# Lithium inhibits glycogen synthase kinase-3 activity and mimics Wingless signalling in intact cells

Vuk Stambolic*† Laurent Ruel* and James R. Woodgett*†

**Background:** Exposing eukaryotic cells to lithium ions (Li+) during development has marked effects on cell fate and organization. The phenotypic consequences of Li+ treatment on *Xenopus* embryos and sporulating *Dictyostelium* are similar to the effects of inhibition or disruption, respectively, of a highly conserved protein serine/threonine kinase, glycogen synthase kinase-3 (GSK-3). In *Drosophila*, the GSK-3 homologue is encoded by *zw3sgg*, a segment-polarity gene involved in embryogenesis that acts downstream of *wg*. In higher eukaryotes, GSK-3 has been implicated in signal transduction pathways downstream of phosphoinositide 3-kinase and mitogen-activated protein kinases.

**Results:** We investigated the effect of Li+ on the activity of the GSK-3 family. At physiological doses, Li+ inhibits the activity of human GSK-3β and *Drosophila* Zw3Sgg, but has no effect on other protein kinases. The effect of Li+ on GSK-3 is reversible *in vitro*. Treatment of cells with Li+ inhibits GSK-3-dependent phosphorylation of the microtubule-associated protein Tau. Li+ treatment of *Drosophila* S2 cells and rat PC12 cells induces accumulation of cytoplasmic Armadillo/β-catenin, demonstrating that Li+ can mimic Wingless signalling in intact cells, consistent with its inhibition of GSK-3.

**Conclusions:** Li+ acts as a specific inhibitor of the GSK-3 family of protein kinases *in vitro* and in intact cells, and mimics Wingless signalling. This reveals a possible molecular mechanism of Li+ action on development and differentiation.

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## Background

Glycogen synthase kinase-3 (GSK-3) is a highly conserved serine/threonine protein kinase implicated in cell-fate determination and hormonal signalling [1]. Unlike many protein kinases, GSK-3 is highly active in resting cells and is primarily regulated by inactivation. Thus, insulin and growth factors inhibit GSK-3 by inducing phosphorylation of an amino-terminal serine residue [2] *via* activation of the extracellular signal-regulated kinase (ERK) cascade and MAP kinase-activated kinase (MAPKAP) kinase-1 [3], or *via* phosphoinositide 3-kinase (PI 3-kinase)-dependent activation of protein kinase B (PKB) [4]. A number of putative GSK-3 substrates have been identified, including glycogen synthase [5], the c-Jun component of the AP-1 transcription factor [6,7], the microtubule-associated protein Tau [8] and the *adenomatous polyposis coli* (APC) gene product [9].

The *Drosophila* GSK-3 homologue, Zeste-white3[Shaggy] (Zw3Sgg), is an essential component of the Wingless signalling pathway. Disruption of the kinase causes defects in segmental organization and cell-fate determination [10–13]. Genetic epistasis experiments have led to a model in which Wingless signalling acts *via* Dishevelled to suppress the activity of Zw3Sgg [10,11,14], resulting in the cytoplasmic accumulation of Armadillo (a homologue of

mammalian β-catenin) [15]. Studies on dorso-ventral axis formation during *Xenopus* embryogenesis revealed a similar requirement for GSK-3 inactivation in Xwnt-8 signalling and β-catenin accumulation. For example, expression of dominant-negative GSK-3 mutants in the ventral side of the embryo induces formation of an ectopic dorsal axis [16,17], an effect similar to that induced by ectopic expression of Xwnt-8, Dishevelled, β-catenin and LEF-1 [18–22]; these mutants also rescue the UV-irradiation-mediated ventralization of embryos [16]. In *Dictyostelium*, the GSK-3 homologue GSK-A also plays a central role in cell-fate determination; disruption of GSK-A leads to the disproportionate differentiation of stalk cells at the expense of spore cells [23].

