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Identification of eukaryotic elongation factor-2 as a novel cellular target of lithium and glycogen synthase kinase-3

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ABSTRACT

Inhibition of glycogen synthase kinase-3 (GSK-3) is thought to be a major consequence of the biological and clinical activity of the mood stabilizer lithium, however, lithium and GSK-3 may activate distinct cellular pathways. We employed a proteomic method to uncover new downstream targets of lithium, and then examined how these proteins are related to GSK-3. Proteomic analysis identified eukaryotic elongation factor-2 (eEF-2) as a cellular target of lithium. This was verified in SH-SY5Y cells and animal models. In cells, lithium decreased eEF-2 phosphorylation at its key inhibitory site, threonine 56, and blocked the enhancement of eEF-2 phosphorylation normally coupled with stress conditions such as nutrient and serum deprivation. Unexpectedly, inhibition of GSK-3 also enhanced eEF-2 phosphorylation, and overexpression of GSK-3 α or GSK-3 β resulted in a strong reduction in eEF-2 phosphorylation. Chronic administration of lithium reduced the hippocampal fraction of phospho-eEF-2 (phospho-eEF-2/total eEF-2) twofold in two different mouse strains. In summary, whereas eEF-2 is activated by both lithium and GSK-3, unexpectedly, lithium treatment and inhibition of GSK-3 have opposing effects on eEF-2.

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Introduction

For decades, lithium salts (lithium) have been used in treatment of bipolar disorder. Lithium has effects on various biological activities including neuroprotection, neurogenesis, and cognition (Manji et al., 1999; Pachet and Wisniewski, 2003; Huang and Klein, 2006; Watase et al., 2007; Macdonald et al., 2008). The downstream pathways regulated by lithium are not fully known. It is believed that inositol monophosphatase, phosphomonoesterase, specific adenylyl cyclase isoforms, β -arrestin-2–Akt complex, and glycogen synthase kinase-3 (GSK-3) are direct targets of lithium (Berridge et al., 1989; De Sarno et al., 2001; Phiel and Klein, 2001; Beaulieu et al., 2008; Bersudsky et al., 2008). Of particular interest in this regard is the protein kinase GSK-3, which has been recently implicated in the pathology of neurodegeneration and in behavior and cognitive function (Lucas et al., 2001; Chen et al., 2004; Gould et al., 2004b; Kaidanovich-beilin et al., 2004; O'Brien et al., 2004; Rockenstein et al., 2007; Terwel et al., 2008; Kaidanovich-Beilin et al., 2009). Lithium is a selective inhibitor of GSK-3; lithium inhibits GSK-3 directly and indirectly through enhanced serine phosphorylation and/or autoregulation (Klein and

Melton, 1996; De Sarno et al., 2001; Zhang et al., 2003; Kirshenboim et al., 2004). Inhibition of GSK-3 has remarkable parallels with the known effects of lithium, including effects on early development, glycogen synthesis, stabilization of β -catenin, and alterations in circadian rhythm (Cheng et al., 1983; Klein and Melton, 1996; Stambolic et al., 1996; Martinek et al., 2001; Gould et al., 2004a; Kim et al., 2006). In behavioral models, deletion of a single copy of GSK-3 β or treatment with pharmacological GSK-3 inhibitors results in anti-depression-like activity that is analogous to lithium administration (Gould et al., 2004b; Kaidanovich-Beilin et al., 2004; O'Brien et al., 2004). Thus, although GSK-3 may be a target of lithium-mediated clinical and biological activity, it is possible that lithium and GSK-3 activate distinct pathways.

The mammalian target of rapamycin, mTOR, is a nutrient and energy sensor that triggers protein synthesis and controls translation initiation and elongation (Proud, 2006). mTOR signaling enhances protein synthesis via phosphorylation and activation of the ribosomal protein S6 kinase (S6K), which, in turn, phosphorylates downstream targets such as the ribosomal protein S6 and eukaryotic initiation factor 4E binding protein-1 (4E-BP1) (Hay and Sonenberg, 2004; Proud, 2006). In addition, the mTOR/S6K pathway activates elongation factor-2 (eEF-2), an essential mediator of the ribosomal elongation step during polypeptide mRNA translation into a polypeptide protein (Proud et al., 2001). Phosphorylation of eEF-2 on threonine 56 (Thr 56) catalyzed by its upstream regulator, eEF-2 kinase, inhibits eEF-2's activity (Nairn and Palfrey, 1987; Ryazanov et

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al., 1988). This unusual calcium/calmodulin-dependent kinase belongs to the alpha kinase family, and may be regulated by several mTOR-dependent and independent kinases including S6K, protein kinase A (PKA), AMPK (AMP activated kinase), p90 RSK (S6 ribosomal activating kinase), and the stress activated protein kinase p38 (Redpath et al., 1993; Knebel et al., 2001; Wang et al., 2001; Browne et al., 2004). eEF-2 phosphorylation plays an important role in coupling protein synthesis with energy metabolism in response to calcium flux, AMP activated protein kinase (AMPK), insulin, nutrient limitation, and endoplasmic reticulum (ER) stress (Ryazanov, 1987; Wang et al., 1998; Patel et al., 2002; Yan et al., 2003; Boyce et al., 2008; Mariappan et al., 2008). Notably, aberrant protein synthesis has been implicated in neurological and cognitive dysfunction including mental retardation, autism, and memory deficits (Bassell and Warren, 2008; Kelleher and Bear, 2008; Narayanan et al., 2008). Hence, modulation of eEF-2 activity may have an important impact on neurological as well as mental behavior. Due to its therapeutic importance, identification of additional new targets of lithium is of central interest. Here we used a hypothesis-free proteomic approach to identify new cellular targets of lithium and explored their relation to GSK-3.

Results

eEF-2 is a downstream target of lithium

We took a proteomic approach based on a 2D gel separation to search for new targets affected by lithium treatment. SH-SY5Y cells were treated with 20 mM lithium for 16 h and total protein extracts were subjected to 2D gel electrophoresis. Comparison of the gel images of control and lithium-treated cells revealed significant changes in several protein spots. One spot, 4711, was consistently lower in the extracts from lithium-treated cells vs. control cells (Fig. 1A). This spot was identified as eEF-2 by mass spectrometry. We verified this result by treating the cells with lithium and determining the expression levels of eEF-2 by conventional western blot analysis. Lithium did not alter the levels of total eEF-2, but significantly reduced levels of phosphorylation at Thr 56 (Fig. 1B), a site known to confer inhibition of eEF-2 *in vivo* (Nairn and Palfrey, 1987; Ryazanov et al., 1988; Redpath et al., 1993; Laitusis et al., 1998; Pavur et al., 2000). Lithium enhanced serine phosphorylation of GSK-3 β (Fig. 1B) as previously described (De Sarno et al., 2001; Gould et al., 2004a; 122

