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Validating GSK3 as an in vivo target of lithium action

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Abstract

Lithium is widely used to treat bipolar disorder, but its mechanism of action in this disorder is unknown. Lithium directly inhibits glycogen synthase kinase-3 (GSK3), a critical regulator of multiple signal transduction pathways. Inhibition of GSK3 provides a compelling explanation for many of the known effects of lithium, including effects on early development and insulin signaling/ glycogen synthesis. However, lithium also inhibits inositol monophosphatase, several structurally related phosphomonoesterases, phosphoglucomutase, and the scaffolding function of ß-arrestin-2. It is not known which of these targets is responsible for the behavioral or therapeutic effects of lithium in vivo. This review discusses basic criteria that can be applied to model systems to validate a proposed direct target of lithium. In this context, we describe a set of simple behaviors in mice that are robustly affected by chronic lithium and are similarly affected by structurally diverse GSK3 inhibitors and by removing one copy of the *Gsk3ß* gene. These observations, from several independent laboratories, support a central role for GSK3 in mediating behavioral responses to lithium.

Keywords

Lithium; Bipolar disorder; glycogen synthase kinase 3 (GSK-3); Wnt; inositol; behavior

Introduction

As has been underlined at the Biochemical Society Focused Meeting on Bipolar Disorder (BPD) at the Royal Holloway (April 2009), several plausible targets of lithium have been described and studied, and a strong case can be made for each of these potential targets as a mediator of lithium action within specific experimental contexts. These targets include inositol monophosphatase (IMPase) and structurally related phosphomonoesterases [1,2], phosphoglucomutase (PGM) [3], glycogen synthase kinase-3 (GSK3) [4], and the recently described ß-arrestin-2/Akt complex [5]. As lithium affects many biological systems, it is likely that each of these potential targets plays an important role in mediating the effects of lithium in specific contexts. However, none have been proven to be relevant drug targets in the therapy of bipolar disorder.

In addition to its therapeutic actions in BPD, lithium alters the function and development of diverse organisms from yeast to vertebrates, and these have provided useful model systems for investigating lithium action. For example, lithium enhances glycogen synthesis, perturbs cell fate decisions in *Dictyostelium*, sea urchins, and vertebrates, stabilizes neuronal growth cones, and stimulates mammalian hematopoiesis (reviewed in depth in [2]). Investigation of lithium action in these model systems has yielded a molecular toolbox for validating putative lithium

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targets in new biological contexts. In this brief review, we will restrict our attention to targets that are directly inhibited by lithium at therapeutically relevant concentrations and discuss a set of criteria that can be applied to validate a putative target of lithium in diverse biological contexts.

Direct targets of lithium

IMPase and the inositol depletion hypothesis

The inositol depletion hypothesis is one of the most compelling and enduring hypotheses to explain lithium action in multiple settings. This hypothesis was enumerated in a seminal paper by Berridge et al [6], and will certainly be addressed in greater detail by others in this issue. In brief, the hypothesis holds that inhibition of IMPase by lithium [7] will limit the regeneration of inositol from IMP, and in settings where the cell is dependent on this source of inositol to synthesize phosphatidylinositols (PIs), lithium should interfere with PI phosphate (PIP) dependent signaling pathways. As originally articulated this would reduce signaling by second messengers generated from signal-induced PIP2 hydrolysis, although it should also interfere with PIP3 dependent pathways (see [8]). An important feature of this hypothesis is that lithium is an uncompetitive inhibitor of IMPase [6] and thus the degree of inhibition increases at high substrate concentration.

Phosphomonoesterases related to IMPase

Members of a family of magnesium-dependent phosphatases structurally related to IMPase have also been proposed as biologically relevant lithium targets [1]. These include inositol polyphosphate 1-phosphatase (IPPase), fructose 1,6 bispohosphatase, bisphosphate nucleotidase (BPNT), and the golgi-resident nucleotidase gPAPP (3′-phosphoadenosine 5′-phosphate 3′ Phosphatase, [9]). Some of these enzymes are significantly more sensitive to lithium than IMPase, and the phenotypes associated with mutations in a few of these genes in metazoan organisms have been described. For example, mutation of the *Drosophila ipp* gene, which encodes IPPase, phenocopies lithium action in the *Drosophila* neuromuscular junction, as discussed below. PGM is not a member of this family, but nevertheless also hydrolyzes a carbohydrate-phosphomonoester linkage as part of its phosphoryltransferase mechanism, and is similarly magnesium-dependent and lithium-sensitive [3].