The consequences of GSK-3 inactivation in these systems have striking parallels with the phenotypic effects of lithium ions (Li+). In isolated rat adipocytes, Li+ specifically activates glycogen synthase and mimics insulin action on glycogen deposition [24]. In *Xenopus*, Li+ is a potent modifier of mesoderm induction that can induce a secondary body axis on the ventral side of the embryo [25,26] and can rescue UV-ventralized embryos [25]. In *Dictyostelium*, Li+ influences cell-fate determination during differentiation, inducing an almost complete redifferentiation of prespore cells into prestalk cells [27–29]. Li+ has recently been shown to inhibit bacterially expressed GSK-3 activity *in*

*vitro* [30]. Here, we demonstrate that physiologically effective concentrations of Li+ specifically inhibit all members of the GSK-3 family both *in vitro* and in intact cells; this raises the possibility that GSK-3 is an important physiological target of Li+ action. We also present evidence that Li+ mimics Wingless signalling in intact cells.

## Results

### Li+ inhibits GSK-3 activity *in vitro*

To determine whether Li+ modulates GSK-3 activity, we tested the ability of purified rat GSK-3β and Zw3Sgg [31] to modify a GSK-3-specific peptide substrate, phospho-GS1, in the presence of LiCl or KCl. Li+ caused a dose-dependent inhibition of both protein kinases with a half-maximal effect occurring at 1–2 mM, whereas equal or greater concentrations of K+ had no effect on GSK-3 activity (Fig. 1a). Li+ had no effect on the activity of other serine kinases tested, including SAPK/JNK (Fig. 1b) and MAPK/ERK (data not shown). Furthermore, Li+ had no effect on the activation of p42 and p44 MAP kinases (ERK2 and ERK1) by phorbol 12-myristate 13-acetate (PMA) and nerve growth factor (NGF) in PC12 cells, indicating that the ion does not affect MEK, Raf or Trk protein kinases (Fig. 1c).

### The effect of Li+ on GSK-3 is reversible

To determine whether the effects of Li+ on GSK-3 could be measured in intact cells, haemagglutinin (HA)-tagged human GSK-3β and GSK-3βS9A (a mutant in which the inhibitory phosphorylation site, Ser 9, was replaced by alanine [3]) were expressed in COS 1 cells by transient transfection. The GSK-3 activity in immunoprecipitates from cells treated with 10 mM Li+ was not significantly different from that from untreated cells (Fig. 2). However, when LiCl was added to immunoprecipitates from untreated cells, the activities of GSK-3β and GSK-3βS9A

were both inhibited. The inhibition could be largely reversed by subsequent washing of the immunoprecipitates (Fig. 2), indicating that the effect of Li+ on GSK-3 is direct, and that post-translational modifications are not necessary.

### Li+ inhibits GSK-3 in intact cells

The reversible nature of the inhibition precluded assessment of the effect of Li+ on GSK-3 in cells by methods that depend on purification. We therefore examined the *in situ* phosphorylation of the Tau protein in cells. GSK-3 phosphorylates several residues on Tau, including Ser 202. Phosphorylation of Ser 202 creates an epitope recognized by the monoclonal antibody AT8, and co-transfection of plasmids encoding GSK-3 and Tau induces AT8 reactivity in COS cells [32]. GSK-3-dependent phosphorylation of Tau was assessed in COS 1 cells that co-expressed HA–GSK-3β and Tau1N4R and were treated with various concentrations of Li+ for 30 minutes. Li+ treatment reduced the AT8 immunoreactivity in a dose-dependent manner (Fig. 3a, AT8 blot, lanes 4–10), without altering the Tau levels, as judged by parallel immunoblotting with the Tau.1 monoclonal antibody, the epitope of which is independent of Ser 202 phosphorylation (Fig. 3a, Tau.1 blot, lanes 4–10) [32] . In contrast, K+ had no effect on AT8 reactivity (Fig. 3a, lanes 11–17). Li+ did not alter expression of GSK-3, as judged by immunoblotting with the 12CA5 monoclonal antibody (Fig. 3a, anti-HA blot, lanes 1–17). Tau phosphorylation was also used to assess the effect of Li+ on GSK-3βS9A and GSK-3α (Fig. 3b). Li+ inhibited both forms of the kinase (Fig. 3b, AT8 blot), without altering the levels of Tau (Fig. 3b, Tau.1 blot), or of GSK-3βS9A and GSK-3α (data not shown). Although GSK-3βS9A was slightly less sensitive to Li+ than GSK-3β (Fig. 3c), this mutant was still effectively inhibited by Li+. These data show that Li+ inhibits both mammalian isoforms of