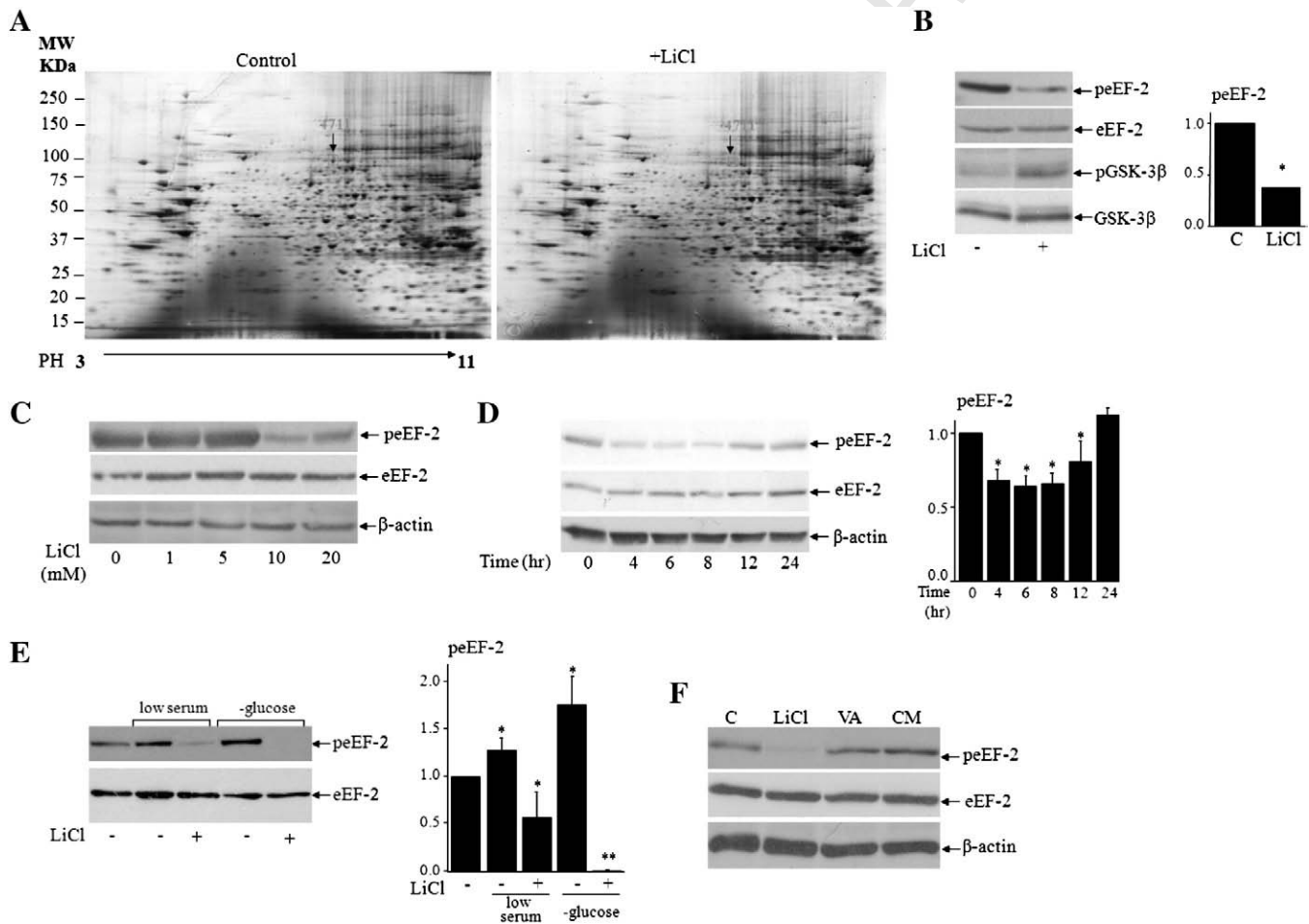


Fig. 1. eEF-2 is a downstream target of lithium. (A) SH-SY5Y cells were treated with 20 mM lithium for 16 h. Cells were lysed and equal amounts of cellular protein (200 μ g) were separated by 2D gel electrophoresis as described in the **Experimental methods**. The indicated spot 4711 was identified as eEF-2 by mass spectrometry. (B) SH-SY5Y cells treated with lithium as described in (A) were lysed and equal amounts of cellular protein (30 μ M) were subjected to gel electrophoresis followed by immunoblot analysis using anti-phospho (Thr 56) eEF-2 (peEF-2), anti-eEF-2, anti-phospho (Ser 9) GSK-3 β (pGSK-3 β), and anti-GSK-3 β antibodies. Densitometry analysis of peEF-2 (mean \pm SEM, $n = 5$) is shown in the right panel. (C) SH-SY5Y cells were treated with various concentrations of lithium for 16 h as indicated. Proteins were processed as described in (B), and peEF-2 and total eEF-2 were determined as described. Equal loading was confirmed by analysis of β -actin. (D) Time-course analysis of eEF-2 phosphorylation in lithium-treated cells. eEF-2 phosphorylation was determined as described. Densitometry analysis of peEF-2 (mean \pm SEM, $n = 3$) is shown in the right panel. (E) SH-SY5Y cells were incubated with a medium containing 0.1% FCS (low serum) or glucose-depleted medium (-glucose) for 16 h followed by treatment with lithium for another 4 h. eEF-2 phosphorylation was determined as described. Densitometry analysis of peEF-2 (mean \pm SEM, $n = 3$) is shown in the right panel. (F) SH-SY5Y cells were treated with 10 mM lithium, 1 mM valproic acid (VA), or 0.1 mM carbamazepine (CM) for 16 h. eEF-2 phosphorylation was determined as described. Representative gels from three to five independent experiments are shown in each panel. * indicates $p < 0.01$; and ** indicates $p < 0.001$.

Kirshenboim et al., 2004; O'Brien et al., 2004), thus verifying the effect of lithium on GSK-3. Since tissue-cultured cells employ mechanisms that exclude toxic cations (Leazer and Klaassen, 2003), and since the IC_{50} for lithium's inhibitory effect on cellular targets is in the millimolar range (Berridge et al., 1989; Klein and Melton, 1996), we expected that high lithium concentrations would be required to achieve an effective intracellular dose. The effective dose of lithium was in the range of 10 to 20 mM, as shown by a dose–response experiment (Fig. 1C, eEF-2 phosphorylation was reduced by $32\% \pm 9.5$ and $56\% \pm 14$ respectively, at 10 mM LiCl). Time–course analysis showed that lithium altered eEF-2 phosphorylation as early as 4 h after the start of treatment, and this effect was still observed after 12 h (Fig. 1D).

We next examined whether lithium affected eEF-2 under conditions known to enhance its phosphorylation, such as nutrient or serum limitation (Wang et al., 1998; Patel et al., 2002; Yan et al., 2003). Indeed, under these conditions, lithium reduced the levels of phosphorylation of eEF-2 compared to cells not treated with lithium (Fig. 1E), suggesting that lithium prevents the attenuation in protein synthesis under stress conditions.