GSK3

GSK3 is a serine/threonine protein kinase that does not share obvious structural features with other lithium sensitive enzymes [4]. Furthermore, GSK3 is the only protein kinase among >70 tested that is inhibited by lithium at therapeutically tolerated concentrations (although several are partially inhibited by lithium at 10 mM [10]). Lithium competes with magnesium [11] and most Ki's reported for GSK3 reflect assays done at superphysiological magnesium concentrations. Thus, the IC_{50} for lithium inhibition of GSK3 is approximately 1.0 mM or lower at typical intracellular magnesium concentrations [2,11].

GSK3 was first described as an antagonist of glycogen synthase, and insulin activates GS in part by Akt/PKB-dependent phosphorylation and inhibition of GSK3 [12]. GSK3 also antagonizes Wnt signaling by constitutively phosphorylating ß–catenin and promoting its degradation [12]. Thus, inhibition of GSK3 by lithium will activate these pathways downstream of GSK3. This downstream activation can explain many of the known effects of lithium on glycogen synthesis, development, circadian rhythm, hematopoiesis, and other responses to lithium [2,4].

In addition to direct inhibition of GSK3 by lithium, several modes of indirect inhibition have been described. Lithium enhances the inhibitory N-terminal phosphorylation of GSK3 by

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increasing Akt activity and by inhibiting the phosphatase that dephosphorylates GSK3 [13, 14]. These indirect effects are a consequence of direct GSK3 inhibition, as GSK3 regulates itself through complex feedback loops that involve activation of protein phosphatase-1 and inhibition of Akt; in addition, in the striatum, lithium disrupts a scaffold of ß-arrestin, Akt, PP2A, and GSK3, leading to enhanced Akt activity [5]. We propose that GSK3 may play a role in stabilizing this complex, so that inhibition of GSK3 could contribute to the disruption of the ß-arrestin/Akt/PP2A/GSK3 complex in vivo, but this has not been tested, and Beaulieu et al presented data to show that lithium can disrupt the interaction of ß-arrestin and Akt in vitro, in the absence of GSK3 [5].

Criteria for validating a potential direct target of lithium in different biological contexts

With multiple plausible targets and numerous biological effects of lithium, it is essential to establish a set of criteria that can be applied in each new context to validate a given lithium target. These criteria may include:

1. Evidence that a therapeutically relevant concentration of lithium inhibits the target in vitro and in vivo. All of the targets described above are inhibited by lithium in vitro, but the challenge has been to show significant inhibition in vivo. Measurement of enzyme activity or level of product is essential to verify in vivo inhibition directly.
2. Pharmacological evidence that structurally distinct inhibitors of the putative target mimic lithium action can provide strong, though not unequivocal, support for a given target. Completely specific enzyme inhibitors are rare, if they exist at all, but it is unlikely that multiple, structurally diverse inhibitors will share “nonspecific” targets.
3. Genetic evidence, for example by gene knockout, RNA interference, or expression of dominant negative constructs, that disruption of gene function mimics lithium action is a powerful approach to validate putative drug targets.
4. Reversal of lithium effect by restoring enzyme function or product in the presence of lithium is a valuable, though not infallible, approach to validate that observed effects of lithium are caused by inhibition of a specific target. This is analogous to validating gene knockout phenotypes by reintroduction of the gene into the mutant background.

Applying the rubric

Cell fate decisions in developing organisms

The ability of lithium to perturb development of a variety of organisms has been recognized since the late 19th century [15]. Lithium shifts cell fate of developing *Dictyostelium* from spore to stalk cell fate [16], vegetalizes animal blastomeres in sea urchins, and dorsalizes *Xenopus* and zebrafish embryos (reviewed in [2]).