## Figure 1

Li+ inhibits the activity of GSK-3β and Zw3Sgg, but does not affect the activity of SAPK or the activation of p42 and p44 MAPK. **(a)** GSK-3β activity in the presence of Li+ and K+ (white and black circles, respectively); Zw3Sgg activity in the presence of Li+ and K+ (white and black squares, respectively). See Materials and methods for details. Activities are expressed as the percentage of those of the untreated controls (100 % is equivalent to 0.1 μmol phosphate transferred per min per mg). **(b)** The activity of SAPK in immunoprecipitates from U937 cells treated with 5 μg ml⁻¹ anisomycin or 200 mM sorbitol for 30 min before lysis. Following immunoprecipitation, the immunocomplexes were treated with 10 mM Li+ (black bars) or 10 mM K+ (white bars) and their activities toward soluble GST–cJun5–89 (residues 5–89 of cJun tagged with a glutathione-S-transferase epitope) were
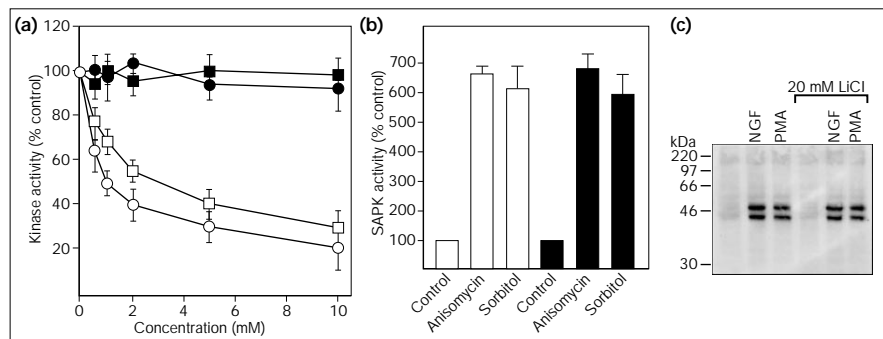


measured in the presence of each ion. SAPK activity is expressed as the percentage of that of the untreated control (100 % is equivalent to 0.8 μmol phosphate per min per mg). **(c)** Cytoplasmic extracts from serum-starved PC12 cells treated as indicated were separated by

SDS–PAGE and immunoblotted using a phosphotyrosine-specific anti-MAPK antibody. The cells were treated with 160 nM PMA or 50 ng ml⁻¹ NGF (Vector Biosystems). The sizes of the molecular weight markers are shown.