Unlike lithium, neither valproic acid nor carbamazepine, two other known mood stabilizers, reduced eEF-2 phosphorylation (Fig. 1F). Thus, reduced eEF-2 phosphorylation is likely to be a specific effect of lithium rather than a common mechanism for mood stabilization.

eEF-2 is a cellular target of GSK-3

Two major hypotheses have been proposed to explain lithium's therapeutic mechanism: inositol depletion and inhibition of GSK-3 (Berridge et al., 1989; Klein and Melton, 1996; Gould et al., 2004a). We examined whether either of these actions could account for lithium's effect on eEF-2. Cells were incubated in inositol-depleted medium. Inositol depletion did not affect eEF-2 phosphorylation, nor did it influence the lithium-induced reduction of eEF-2 phosphorylation (Fig. 2A). Unexpectedly, inhibition of GSK-3 did not reduce, but rather enhanced, eEF-2 phosphorylation (Fig. 2B). This was observed in cells that were treated with three structurally dissimilar GSK-3 inhibitors: an ATP-competitive inhibitor, BIO-6 (Meijer et al., 2003), an allosteric non-ATP-competitive inhibitor, TDZD8 (Martinez et al., 2002), and a substrate-competitive inhibitor, L803-mts (Plotkin et al., 2003). Levels of β -catenin, an established target of GSK-3 (Liu et al., 2002; Gould et al., 2004a; O'Brien et al., 2004), were elevated in these cells, verifying that the GSK-3 inhibitors were active in these cells (Fig. 2B). Time–course analysis of cells treated with SB216763, another ATP-competitive inhibitor of GSK-3 (Coghlan et al., 2000), further indicated that inhibition of GSK-3 increased eEF-2 phosphorylation and that this effect was sustained for at least 24 h post-treatment (1.5 ± 0.2 fold increase in a eEF-2 phosphorylation after 24 h treatment) (Fig. 2C, left panel). This elevation in eEF-2 phosphorylation was accompanied by reduced phosphorylation of ribosomal protein S6 (Fig. 2C, right panel), which is correlated with increased protein synthesis (Peterson and Schreiber, 1998). On the other hand, lithium had the opposite effect: lithium treatment resulted in enhanced S6 phosphorylation compared to untreated cells (Fig. 2C, right panel). Overexpression of GSK-3 α or GSK-3 β using recombinant adenovirus led to a strong decrease in eEF-2 phosphorylation (about 50% reduction), that was accompanied by enhanced phosphorylation of S6 (Fig. 2D). When cells were subjected to a combined treatment of GSK-3 inhibitors and lithium, there was still a reduction in eEF-2 phosphorylation, suggesting that lithium overcame the opposing, phosphorylation-promoting effect of GSK-3 inhibition (Fig. 2E).

Finally, we examine whether lithium or GSK-3 impacted eEF-2 kinase (eEF-2 K) activity. eEF-2 kinase is responsible for phosphorylation of eEF-2 at Thr 56 (Nairn and Palfrey, 1987; Ryazanov et al., 1988). Phosphorylation at of eEF-2 kinase at serine 366 (Ser 366) by

S6 kinase inhibits the kinase activity (Wang et al., 2001). No significant or reproducible differences compared to untreated cells were observed in Ser 366 phosphorylation of eEF-2 kinase following treatment with lithium, GSK-3 inhibitors, or upon overexpression of GSK-3 α or GSK-3 β (Fig. 2F). Reduced phosphorylation of eEF-2 kinase at Ser 366 when cells were incubated in low glucose served as a 'positive' control (Fig. 2G). Thus, activation of S6 kinase (by lithium or by GSK-3 inhibitors) did not result in increased phosphorylation of eEF-2 kinase. It is possible that such activation was not sufficient to promote eEF-2 kinase phosphorylation. Alternatively, phosphorylated Ser 366 may not be stable but may undergo a rapid de-phosphorylation making it difficult to detect changes in its phosphorylation levels under these conditions. Finally, it is possible, that additional pathways activated by lithium or by GSK-3 inhibitors oppose this S6 kinase effect resulting in no detectable changes in eEF-2 kinase phosphorylation. Taken together, our data indicate that eEF-2 is a downstream cellular target of both lithium and GSK-3. However, inhibition of GSK-3 opposes lithium's effect on eEF-2 phosphorylation.

Chronic treatment with lithium reduces the phosphorylated eEF-2 fraction in the hippocampus

We next examined the impact of lithium on eEF-2 *in vivo*. We chose to study lithium's effect in the hippocampus since many studies have implicated this brain area in the pathogenesis of bipolar disorder and lithium's mood-stabilizing effect (Beyer et al., 2004; Strakowski et al., 2005; Scherk et al., 2008; Chepenik et al., 2009; Kim and Thayer, 2009). Two mice strains, ICR and Sabra mice, were fed a lithium-containing diet for 2 weeks in a regime that results in therapeutically relevant lithium levels (0.5–1.0 mM) (O'Brien et al., 2004; Bersudsky et al., 2008). This treatment resulted in upregulation of β -catenin and of GSK-3 β phosphorylation at Ser 9 (shown for the ICR mice, Fig. 3A, data not shown for Sabra mice). These are two well established *in vivo* effects of lithium (De Sarno et al., 2001; Gould et al., 2004a; Kirshenboim et al., 2004; O'Brien et al., 2004), validating lithium's *in vivo* impact in our models. In agreement with the *in vitro* results (Fig. 1B), lithium enhanced S6 phosphorylation in the hippocampus (shown for the ICR Fig. 3A). Consequently, although lithium treatment did not change phospho-eEF-2 levels *per se*, it significantly upregulated levels of total eEF-2 (Fig. 3B and C), resulting in a reduced phospho-eEF-2 fraction (the phospho-eEF-2/total eEF-2 ratio) (Fig. 3B and C).