In this section, we will apply the above criteria to review the evidence that the developmental effects of lithium are mediated by inhibition of GSK3: 1) We and others have shown that lithium indeed inhibits GSK3 in *Xenopus* oocytes and embryonic extracts. 2) Alternative GSK3 inhibitors, including 6BIO, the 25-mer peptide GID, and a ruthenium-based organometallic (RuOH) mimic the developmental effects of lithium [17-19], as does the GSK3 inhibitor protein FRAT/GBP [20]. 3) GSK3 loss of function mimics the developmental effects of lithium in *Dictyostelium* [21] and dominant negative GSK3 mimics lithium action in sea urchins, frogs, and zebrafish (reviewed in [2]). 4) Overexpression of GSK3 suppresses the dorsalizing effects of lithium in *Xenopus* (P. Klein, unpublished). In metazoan embryos, the developmental effects of lithium inhibition of GSK3 are mediated through activation of canonical Wnt signaling, and thus blocking downstream Wnt signaling, for example by removing ß-catenin, blocks the developmental effects of lithium (Janet Heasman, personal communication) and conversely in vivo lithium treatment mimics the developmental phenotype of Wnt gain of function mutations

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in the mouse anterior heart field, leading to expansion of right heart and cardiac outflow precursors [22]. Interestingly, lithium exposure in human embryos has been associated with increased risk for Ebstein's anomaly, a downward displacement of the tricuspid valve and other right heart anomalies proposed to be a consequence of lithium-mediated activation of Wnt signaling [23]. Taken together these data strongly support GSK3 as a critical target of lithium action in the development of diverse organisms.

Lithium also inhibits IMPase in vivo in *Xenopus* embryos [4]. However, an alternative and much more potent IMPase inhibitor (L690, 330) has no obvious effect on embryo development [4], indicating that inhibition of IMPase is not sufficient to account for the developmental effects of lithium. This is one of the few examples in which a specific IMPase inhibitor has been tested in an in vivo setting, and was possible because the compound, which does not readily cross plasma membranes, could be injected directly into embryonic *Xenopus* blastomeres. Genetic tests of IMPase function have not been carried out in *Xenopus* or zebrafish. Lithium effects in *Xenopus* can be blocked by coinjection of 0.3M inositol, supporting the inositol depletion hypothesis; however, this concentration of inositol also reverses the dorsalizing effect of dominant negative GSK3 [24], suggesting either that inositol rescue has an indirect effect on dorsalizing pathways, or, interestingly, that GSK3 somehow regulates inositol levels, as proposed by Greenberg and colleagues and discussed below.

Effects of lithium on neuronal function

Loss of function of the *ipp* gene in *Drosophila* leads to defects in synaptic transmission in the larval neuromuscular junction, as well as accumulation of inostiol phosphate species [25]. Lithium inhibits the *Drosophila* IPPase completely at 2mM and phenocopies the synaptic defects of the *ipp* mutants. Alternative IPPase inhibitors are not available and rescue of lithium effects by overexpressing IPPase was not tested, but these results nevertheless strongly support that the effect of lithium in the neuromuscular junction of *Drosophila* larvae indeed occurs through inhibition of IPPase. A similar phenotype in other organisms has not been reported, however.

Mutations in the IMPase gene *ttx-7* disrupts thermotaxis in *C. elegans*, and lithium also phenocopies this defect [26]. This work was especially elegant as either exogenous inositol or overexpression of IMPase rescues normal thermotaxis. These data make a strong case that disruption of this behavior by lithium is mediated by inhibition of IMPase. It will be interesting to see whether lithium or loss of the IMPase gene also reduces synthesis of PI, PIP, or PIP2, as reduction in PIP2 by lithium has been observed in *Dictyostelium* and most likely accounts for the lithium-induced chemotactic defects observed in this organism [8].