**Figure 2**
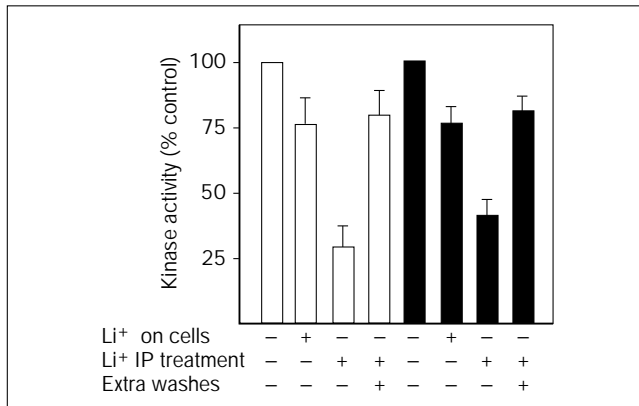


Inhibition of GSK-3 by Li+ is reversible and is unaffected by mutation of an inhibitory phosphorylation site. GSK-3β (white bars) and GSK-3βS9A (black bars) were immunoprecipitated (IP) from transfected COS 1 cells pretreated with or without 10 mM LiCl, as indicated; activities were measured as described in Materials and methods. Where noted, two extra washes of the immunoprecipitates were performed with 500 μl of kinase buffer.
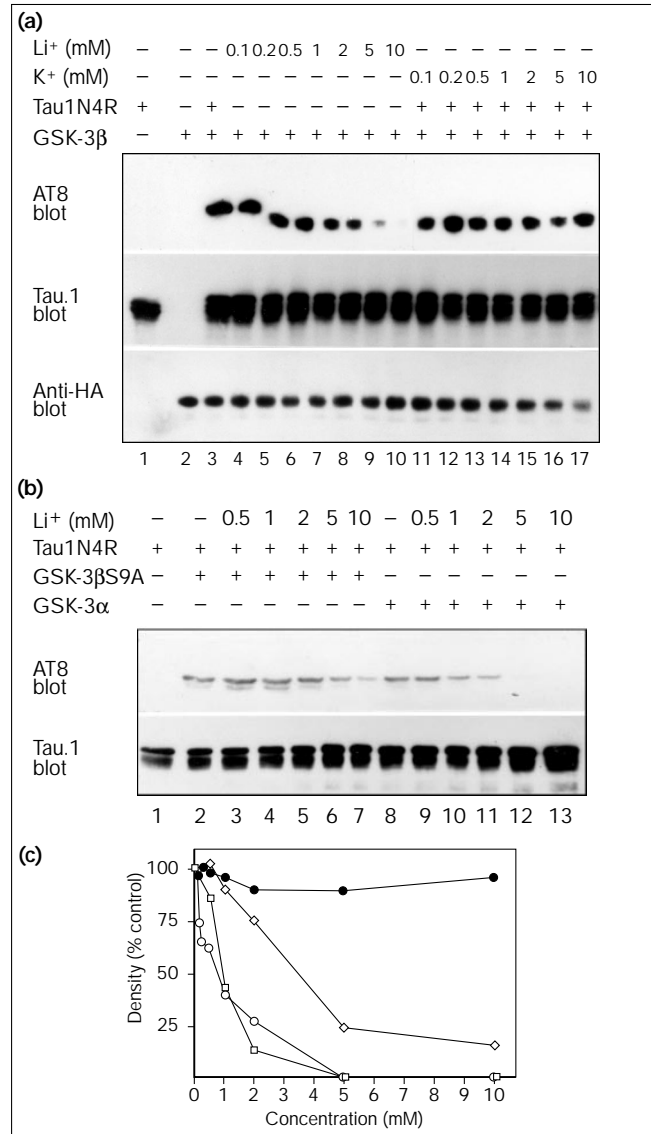
GSK-3 and, in the case of GSK-3β, inhibition is independent of the Ser 9.

## Li+ mimics Wingless signalling in intact cells

In intact *Drosophila* cells, Zw3[Sgg] is thought to act downstream of the diffusible factor Wingless, with Wingless inactivating Zw3[Sgg], resulting in an increase in the levels of Armadillo. If this model is correct, inhibition of Zw3[Sgg] by Li+ would be expected to mimic the Wingless signal, even in the absence of the key component of the Wingless receptor, Frizzled. To test this hypothesis, S2 cells, which do not express Frizzled [33], were incubated in the presence of Li+ and the levels of cytoplasmic Armadillo were determined by immunoblotting (Fig. 4). Li+ induced time-dependent accumulation of cytoplasmic Armadillo (Fig. 4a, lanes 2 and 4) without altering the level of Zw3[Sgg]; treatment with K+ did not affect Armadillo levels (Fig. 4a, lanes 3 and 5). The effect of Li+ on the level of cytoplasmic Armadillo is comparable to the effect of overexpression of Dishevelled from plasmid pPAC-Dsh in S2 cells (Fig. 4, lanes 6 and 7). SDS–PAGE analysis showed that Li+ treatment specifically caused the accumulation of a faster-migrating form of Armadillo (Fig. 4a, lower arrow). Induction of this form — which may represent hypophosphorylated protein — has previously been found after exposure to Wingless [32].