Discussion

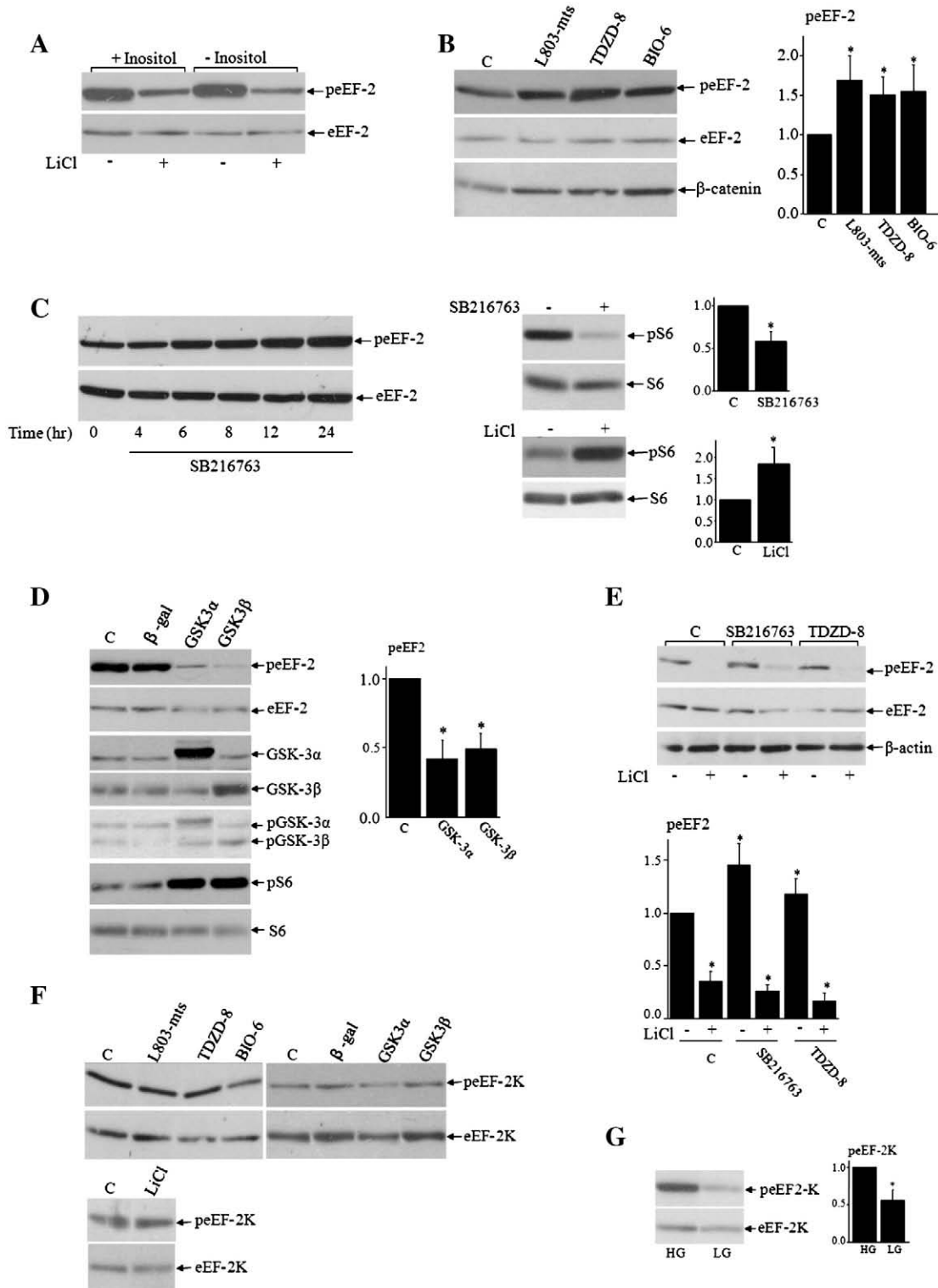
This is the first report that eEF-2, a mammalian elongation factor essential for protein synthesis, is a target of lithium and GSK-3. We showed that, *in vitro*, acute lithium administration reduced phosphorylation of eEF-2 at Thr 56, a key inhibitory site for this enzyme's activity. Lithium treatment also counteracted the inhibitory effect on eEF-2 induced by stress conditions such as nutrient limitation. Reduction of the phosphorylated fraction of eEF-2 was observed in the mouse hippocampus after chronic *in vivo* administration of lithium. Given that upregulation of phospho-eEF-2 directly inhibits protein synthesis (Marin et al., 1997; Boyce et al., 2008), it is conceivable that lithium enhances protein synthesis. This may be the mechanism of the therapeutically beneficial effects of lithium in neuroprotective activity, as well as its ability to reverse the detrimental physiological and behavioral consequences of stress (Hennion et al., 2002; Vasconcellos et al., 2003; Hiroi et al., 2005). It should be noted that increased phosphorylation of eEF-2 has been found in pathological conditions associated with cell death, brain damage, and Alzheimer's disease (Johnson et al., 1992; Althausen et al., 2001; Boyce et al., 2008). Reduced mRNA translation has also been recognized in patients with neuronal damage and bipolar disorder (MacQueen et al., 2003; Sheline et al., 2003). Hence, lithium may

249 prevent or attenuate the biological consequences that are associated
250 with these pathologies.

251 Surprisingly, GSK-3 inhibition did not mimic lithium's effect on
252 eEF-2 phosphorylation, as we expected. In contrast, inhibition of GSK-
253 3 resulted in enhanced eEF-2 phosphorylation, whereas overexpression
254 of either GSK-3 α or GSK-3 β reduced eEF-2 phosphorylation
255 compared with the levels in untreated cells. Thus, eEF-2 is a cellular
256 target affected by both lithium and GSK-3, but lithium's reduction of
257 eEF-2 phosphorylation does not appear to be mediated through its

258 ability to inhibit GSK-3. Inhibition of GSK-3 by lithium is not sufficient
259 to enhance eEF-2 phosphorylation (see Fig. 2E). Marriappan et al.
260 showed that inhibition of GSK-3 by insulin correlates with enhanced
261 phosphorylation of eEF-2 (Mariappan et al., 2008). This is the opposite
262 of what we observed. It is likely that regulation of eEF-2 by GSK-3 is
263 dependent on cell type and stimulation type.

264 The precise molecular mechanism underlying lithium's reduction
265 of eEF-2 phosphorylation remains unknown. It is possible that lithium
266 influences eEF-2 kinase, which is the only kinase known thus far to



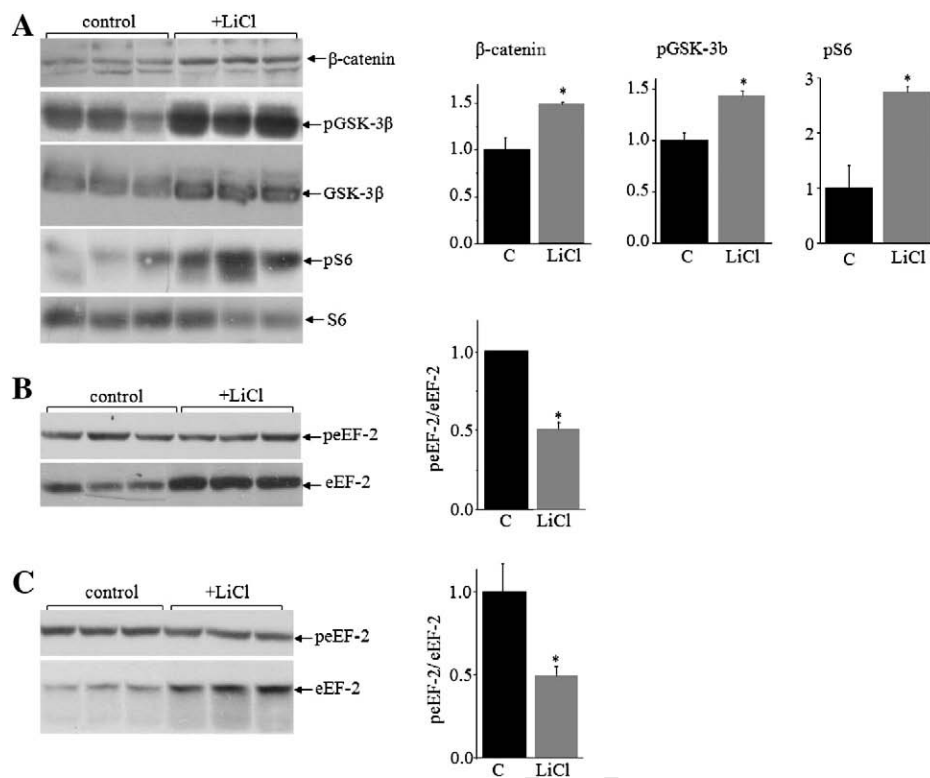


Fig. 3. *In vivo* treatment of lithium affects eEF-2 in the hippocampus. (A) ICR mice received lithium in their food for 2 weeks as described in the [Experimental methods](#). Hippocampal proteins were extracted, and equal amounts of protein (30 μg) were subjected to gel electrophoresis, followed by immunoblot analysis using anti-β-catenin, anti-phospho (Ser 9) GSK-3β, anti-phospho S6, anti-GSK-3β and anti-S6 antibodies. Representative gels from three different hippocampal samples are shown in the left panel. Densitometry analysis of β-catenin and phosphorylated proteins is shown in the right panel. Results present the means of 12 animals/group ± SEM; * indicates $p < 0.01$. (B) Same as in (A) except that antibodies against phospho-eEF-2 and total eEF-2 were used. The ratio of phosphorylated eEF-2/total eEF-2 as calculated from densitometry analysis is shown in the right panel. Results are means of 12 animals/group ± SEM; * indicates $p < 0.01$. (C) Same as in (A) except that Sabra mice were used. Densitometry results are shown in the right panel and are means ± SEM of 10 animals; * indicates $p < 0.01$.

