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. *Proc Natl Acad Sci U S A*. 2004;101(14):5099–5104.
35. Gould TD, Einat H, Bhat R, Manji HK. AR-A014418, a selective GSK-3 inhibitor, produces antidepressant-like effects in the forced swim test. *Int J Neuropsychopharmacol*. 2004;7(4):387–390.
36. Kaidanovich-Beilin O, Milman A, Weizman A, Pick CG, Eldar-Finkelman H. Rapid antidepressive-like activity of specific glycogen synthase kinase-3 inhibitor and its effect on beta-catenin in mouse hippocampus. *Biol Psychiatry*. 2004;55(8):781–784.
37. Weiner I, Schiller D, Gaisler-Salomon I, Green A, Joel D. A comparison of drug effects in latent inhibition and the forced swim test differentiates between the typical antipsychotic haloperidol, the atypical antipsychotics clozapine and olanzapine, and the antidepressants imipramine and paroxetine. *Behav Pharmacol*. 2003;14(3):215–222.
38. Beaulieu JM, Caron MG. Looking at lithium: molecular moods and complex behaviour. *Mol Interv*. 2008;8(5):230–241.
39. Borchelt DR, et al. A vector for expressing foreign genes in the brains and hearts of transgenic mice. *Genet Anal*. 1996;13(6):159–163.
40. Ma T, et al. Statin's excitoprotection is mediated by sAPP and the subsequent attenuation of calpain-induced truncation events, likely via rho-ROCK signaling. *J Neurosci*. 2009;29(36):11226–11236.
41. Goñi-Oliver P, Lucas JJ, Avila J, Hernández F. N-terminal cleavage of GSK-3 by calpain: a new form of GSK-3 regulation. *J Biol Chem*. 2007;282(31):22406–22413.
42. Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther*. 1977;229(2):327–336.
43. Beaulieu JM, et al. Reply to Belmaker et al.: GSK3 haploinsufficiency results in lithium-like effects in the forced-swim test. *Proc Natl Acad Sci U S A*. 2008;105(20):E24.
44. Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell*. 2005;122(2):261–273.
45. Beaulieu JM, et al. Regulation of Akt signaling by D2 and D3 dopamine receptors in vivo. *J NeuroSci*. 2007;27(4):881–885.
46. Kovacs JJ, Hara MR, Davenport CL, Kim J, Lefkowitz RJ. Arrestin development: emerging roles for beta-arrestins in developmental signaling pathways. *Dev Cell*. 2009;17(4):443–458.
47. DeFea KA. Beta-arrestins as regulators of signal termination and transduction: how do they determine what to scaffold? *Cell Signal*. 2011;23(4):621–629.
48. Doble BW, Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci*. 2003; 116(pt 7):1175–1186.
49. Sim AT, Scott JD. Targeting of PKA, PKC and protein phosphatases to cellular microdomains. *Cell Calcium*. 1999;26(5):209–217.
50. Kikuchi A. Roles of axin in the Wnt signalling pathway. *Cell Signal*. 1999;11(11):777–788.
51. Li X, et al. Lithium regulates glycogen synthase kinase-3beta in human peripheral blood mononuclear cells: implication in the treatment of bipolar disorder. *Biol Psychiatry*. 2007;61(2):216–222.
52. Mohammad MK, et al. Olanzapine inhibits glycogen synthase kinase-3beta: an investigation by docking simulation and experimental validation. *Eur J Pharmacol*. 2008;584(1):185–191.
53. Prickaerts J, et al. Transgenic mice overexpressing glycogen synthase kinase 3beta: a putative model of hyperactivity and mania. *J NeuroSci*. 2006;26(35):9022–9029.
54. Lucas JJ, Hernandez F, Gomez-Ramos P, Moran MA, Hen R, Avila J. Decreased nuclear beta-catenin, tau hyperphosphorylation, and neurodegeneration in GSK-3beta conditional transgenic mice. *EMBO J*. 2001;20(1–2):27–39.
55. Ackermann TF, Kempe DS, Lang F, Lang UE. Hyperactivity and enhanced curiosity of mice expressing PKB/SGK-resistant glycogen synthase kinase-3 (GSK-3). *Cell Physiol Biochem*. 2010;25(6):775–786.
56. Chalecka-Franaszek E, Chuang DM. Lithium activates the serine/threonine kinase Akt-1 and suppresses glutamate-induced inhibition of Akt-1 activity in neurons. *Proc Natl Acad Sci U S A*. 1999;96(15):8745–8750.
57. Zhang F, Phiel CJ, Spece L, Gurvich N, Klein PS. Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. *J Biol Chem*. 2003;278(35):33067–33077.
58. Bersudsky Y, Shaldubina A, Kozlovsky N, Woodgett JR, Agam G, Belmaker RH. Glycogen synthase kinase-3beta heterozygote knockout mice as a model of findings in postmortem schizophrenia brain or as a model of behaviors mimicking lithium action: negative results. *Behav Pharmacol*. 2008; 19(3):217–224.
59. Gould TD, O'Donnell KC, Picchini AM, Manji HK. Strain differences in lithium attenuation of d-amphetamine-induced hyperlocomotion: a mouse model for the genetics of clinical response to lithium. *Neuropsychopharmacology*. 2007;32(6):1321–1333.
60. Sasaki Y, Vohra BP, Baloh RH, Milbrandt J. Transgenic mice expressing the Nmnat1 protein manifest robust delay in axonal degeneration in vivo. *J NeuroSci*. 2009;29(20):6526–6534.
61. Tao YS, Edwards RA, Tubb B, Wang S, Bryan J, McCrea PD. beta-Catenin associates with the actin-bundling protein fascin in a noncadherin complex. *J Cell Biol*. 1996;134(5):1271–1281.