In vertebrates, expression of the Wingless homologue Wnt-1 results in increased cytoplasmic levels of β-catenin [34]. In *Xenopus*, expression of the dominant-negative mutant of GSK-3 also results in cytoplasmic accumulation of β-catenin [35]. To determine whether this role of GSK-3 was conserved in mammalian cells, we examined the

**Figure 3**



Li+ inhibits GSK-3-dependent phosphorylation of Tau in intact cells. **(a)** Inhibition of GSK-3β by Li+ but not K+. GSK-3β activity in cells cotransfected with plasmids encoding GSK-3β and Tau1N4R was assessed by measuring AT8 immunoreactivity following incubation with the indicated concentrations of Li+ (lanes 4–10) and K+ (lanes 11–17). The Tau.1 and anti-HA immunoblots are controls for Tau1N4R and HA-GSK-3β expression, respectively. **(b)** Inhibition of GSK-3βS9A and GSK-3α by Li+; activity was assessed as in (a). **(c)** Scanning densitometry of AT8 blots in (a,b): cells expressed Tau1N4R and GSK-3β and were treated with Li+ (open circles) or K+ (filled circles), or expressed Tau1N4R and GSK-3βS9A and were treated with Li+ (open diamonds) or Li+ (open squares). Densities are normalized to those of untreated controls.

effect of Li+ on β-catenin levels in PC12 cells. In contrast to the K+ control (Fig. 4b, lanes 3 and 5), Li+ induced the accumulation of cytoplasmic β-catenin (Fig. 4b, lanes 2 and 4) and did not effect the levels of GSK-3α and GSK-3β (Fig. 4b). Taken together, these data indicate that

**Figure 4**

Li+ mimics Wingless/Wnt signalling in intact cells. **(a)** Cytoplasmic extracts from S2 cells treated with 10 mM LiCl or 10 mM KCl, or transfected with the indicated constructs, were separated by SDS–PAGE. Samples were transferred to a PVDF membrane which was probed with the monoclonal antibodies 7A1 (anti-Armadillo) and 2G2C5 (anti-Zw3sgg). The sizes of the molecular weight markers (in kDa) are indicated. **(b)** Cytoplasmic extracts from rat PC12 cells treated with 10 mM LiCl or 10 mM KCl were similarly probed with monoclonal antibodies against β-catenin or GSK-3α and GSK-3β (4G1E11).
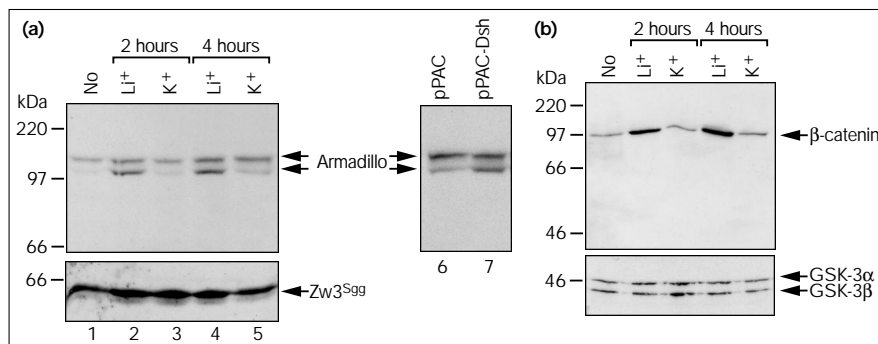


Li+ mimics Wingless/Wnt signalling by specifically inhibiting the activity of GSK-3 family members.

## Discussion

The effects of Li+ on invertebrate embryogenesis are well established. Li+ is an effective inhibitor of inositol phosphatase (IMPase), and phosphoinositides accumulate in Li+-treated cells. However, in *Xenopus*, a potent chemical inhibitor of IMPase does not phenocopy the effects of Li+ on formation of the dorso-ventral axis [30], strongly suggesting that Li+ exerts its effects through other mechanisms. The remarkable similarities between the effects of Li+ on *Xenopus* embryogenesis, *Dictyostelium* sporulation and glycogen synthesis, and the known effects of inhibition/disruption of GSK-3, suggest that this kinase, or a regulator of it, is an appealing candidate for mediating Li+ action. We have shown that Li+ specifically inhibits GSK-3 family members *in vitro* and in intact cells, and that Li+ can mimic the actions of Wnt/Wingless on β-catenin/Armadillo in mammalian and *Drosophila* cells. These data support and strengthen the hypothesis, recently proposed by Klein and Melton [30], that inhibition of GSK-3 may contribute to the mechanism of action of Li+ . Indeed, the extraordinary degree of cross-species similarity between the effects of Li+ and the consequences of disruption of GSK-3 function implies that dominant cellular action of the ion may be to inhibit GSK-3. Assessment of the relative importance of the inhibition of GSK-3 to physiological effects of Li+ will require the generation of Li+-insensitive mutants, which, upon expression in cells or embryos, should confer phenotypic resistance.