phosphorylate eEF-2 on Thr 56 (Ryazanov et al., 1988). eEF-2 kinase may be regulated by several mTOR-dependent kinases including S6K, protein kinase A (PKA), AMPK (AMP activated kinase), and p90 RSK (S6 ribosomal activating kinase) (Redpath et al., 1993; Wang et al., 2001; Browne et al., 2004). In addition, lithium may inhibit eEF-2 kinase via activation of p38 (Nemeth et al., 2002), through phosphorylation of eEF-2 kinase at ser 359 (Knebel et al., 2001). Hence, lithium may inhibit eEF-2 kinase by regulating one or more of these protein kinases, except for Ser 366, that we found to be insensitive to lithium. Reduced eEF-2 phosphorylation could be also mediated by elevated protein phosphatase activity; yet lithium was shown to inhibit rather than to activate a variety of phosphatases, particularly the protein phosphatases PP2A and PP1 (Mora et al., 2002; Lahne et al., 2006). Lithium's effect on eEF-2 phosphorylation

could also be mediated by its effect on cellular pH (Aronson, 1985). This, in turn, might inhibit eEF-2 kinase activity (Dorovkov et al., 2002).

GSK-3 may influence eEF-2 phosphorylation through pathways that are similar to, or distinct from, those discussed above. Interestingly, GSK-3 has been characterized as a PP1 activator (Vandenhede et al., 1985; Picking et al., 1991), and it is therefore possible that GSK-3-reduced phosphorylation of eEF-2 is mediated through activation of PP1. Both lithium and GSK-3 have been previously implicated in the regulation of protein synthesis: lithium was shown to alleviate translational repression of TOP mRNA (mRNA with 5'-terminal oligopyrimidine tract) (Stolovich et al., 2005), and GSK-3 was implicated as a negative regulator of translation via phosphorylation of eukaryotic initiation factor-2B (eIF-2B) (Welsh

Fig. 2. eEF-2 is a downstream target of GSK-3. (A) SH-SY5Y cells were incubated with or without inositol (16 h) prior to treatment with 10 mM lithium for 6 h. Cells were lysed and equal amounts of cellular protein (30 μM) were subjected to gel electrophoresis followed by immunoblot analysis using anti-phospho (Thr 56) eEF-2 (peEF-2) and anti-eEF-2 antibodies. (B) SH-SY5Y cells were treated with GSK-3 inhibitors: L803-mts (40 μM), TDZD8 (20 μM), and BIO-6 (10 μM) for 4 h. Proteins were processed as described in (A). Amounts of phosphorylated eEF-2, total eEF-2 and β-catenin were determined. Densitometry analysis of peEF-2 (mean ± SEM, $n = 3-6$) is shown in the right panel. (C) The left panel shows a time-course analysis of eEF-2 phosphorylation in cells treated with GSK-3 inhibitor SB216763 (10 μM). eEF-2 phosphorylation was determined as described above. The right panel shows levels of S6 phosphorylation (pS6, ser 240/244) and total S6 as determined by western blot in cells were treated with SB216763 (10 μM) or lithium (10 mM) for 6 h. Densitometry analysis of pS6 is presented (mean ± SEM, $n = 5-6$). (D) SH-SY5Y cells were infected with recombinant adenovirus coding for GSK-3β or GSK-3α as described in the [Experimental methods](#). Cells were harvested 30 h post-infection and proteins were processed as described in (A). Tyrosine phosphorylation of GSK-3α and GSK-3β (Tyr216/274) and phosphorylation of eEF-2 and S6 were analyzed. Densitometry analysis of peEF-2 (mean ± SEM, $n = 3$) is shown in the right panel. (E) SH-SY5Y cells were treated with GSK-3 inhibitors SB216763 (10 μM) or TDZD-8 (10 μM) for 2 h followed by addition of lithium (10 mM) for an additional 4 h. Phosphorylation of eEF-2 was determined as described. Densitometry analysis of peEF-2 (mean ± SEM, $n = 3$) is shown in the lower panel. (F) Cells were treated with GSK-3 inhibitors or lithium (10 mM) for 4 h as indicated. Phosphorylation of eEF-2 kinase (Ser 366, peEF-2-K) was determined in inhibitor-treated cells or in cells overexpressing GSK-3α or GSK-3β. (G) A 'positive' control for the experiment shown in (F) indicated that phosphorylation of eEF-2 kinase at Ser 366 is significantly reduced by low glucose (high glucose (HG), 11 mM; low glucose (LG), 1 mM). Right panel shows densitometry analysis of phosphorylated eEF-2 kinase (mean ± SEM, $n = 3$). All panels show representative gels from three to five independent experiments. * indicates $p < 0.01$.

and Proud, 1993; Proud, 2006). Lithium appears to enhance protein synthesis via its effect on eEF-2 in mechanism that is not mediated through GSK-3. The impact of GSK-3 is more complex since it mediates opposing effects on different components of the translation machinery.

Experimental methods

Reagents

GSK-3 inhibitors SB216763 and TDZD8 were purchased from Calbiochem (La Jolla, CA, USA). BIO-6, valproic acid, and carbamazepine were from Sigma (Rehovot, Israel). A substrate-competitive inhibitor of GSK-3, L803-mts, was previously described by us (Plotkin et al., 2003). Anti-phospho-GSK-3 β (Ser 9), anti-phospho-eEF-2 (Thr 56), anti-phospho-eEF-2 kinase (Ser 366), and anti-phospho S6 protein (Ser 240/244) antibodies were from Cell Signaling Technologies (Beverly, MA, USA). Anti-GSK-3 β antibody was from Transduction Laboratories (Lexington, KY, USA).

Two-dimensional gel separation and proteomic analysis

Human neuroblastoma SH-SY5Y cells were treated with 20 mM LiCl for 16 h. Cells were lysed with guanidinium isothiocyanate-phenol (TRIZOL). Two-dimensional (2D) gel separation and mass spectrometry analysis were performed as an outsource service at the Smoler Proteomics Center of the Technion in Haifa, Israel. (<http://biology.technion.ac.il/proteomics/index.htm>).