Lithium, VPA, and carbamazepine, all of which are used to treat BPD, stabilize neuron growth cones in cultured sensory neurons, suggesting that each drug modulates a common pathway that regulates growth cone dynamics [52]. Furthermore, the effect of all three drugs was blocked by adding inositol. A reasonable hypothesis, proposed by the authors, is that growth cone stabilization by these BPD drugs arises due to reduction in endogenous inositol [52]. To test this hypothesis, it will be important to measure inositol, but this has not yet been done in this setting. However, lithium (directly) and valproic acid (indirectly) also inhibit GSK3 in neurons [27,28], alternative direct GSK3 inhibitors stabilize growth cones [29], and knock down of *Gsk3* also stabilizes growth cones [29], providing strong evidence that the lithium effect on neuronal growth cones occurs through inhibition of GSK3. An interesting hypothesis that could reconcile these findings, as articulated by Greenberg and colleagues, is that GSK3 may indirectly regulate inositol synthesis [30], so that inhibition would reduce inositol synthesis and addition of inositol would then rescue this defect whether arising from GSK3 inhibition or other means of reducing cellular inositol. Greenberg and colleagues provided strong data to

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support this model in yeast, and it will be intriguing to see whether similar regulation occurs in neurons.

Lithium sensitive behaviors in mouse

Surprisingly few behaviors had been described for chronic lithium treatment. We therefore examined multiple established and reproducible behaviors in mice placed on a simple treatment protocol (lithium in the food) that achieves serum lithium levels of 1 mEq/L. In addition to the known attenuation of amphetamine-induced hyperactivity and enhancement of pilocarpine-induced seizures [31], we found that the forced swim test, the elevated zero maze, and holeboard/exploratory behaviors were affected by long-term dietary lithium [32]. Importantly, overall activity, coordination, and general measures of the state of the animal were unaffected. Furthermore, Beaulieu et al reported that the tail suspension test and dark to light emergence are affected by parenteral lithium administration [5].

Evidence that GSK3 inhibition mediates the behavioral effects of lithium—

Lithium inhibits GSK3 in vivo in mouse and rat brain [2,33,34], and alternative GSK3 inhibitors mimic many of the behavioral effects of lithium (Table I). Both AR-A014418, an ATP competitive GSK3 inhibitor that crosses the blood brain barrier, and the peptide inhibitor L803mts, which was injected into the ventricles, reduce immobility in the forced swim test [35,36]. The thiadiazolidinone TDZD-8 reduces immobility in a related behavior, the tail suspension test, and reduces latency to emerge from dark to light areas [5]. Lithium attenuates hyperactivity induced by amphetamine [31]; this amphetamine-induced behavior is believed to be due to increased dopamine signaling, and is mimicked in dopamine transporter knockout mice (DAT-KO) [37]. Furthermore, lithium and several GSK3 inhibitors, including SB216763, alsterpaullone, 6-bromo-5′-indirubin-3′-oxime (6BIO), and TDZD, reduce hyperactivity in DAT-KO mice. Open field activity is also attenuated in wild-type mice by IP injection of lithium (as LiCl), TDZD, or AR-A014418 [5,35]; as the open field is generally a measure of the overall state of the animal, these observations could suggest a nonspecific effect of this mode of drug delivery on the state of the animal. However, an alternative explanation, which we favor, is that IP injection achieves higher peak concentrations of lithium or other inhibitors, and that the reduced activity is a specific consequence of more potent inhibition of GSK3. Support for this explanation comes from our observation that, although neither oral lithium nor *Gsk3ß* haploinsufficiency alone alters open field activity, oral lithium treatment of *Gsk3ß*$^{+/-}$ mice does reduce activity in the open field (and also markedly reduces immobility in the FST [32]). Furthermore, overexpression of a phosphorylation-resistant *Gsk3* mutant increases locomotor activity and was proposed as a model of manic hyperactivity [38].