The mechanism underlying the stabilization of β-catenin by inhibition of GSK-3 is unknown. Yost *et al.* [35] have suggested that β-catenin is directly phosphorylated by GSK-3, consistent with the finding that phosphorylation of Armadillo is decreased in Zw3sgg mutant *Drosophila* embryos [36] and that, after treatment with Li+, the migration of Zw3sgg is increased on gels (Fig. 4a). Phosphorylation of β-catenin/Armadillo may destabilize the protein and limit its interaction with LEF-1-like factors. Because overexpression of either LEF-1 or β-catenin causes duplication of the dorsal axis in *Xenopus* [18,22], the concentrations of these proteins appear to be critical. However, in our hands, neither β-catenin nor Armadillo is a good *in vivo* substrate for GSK-3 or Zw3sgg, respectively (V.S. and J.R.W., unpublished observations). The recent demonstration that the APC protein is directly phosphorylated by GSK-3 [9] and is linked to catenin stabilization [34], raises the possibility that phosphorylated APC targets β-catenin for phosphorylation by a distinct protein kinase, which leads to its degradation. In addition, the intriguing possibility that inhibition of GSK-3 contributes to the therapeutic effects of Li+ in bipolar/manic-depressive disorder deserves further investigation.

## Materials and methods

*Materials, cell culture and transfections*
All reagents were purchased from Sigma Chemical Co. unless indicated otherwise. COS 1, U937 and PC12 cell lines were cultured in DMEM without sodium pyruvate, containing 10 % FBS (Gibco-BRL), penicillin and streptomycin. S2 cells were cultured in Schneider's medium (Gibco-BRL) containing 10 % FBS, penicillin and streptomycin. *Eco*RI fragments encoding HA–GSK-3β, HA–GSK-3βS9A and GSK-3α [3] were subcloned into the mammalian expression vector pMT2. pSG5/Tau1N4R was a gift from B. Anderton. The *Drosophila dishevelled* cDNA (pdc2.6; a gift from J. Axelrod and N. Perrimon) was subcloned into the *Drosophila* expression vector pPAC, generating pPAC-Dsh. COS 1 and S2 cells were transfected using the calcium phosphate procedure.

*Immunological procedures*
For western-blot analysis of Tau phosphorylation, transfected COS 1 cells were lyzed in a MES-based buffer as described [32] and the lysates were analyzed. For western-blot analysis of MAPK, Armadillo, β-catenin, GSK-3 and Zw3sgg, S2 or PC12 cells, as appropriate, were lyzed by incubation on ice for 15 min in a hypotonic lysis buffer containing 50 mM Tris (pH 7.4), 0.5 mM NaF, 100 µM Na-vanadate, 1 mM benzamidine and 5 µg ml–1 leupeptin; the insoluble fraction was removed by centrifugation and the supernatant was analyzed. Cell lysates were normalized for total protein, SDS–PAGE sample buffer was added, and samples were separated by 7.5 % SDS–PAGE (Armadillo and β-catenin blots) or 12.5 % SDS–PAGE (MAPK, GSK-3, Zw3sgg and Tau blots). Separated proteins were transferred to PVDF membranes (NEN–Dupont), which were blocked and probed with the appropriate antibodies. Antibody AT8 was from Innogenetics, Belgium; the Tau.1 antibody from B. Anderton;

*Protein purification and kinase assays*
For GSK-3 assays, we used rat GSK-3β and histidine-tagged *Drosophila* Zw3$^{Sgg}$ purified from baculovirus-infected Sf9 cells [31], or immunoprecipitates from transfected COS 1 cells, as described [3]. The activities of the GSK-3 proteins towards the phospho-GS1 peptide substrate and of SAPK toward GST–cJun5–89 were analyzed as described previously [3,38].