Cell culture and protein analysis

SH-SY5Y cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 5 mM L-glutamine, 0.15% sodium bicarbonate, and 0.08% gentamycin. Cells were treated with lithium and other inhibitors as indicated in the text. In some experiments, glucose concentrations were changed as indicated over the range from 0 to 11 mM. For the amino-acid-depletion experiments, cells were incubated in a Krebs-Ringer-HEPES buffer (12 mM NaCl, 0.4 mM KH₂PO₄, 0.1 mM MgSO₄, 0.1 mM CaCl₂, 1 mM NaHCO₃, and 3 mM HEPES, pH 7.4) containing 11 mM glucose. In all experiments, serum levels were 0.5% unless otherwise indicated. At the end of the experiments, cells were collected and lysed in an ice-cold buffer G (20 mM Tris, pH 7.3, 10 mM β -glycerophosphate, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate (NaPPi), 25 μ g/ml leupeptin, 25 μ g/ml aprotinin, 1 mM DTT, 500 nM microcystine LR, and 1% Triton X-100). Cell extracts were centrifuged at 15,000 \times g for 20 min, and supernatants were collected. Equal amounts of protein (30 μ g), as determined by Bradford analysis, were boiled with Laemmli sample buffer and subjected to gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted with a specific antibody (1:1000 dilution) as indicated. For infection, cells were incubated with recombinant adenovirus coding for GSK-3 α or GSK-3 β (generated by Ziva Liberman using the AdEasy™ Adenoviral Vector System, Stratagene, La Jolla, CA, USA) at 1:1000 dilution for 24 h. Cells were washed and incubated with fresh growth medium for an additional 6 to 8 h.

Animals

Male ICR or Sabra mice (Harlan, Jerusalem, Israel) aged 12–13 or 8 weeks, respectively, were housed in individual cages in a temperature-controlled facility with a 12-h light/dark cycle. The mice were randomly assigned to two groups (12 ICR or 10 Sabra mice each), given tap water *ad libitum* and fed regular powdered chow for rodents (Harlan, Teklad, Jerusalem, Israel). Additional drinking water containing 0.9% NaCl was provided to prevent electrolyte imbalance. The

treatment with lithium was adapted from O'Brien et al. (O'Brien et al., 2004) to produce serum levels of 0.5 to 1 mM. In brief, mice received the powdered chow mixed with 0.2% LiCl for 5 days followed by 0.4% LiCl for 10 additional days. On day 15, mice were decapitated and the hippocampus was removed and immediately frozen in liquid nitrogen. Trunk blood was collected and serum lithium levels were measured using an AVL 9180 Electrolyte Analyzer (Hoffmann-La Roche, Basel, Switzerland). The experimental procedures were approved by the Ben-Gurion University of the Negev (Beer-Sheva, Israel) and Ariel University Center (Ariel, Israel) Institutional Review Committee for the Use of Animals and were carried out in compliance with the declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Tissue extracts

Hippocampi were homogenized in ice-cold buffer H (150 mM β -glycerophosphate, pH 7.3, 50% glycerol, 5 mM EGTA, 5 mM EDTA, 40 mM NaF, 25 mM NaPPi, 25 μ g/ml leupeptin, 25 μ g/ml aprotinin, 1 mM DTT, and 1% NP40). The extracts were centrifuged at \times 15,000 g for 20 min and supernatants were collected. Equal amounts of protein (30–50 μ g) were subjected to western blot analysis as described above.

Statistical analysis

Data were analyzed with Origin Professional 6.0 software using the Student's *t*-test to compare control and lithium-treated animals. Data were considered significant at $p < 0.05$.

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References

- Althausen, S., Mengesdorf, T., Mies, G., Olah, L., Nairn, A.C., Proud, C.G., Paschen, W., 2001. Changes in the phosphorylation of initiation factor eIF-2 α , elongation factor eEF-2 and p70 S6 kinase after transient focal cerebral ischaemia in mice. *J. Neurochem.* 78, 779–787. 384
- Aronson, P.S., 1985. Properties of the renal Na⁺ – H + exchanger. *Ann. NY Acad. Sci.* 456, 220–228. 387
- Bassell, G.J., Warren, S.T., 2008. Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. *Neuron* 60, 201–214. 389
- Beaulieu, J.M., Marion, S., Rodriguiz, R.M., Medvedev, I.O., Sotnikova, T.D., Ghisi, V., Wetsel, W.C., Lefkowitz, R.J., Gainetdinov, R.R., Caron, M.G., 2008. A beta-arrestin 2 signaling complex mediates lithium action on behavior. *Cell* 132, 125–136. 392
- Berridge, M.J., Downes, C.P., Hanley, M.R., 1989. Neural and developmental actions of lithium: a unifying hypothesis. *Cell* 59, 411–419. 394
- Bersudsky, Y., Shaldubina, A., Kozlovsky, N., Woodgett, J.R., Agam, G., Belmaker, R.H., 2008. Glycogen synthase kinase-3 β 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.* 19, 217–224. 398
- Beyer, J.L., Kuchibhatla, M., Payne, M.E., Moo-Young, M., Cassidy, F., Macfall, J., Krishnan, K.R., 2004. Hippocampal volume measurement in older adults with bipolar disorder. *Am. J. Geriatr. Psychiatry* 12, 613–620. 401
- Boyce, M., Py, B.F., Ryazanov, A.G., Minden, J.S., Long, K., Ma, D., Yuan, J., 2008. A pharmacoproteomic approach implicates eukaryotic elongation factor 2 kinase in ER stress-induced cell death. *Cell Death Differ.* 15, 589–599. 404
- Browne, G.J., Finn, S.G., Proud, C.G., 2004. Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398. *J. Biol. Chem.* 279, 12220–12231. 407
- Chen, G., Bower, K.A., Ma, C., Fang, S., Thiele, C.J., Luo, J., 2004. Glycogen synthase kinase 3 β (GSK3 β) mediates 6-hydroxydopamine-induced neuronal death. *FASEB J.* 18, 1162–1164. 410
- Cheng, K., Creacy, S., Lerner, J., 1983. ‘Insulin-like’ effects of lithium ion on isolated rat adipocytes. I. Stimulation of glycogenolysis beyond glucose transport. *Mol. Cell. Biochem.* 56, 177–182. 413
- Chenopik, L.G., Fredericks, C., Papademetris, X., Spencer, L., Lacadie, C., Wang, F., Pittman, B., Duncan, J.S., Staib, L.H., Duman, R.S., Gelernter, J., Blumberg, H.P., 2009. Effects of the brain-derived neurotrophic growth factor val66met variation on hippocampus morphology in bipolar disorder. *Neuropsychopharmacology* 34, 944–951. 418