To provide genetic evidence to support GSK3 as the target of lithium, we and others have tested whether deletion of the *Gsk3ß* gene in mice affects behavior in a manner similar to lithium [5,32,37]. (Careful attention to the genetic background of the mice to be tested is essential to obtain reliable results [39], and therefore our behavioral studies with *Gsk3ß* KO mice were performed in mice that had been backcrossed into a defined strain (C57/Bl6) >10 generations). *Gsk3ß*$^{-/-}$ mice die during gestation but the heterozygotes are viable and develop apparently normally, despite 50% reduction in GSK3ß protein levels throughout the cortex, hippocampus, hypothalamus, and cerebellum [32]. *Gsk3ß*$^{+/-}$ mice behave similarly to lithium treated mice in the FST, TST, and amphetamine-induced hyperactivity, exploratory behavior, and elevated zero maze [5,32,37]. Furthermore, expression in the brain of a ß-catenin mutant that lacks the GSK3 phosphorylation sites (and is therefore stabilized) mimics the effects of lithium in the FST and amphetamine-induced hyperactivity [40], though conditional loss of ß-catenin has so far had only modest effects on lithium sensitive behaviors [41]. Finally, preliminary data, to be presented in detail elsewhere, show that the effects of lithium on the FST, exploratory

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behavior, and elevated zero maze are reversed by overexpression of *Gsk3β* in the brain, strongly supporting that GSK3 is the specific target of lithium in these behaviors.

Evidence for inositol depletion in the behavioral effects of lithium—Chronic lithium also reduces inositol in mouse brain by 10-25% [42,43]. To test whether this partial reduction in inositol can account for the observed behavioral changes, an alternative approach to reducing inositol in the brain would be extremely helpful, but IMPase inhibitors that cross the blood brain barrier are not currently available. However, homozygous knockout of the sodium myo-inositol transporter (*SMIT1*) gene in mouse reduces inositol levels in fetal brain by >90%, and yet has no effect on global PI levels, indicating that the more modest inositol reduction observed with lithium is unlikely to reduce PI synthesis [44]. Consistent with this, lithium has never been shown to reduce PI or PIP2 in vivo [45,46]. Nevertheless, a reduction in PI/PIP2 could be restricted to specific areas within the brain that would not be detected by global PI measurement. Therefore it is still worthwhile to test behaviors in *SMIT*1 KO mice. Although *SMIT1*$^{-/-}$ mice die at gestation, the *SMIT1*$^{+/-}$ heterozygotes are viable. Inositol levels are reduced in the brains of adult *SMIT1*$^{+/-}$ mice by 33-37% (i.e. a greater reduction than with lithium) [43]. Under these conditions of inositol depletion, there is no effect in the forced swim test, amphetamine-induced hyperactivity, or sensitivity to pilocarpine-induced seizures [43,47], demonstrating that global inositol depletion to an extent greater than observed with lithium is not sufficient to cause lithium sensitive behaviors. One caveat to these experiments, however, is that it is not clear that *SMIT1* is expressed in neurons [48] nor that inositol is specifically reduced in neurons of *SMIT1* mutant mice.

More recent experiments show that the *SMIT1* homozygous KO can be raised to adulthood if the mothers are supplemented with inositol. When inositol supplementation is discontinued, the adults show a ~60% reduction in brain inositol. Under these conditions of more severe inositol depletion, the animals demonstrate reduced immobility (increased swimming activity) in the FST and increased sensitivity to pilocarpine induced seizures, paralleling the effects of lithium [49]; how this mutation affects animal state, for example in the open field, was not reported, and is an important concern, as increased baseline activity is a potential confounding factor in interpreting the FST in *IMPA1* mutant mice (see below). Furthermore, lithium treatment does not achieve this degree of inositol depletion, so the relevance of these observations to lithium action will need to be further explored.

Mouse knockouts have been reported for both IMPase genes, *IMPA1* and *IMPA2*. Homozygous knockout of *IMPA1* is lethal but can be rescued by inositol supplementation of pregnant mothers [50]. Although this mutation does not affect inositol levels in the adult brain, it does reduce IMPase activity by up to 65%. Therefore, while this mouse line is not a model of global inositol depletion, they may have localized reductions in inositol that would be difficult to measure but could still mimic lithium-mediated inhibition of IMPase. These mice demonstrate increased swimming activity in the FST and increased sensitivity to pilocarpine, similar to lithium (Table I). However, deletion of *IMPA1* also causes marked hyperactivity in the open field (and in the home cage); as lithium *increases* swimming activity in the FST (traditionally reported as reduced immobility), the baseline hyperactivity in *IMPA1*$^{-/-}$ mice is a significant confounding factor in interpreting the FST in this mutant line. Knockout of *IMPA2* does not mimic lithium sensitive behaviors, and does not reduce inositol or IMPase activity in brain [51], implying redundancy with *IMPA1*.