## Acknowledgements

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# Erratum
## Lithium inhibits glycogen synthase kinase-3 activity and mimics Wingless signalling in intact cells
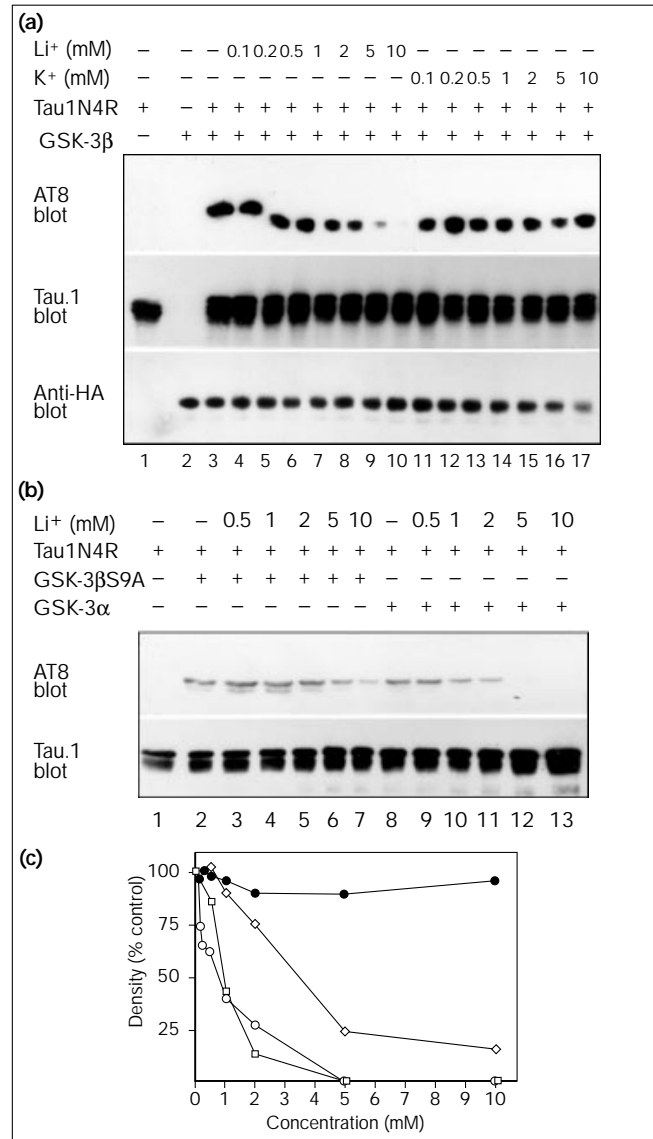Vuk Stambolic, Laurent Ruel and
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In this paper, which appeared in the 1 December 1996 issue of *Current Biology*, an error was inadvertently printed in Figure 3a. Tau1N4R was incorrectly shown as not expressed (– symbols) in lanes 4–10; the correct version of the figure, in which Tau1N4R is expressed in all lanes except for lane 2, can be seen in the opposite column.

In addition, an error appears in the second paragraph of the Discussion section on page 1667. The second sentence of this paragraph should read: "Yost *et al.* [35] have suggested that β-catenin is directly phosphorylated by GSK-3, consistent with the finding that phosphorylation of Armadillo is decreased in Zw3[sgg] mutant *Drosophila* embryos [36] and that, after treatment with Li^{+}, the migration of *Arm* is increased in gels (Fig. 4a)" and not "the migration of Zw3[sgg] is increased in gels" as stated.

**Figure 3**



Li^{+} inhibits GSK-3-dependent phosphorylation of Tau in intact cells. **(a)** Inhibition of GSK-3β by Li^{+} but not K^{+}. GSK-3β activity in cells cotransfected with plasmids encoding GSK-3β and Tau1N4R was assessed by measuring AT8 immunoreactivity following incubation with the indicated concentrations of Li^{+} (lanes 4–10) and K^{+} (lanes 11–17). The Tau.1 and anti-HA immunoblots are controls for Tau1N4R and HA-GSK-3β expression, respectively. **(b)** Inhibition of GSK-3βS9A and GSK-3α by Li^{+}; activity was assessed as in (a). **(c)** Scanning densitometry of AT8 blots in (a,b): cells expressed Tau1N4R and GSK-3β and were treated with Li^{+} (open circles) or K^{+} (filled circles), or expressed Tau1N4R and GSK-3βS9A and were treated with Li^{+} (open diamonds) or Li^{+} (open squares). Densities are normalized to those of untreated controls.