- Coghlan, M.P., Culbert, A.A., Cross, D.A., Corcoran, S.L., Yates, J.W., Pearce, N.J., Rausch, O.L., Murphy, G.J., Carter, P.S., Roxbee Cox, L., Mills, D., Brown, M.J., Haigh, D., Ward, R.W., Smith, D.G., Murray, K.J., Reith, A.D., Holder, J.C., 2000. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem. Biol.* 7, 793–803.
- De Sarno, P., Li, X., Jope, R.S., 2001. Regulation of Akt and glycogen synthase kinase-3 β phosphorylation by sodium valproate and lithium. *Neuropharmacology* 43, 1158–1164.
- Dorovkov, M.V., Pavur, K.S., Petrov, A.N., Ryazanov, A.G., 2002. Regulation of elongation factor-2 kinase by pH. *Biochemistry* 41, 13444–13450.
- Gould, T.D., Chen, G., Manji, H.K., 2004a. In vivo evidence in the brain for lithium inhibition of glycogen synthase kinase-3. *Neuropsychopharmacology* 29, 32–38.
- Gould, T.D., Einat, H., Bhat, R., Manji, H.K., 2004b. AR-A014418, a selective GSK-3 inhibitor, produces antidepressant-like effects in the forced swim test. *Int. J. Neuropsychopharmacol.* 7, 387–390.
- Hay, N., Sonenberg, N., 2004. Upstream and downstream of mTOR. *Genes Dev.* 18, 1926–1945.
- Hennion, J.P., el-Masri, M.A., Huff, M.O., el-Mailakh, R.S., 2002. Evaluation of neuroprotection by lithium and valproic acid against ouabain-induced cell damage. *Bipolar Disord.* 4, 201–206.
- Hiroi, T., Wei, H., Hough, C., Leeds, P., Chuang, D.M., 2005. Protracted lithium treatment protects against the ER stress elicited by thapsigargin in rat PC12 cells: roles of intracellular calcium, GRP78 and Bcl-2. *Pharmacogenomics* 5, 102–111.
- Huang, H.C., Klein, P.S., 2006. Multiple roles for glycogen synthase kinase-3 as a drug target in Alzheimer's disease. *Curr. Drug Targets* 7, 1389–1397.
- Johnson, G., Gotlib, J., Haroutyan, V., Bierer, L., Nairn, A.C., Merrill, C., Wallace, W., 1992. Increased phosphorylation of elongation factor 2 in Alzheimer's disease. *Brain Res. Mol. Brain Res.* 15, 319–326.
- Kaidanovich-beilin, O., Milman, A., Weizman, A., Pick, C., Eldar-Finkelman, H., 2004. Rapid anti-depressive like activity of specific GSK-3 inhibitor, and its effect on -catenin in the mouse hippocampus. *Biol. Psychiatry*.
- Kaidanovich-Beilin, O., Lipina, T.V., Takao, K., van Eede, M., Hattori, S., Laliberte, C., Khan, M., Okamoto, K., Chambers, J.W., Fletcher, P.J., Macaulay, K., Doble, B.W., Henkelman, M., Miyakawa, T., Roder, J., Woodgett, J.R., 2009. Abnormalities in brain structure and behavior in GSK-3 α mutant mice. *Mol. Brain* 2, 35.
- Kelleher 3rd, R.J., Bear, M.F., 2008. The autistic neuron: troubled translation? *Cell* 135, 401–406.
- Kim, H.J., Thayer, S.A., 2009. Lithium increases synapse formation between hippocampal neurons by depleting phosphoinositides. *Mol. Pharmacol.* 75, 1021–1030.
- Kim, W.Y., Zhou, F.Q., Zhou, J., Yokota, Y., Wang, Y.M., Yoshimura, T., Kaibuchi, K., Woodgett, J.R., Anton, E.S., Snider, W.D., 2006. Essential roles for GSK-3 α and GSK-3 β in neurotrophin-induced and hippocampal axon growth. *Neuron* 52, 981–996.
- Kirshenboim, N., Plotkin, B., Ben Shlomo, S., Kaidanovich-Beilin, O., Eldar-Finkelman, H., 2004. Lithium-mediated phosphorylation of glycogen synthase kinase-3 involves PI3 Kinase-dependent activation of protein kinase C- α . *J. Mol. Neurosci.* 24, 199–207.
- Klein, P.S., Melton, D.A., 1996. A molecular mechanism for the effect of lithium on development. *Proc. Nat. Acad. Sci. U.S.A.* 93, 8455–8459.
- Knebel, A., Morrice, N., Cohen, P., 2001. A novel method to identify protein kinase substrates: eEF2 kinase is phosphorylated and inhibited by SAPK4/p38 δ . *EMBO J.* 20, 4360–4369.
- Lahne, H.U., Kloster, M.M., Lefdal, S., Blomhoff, H.K., Naderi, S., 2006. Degradation of cyclin D3 independent of Thr-283 phosphorylation. *Oncogene* 25, 2468–2476.
- Laitusis, A.L., Brostrom, C.O., Ryazanov, A.G., Brostrom, M.A., 1998. An examination of the role of increased cytosolic free Ca²⁺ concentrations in the inhibition of mRNA translation. *Arch. Biochem. Biophys.* 354, 270–280.
- Leazer, T.M., Klaassen, C.D., 2003. The presence of xenobiotic transporters in rat placenta. *Drug Metab. Dispos.* 31, 153–167.
- Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G.H., Tan, Y., Zhang, Z., Lin, X., He, X., 2002. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* 108, 837–847.
- Lucas, J.J., Hernandez, F., Gomez-Ramos, P., Moran, M.A., Hen, R., Avila, J., 2001. Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3 β conditional transgenic mice. *EMBO J.* 20, 27–39.
- Macdonald, A., Briggs, K., Poppe, M., Higgins, A., Velayudhan, L., Lovestone, S., 2008. A feasibility and tolerability study of lithium in Alzheimer's disease. *Int. J. Geriatr. Psychiatry*.
- MacQueen, G.M., Campbell, S., McEwen, B.S., Macdonald, K., Amano, S., Joffe, R.T., Nahmias, C., Young, L.T., 2003. Course of illness, hippocampal function, and hippocampal volume in major depression. *Proc. Nat. Acad. Sci. U.S.A.* 100, 1387–1392.
- Manji, H.K., Moore, G.J., Chen, G., 1999. Lithium at 50: have the neuroprotective effects of this unique cation been overlooked? *Biol. Psychiatry* 46, 929–940.
- Mariappan, M.M., Shetty, M., Sataranatarajan, K., Choudhury, G.G., Kasinath, B.S., 2008. Glycogen synthase kinase 3 β is a novel regulator of high glucose- and high insulin-induced extracellular matrix protein synthesis in renal proximal tubular epithelial cells. *J. Biol. Chem.* 283, 30566–30575.
- Marin, P., Nastiuik, K.L., Daniel, N., Girault, J.A., Czernik, A.J., Glowinski, J., Nairn, A.C., Premont, J., 1997. Glutamate-dependent phosphorylation of elongation factor-2 and inhibition of protein synthesis in neurons. *J. Neurosci.* 17, 3445–3454.
- Martinek, S., Inonog, S., Manoukian, A.S., Young, M.W., 2001. A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock. *Cell* 105, 769–779.
- Martinez, A., Alonso, M., Castro, A., Perez, C., Moreno, F.J., 2002. First non-ATP competitive glycogen synthase kinase 3 β (GSK-3 β) inhibitors: thiazolidinones (TDZD) as potential drugs for the treatment of Alzheimer's disease. *J. Med. Chem.* 45, 1292–1299.
- Mora, A., Sabio, G., Risco, A.M., Cuenda, A., Alonso, J.C., Soler, G., Centeno, F., 2002. Lithium blocks the PKB and GSK3 dephosphorylation induced by ceramide through protein phosphatase-2A. *Cell. Signal.* 14, 557–562.