Summary

The study of lithium action in diverse model systems has led to a number of approaches to probe potential targets of lithium action. In this review we have focused on direct molecular targets and have reviewed the various approaches in terms of a rubric to validate these enzymes

as relevant targets of lithium in selected biological contexts. The evidence supporting GSK3 as the relevant target of lithium in developing organisms including *Dictyostelium*, sea urchins, *Xenopus*, and zebrafish is very strong, and in metazoans it is highly likely that lithium exerts its effects on cell fate and patterning by inhibiting GSK3 and activating the Wnt signaling pathway. Conversely, inhibition of inositol phosphatases is a very likely explanation for the in vivo effects of lithium on synaptic transmission in invertebrates such as *Drosophila* (*ipp*) and *C. elegans* (*ttx-7*), and it will be interesting to see whether these observations apply to mammalian synaptic function. The direct target(s) responsible for the effects of lithium and other BPD drugs on axonal growth cones in cultured neurons remain(s) unclear, with support for both inositol depletion and inhibition of GSK3; an intriguing hypothesis to reconcile these observations in this system is that GSK3 regulates inositol synthesis, an idea that is supported by data from yeast but not yet tested in mammalian cells. Finally, global reduction in cerebral inositol to an extent greater than that observed with lithium does not recapitulate lithium effects in mouse behavior, and while the *SMIT1* and *IMPA1* knockouts show some behavioral parallels to lithium treatment, we feel that the weight of the pharmacological and genetic evidence, including the effects of multiple GSK3 inhibitors and the *Gsk3β*$^{+/-}$ knockout, strongly supports GSK3 as the critical target of lithium action in multiple mouse behaviors.

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TABLE I

Lithium sensitive behaviors and target inhibition in mice

Behavior	Li[1]	Alt. GSK3 inhibitors	*Gsk3β*$^{+/-}$	*IMPA1*$^{-/-}$	*SMIT1*$^{+/-}$	*SMIT1*$^{-/-}$
Forced swim test (time immobile)	↓	↓[2]	↓	↓[7]	No Δ	↓
Tail suspension test (time immobile)	↓	↓[3]	↓			
Amphetamine-induced Hyperactivity	↓	↓[4]	↓		No Δ	
Open field (overall activity)	No Δ	↓[5]	No Δ	↑↑[7]	No Δ	
Exploratory/Holeboard	↓		↓			
Elevated zero/plus maze (time in open area)	↑		↑			
Light/dark emergence (latency to cross)	↓	↓[6]				
Pilocarpine-induced seizures (sensitization)	↑			↑	No Δ	↑
[Inositol][8]	↓22-25%			No Δ	↓ 33-37%	↓ 55-60%

[1] Most of the behaviors in this column were done with chronic lithium given in food, which achieves serum Li = 1.0 mEq/L [32]. The TST and light/dark emergence were performed with IP injections of lithium [5].

[2] Reported for both AR-A014418 [35], an ATP competitor, and L803-mts, a myristoylated peptide inhibitor of GSK3 [36].

[3] TDZD (thiadiazolidinone-8) [5].

[4] AR-A014418 [35]. The GSK3 inhibitors SB216763, alsterpaullone, 6-bromo-5′-indirubin-3′-oxime (6BIO), and TDZD (as well as lithium) reduce hyperactivity in dopamine transporter knockout (DAT-KO) mice, which is thought to mimic amphetamine-induced hyperactivity [37].

[5] AR-A014418 [35], TDZD [5], and IP injection of LiCl [5]. We have also observed reduced activity in *Gsk3*$^{+/-}$ mice treated with oral LiCl [32].

[6] TDZD [5].

[7] Increased baseline activity (open field) makes it difficult to interpret increased activity (typically reported as reduced immobility) in the forced swim test [50].