- Nairn, A.C., Palfrey, H.C., 1987. Identification of the major Mr 100,000 substrate for calmodulin-dependent protein kinase III in mammalian cells as elongation factor-2. *J. Biol. Chem.* 262, 17299–17303.
- Narayanan, U., Nalavadi, V., Nakamoto, M., Thomas, G., Ceman, S., Bassell, G.J., Warren, S.T., 2008. S6K1 phosphorylates and regulates fragile X mental retardation protein (FMRP) the neuronal protein synthesis-dependent mammalian target of rapamycin (mTOR) signaling cascade. *J. Biol. Chem.* 283, 18478–18482.
- Nemeth, Z.H., Deitch, E.A., Szabo, C., Hauser, C.J., Hasko, G., 2002. Lithium induces NF- κ B activation and interleukin-8 production in human intestinal epithelial cells. *J. Biol. Chem.* 277, 7713–7719.
- O'Brien, W.T., Harper, A.D., Jove, F., Woodgett, J.R., Maretto, S., Piccolo, S., Klein, P.S., 2004. Glycogen synthase kinase-3 β haploinsufficiency mimics the behavioral and molecular effects of lithium. *J. Neurosci.* 24, 6791–6798.
- Pachet, A.K., Wisniewski, A.M., 2003. The effects of lithium on cognition: an updated review. *Psychopharmacology (Berl)* 170, 225–234.
- Patel, J., McLeod, L.E., Vries, R.G., Flynn, A., Wang, X., Proud, C.G., 2002. Cellular stresses profoundly inhibit protein synthesis and modulate the states of phosphorylation of multiple translation factors. *Eur. J. Biochem.* 269, 3076–3085.
- Pavur, K.S., Petrov, A.N., Ryazanov, A.G., 2000. Mapping the functional domains of elongation factor-2 kinase. *Biochemistry* 39, 12216–12224.
- Peterson, R.T., Schreiber, S.L., 1998. Translation control: connecting mitogens and the ribosome. *Curr. Biol.* 8, R248–R250.
- Phiel, C.J., Klein, P.S., 2001. Molecular targets of lithium action. *Ann. Rev. Pharm. Toxicol.* 41, 789–813.
- Picking, W.D., Kudlicki, W., Kramer, G., Hardesty, B., Vandenhede, J.R., Merlevede, W., Park, I.K., DePaoli-Roach, A., 1991. Fluorescence studies on the interaction of inhibitor 2 and okadaic acid with the catalytic subunit of type 1 phosphoprotein phosphatases. *Biochemistry* 30, 10280–10287.
- Plotkin, B., Kaidanovich, O., Talior, I., Eldar-Finkelman, H., 2003. Insulin mimetic action of synthetic phosphorylated peptide inhibitors of glycogen synthase kinase-3. *J. Pharmacol. Exp. Ther.* 974–980.
- Proud, C.G., 2006. Regulation of protein synthesis by insulin. *Biochem. Soc. Trans.* 34, 213–216.
- Proud, C.G., Wang, X., Patel, J.V., Campbell, L.E., Kleijn, M., Li, W., Browne, G.J., 2001. Interplay between insulin and nutrients in the regulation of translation factors. *Biochem. Soc. Trans.* 29, 541–547.
- Redpath, N.T., Price, N.T., Severinov, K.V., Proud, C.G., 1993. Regulation of elongation factor-2 by multisite phosphorylation. *Eur. J. Biochem.* 213, 689–699.
- Rockenstein, E., Torrance, M., Adame, A., Mante, M., Bar-on, P., Rose, J.B., Crews, L., Masliah, E., 2007. Neuroprotective effects of regulators of the glycogen synthase kinase-3 β signaling pathway in a transgenic model of Alzheimer's disease are associated with reduced amyloid precursor protein phosphorylation. *J. Neurosci.* 27, 1981–1991.
- Ryazanov, A.G., 1987. Ca²⁺/calmodulin-dependent phosphorylation of elongation factor 2. *FEBS Lett.* 214, 331–334.
- Ryazanov, A.G., Shestakova, E.A., Natapov, P.G., 1988. Phosphorylation of elongation factor 2 by EF-2 kinase affects rate of translation. *Nature* 334, 170–173.
- Scherk, H., Backens, M., Schneider-Axmann, T., Kemmer, C., Usher, J., Reith, W., Falkai, P., Gruber, O., 2008. Neurochemical pathology in hippocampus in euthymic patients with bipolar I disorder. *Acta Psychiatr. Scand.* 117, 283–288.
- Sheline, Y.I., Gado, M.H., Kraemer, H.C., 2003. Untreated depression and hippocampal volume loss. *Am. J. Psychiatry* 160, 1516–1518.
- Stambolic, V., Ruel, L., Woodgett, J.R., 1996. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr. Biol.* 6, 1664–1668.
- Stolovich, M., Lerer, T., Bolkier, Y., Cohen, H., Meyuhas, O., 2005. Lithium can relieve translational repression of TOP mRNAs elicited by various blocks along the cell cycle in a glycogen synthase kinase-3- and S6-kinase-independent manner. *J. Biol. Chem.* 280, 5336–5342.
- Strakowski, S.M., Adler, C.M., Holland, S.K., Mills, N.P., DelBello, M.P., Eliassen, J.C., 2005. Abnormal FMRI brain activation in euthymic bipolar disorder patients during a counting Stroop interference task. *Am. J. Psychiatry* 162, 1697–1705.
- Terwel, D., Muyllaert, D., Dewachter, I., Borghgraef, P., Croes, S., Devijver, H., Van Leuven, F., 2008. Amyloid activates GSK-3 β to aggravate neuronal tauopathy in bigenic mice. *Am. J. Pathol.* 172, 786–798.
- Vandenhede, J.R., Yang, S.D., Merlevede, W., Jurgensen, S., Chock, P.B., 1985. Kinase FA-mediated regulation of rabbit skeletal muscle protein phosphatase. Reversible phosphorylation of the modulator subunit. *J. Biol. Chem.* 260, 10512–10516.
- Vasconcelos, A.P., Tabajara, A.S., Ferrari, C., Rocha, E., Dalmaz, C., 2003. Effect of chronic stress on spatial memory in rats is attenuated by lithium treatment. *Physiol. Behav.* 79, 143–149.
- Wang, X., Campbell, L.E., Miller, C.M., Proud, C.G., 1998. Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem. J.* 334 (Pt 1), 261–267.
- Wang, X., Li, W., Williams, M., Terada, N., Alessi, D.R., Proud, C.G., 2001. Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. *EMBO J.* 20, 4370–4379.
- Watake, K., Gatchel, J.R., Sun, Y., Emamian, E., Atkinson, R., Richman, R., Mizusawa, H., Orr, H.T., Shaw, C., Zoghbi, H.Y., 2007. Lithium therapy improves neurological function and hippocampal dendritic arborization in a spinocerebellar ataxia type 1 mouse model. *PLoS Med.* 4, e182.
- Welsh, G.L., Proud, C.G., 1993. Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochem. J.* 294, 625–629.
- Yan, L., Nairn, A.C., Palfrey, H.C., Brady, M.J., 2003. Glucose regulates EF-2 phosphorylation and protein translation by a protein phosphatase-2A-dependent mechanism in INS-1-derived 832/13 cells. *J. Biol. Chem.* 278, 18177–18183.
- Zhang, F., Phiel, C.J., Spece, L., Gurvich, N., Klein, P.S., 2003. Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. *J. Biol. Chem.* 278, 33067–33077.