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Deletion or inhibition of prolyl oligopeptidase blocks lithium-induced phosphorylation of GSK3b and Akt by activation of protein phosphatase 2A

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Abstract

Alterations in prolyl oligopeptidase (PREP) activity have been connected, for example, with bipolar and major depressive disorder, and several studies have reported that lack or inhibition of PREP blocks the effects of lithium on inositol 1,4,5-triphosphate (IP_3) levels. However, the impact of PREP modulation on other intracellular targets of lithium, such as glycogen synthase kinase 3 beta (GSK3b) or protein kinase B (Akt), has not been studied. We recently found that PREP regulates protein phosphatase 2A (PP2A), and because GSK3b and Akt are PP2A substrates, we studied if PREP-related lithium insensitivity is dependent on PP2A. To assess this, HEK-293 and SH-SY5Y cells with PREP deletion or PREP inhibition (KYP-2047) were exposed to lithium, and thereafter, the phosphorylation levels of GSK3b and Akt were measured by Western blot. As expected, PREP deletion and inhibition blocked the lithium-induced phosphorylation on GSK3b and Akt in both cell lines. When lithium exposure was combined with okadaic acid, a PP2A inhibitor, KYP-2047 did not have effect on lithium-induced GSK3b and Akt phosphorylation. Therefore, we conclude that PREP deletion or inhibition blocks the intracellular effects of lithium on GSK3b and Akt via PP2A activation.

KEYWORDS
bipolar disorder, depression, glycogen synthase kinase 3 beta, lithium, prolyl oligopeptidase

1 | INTRODUCTION AND BACKGROUND

Lithium has been in use for more than 70 years as a mood stabilizer, and it is still commonly used for bipolar disorder.[1] It has also beneficial effects on drug-resistant major depression.[2] One main mechanism for lithium is considered to be direct inhibition of glycogen synthase kinase 3β (GSK3b) via phosphorylation that has been comprehensively presented in various models (for reviews, see Freland and Beaulieu and Malhi and Outhred[3,4]) and in patients with bipolar disorder.[5] Additionally, dysregulated GSK3b has been connected with several mental disorders where lithium has therapeutic effect, but the results are not unambiguous.[6–9] GSK3b is an important kinase in the cell, regulating, for example, glucose response, neuronal plasticity and apoptosis, and GSK3b inhibition by lithium has been shown

to reduce apoptosis and to induce neuronal plasticity and growth via brain-derived neurotrophic factor (BDNF; for review, see Freland and Beaulieu[3]). Another important target for biological effects of lithium is protein kinase B (Akt, PKB),[10] and lithium induces the activation of Akt by phosphorylating it.[11] A recent study discovered hypoactive Akt in prefrontal cortex of bipolar patients that can in part explain the beneficial effects of lithium in this disease.[12] However, lithium regulates also several other cellular pathways, and the exact mechanism-of-action for lithium is not known.[13]

Although lithium is still an effective treatment for bipolar disorder, there is still a substantial number of patients who do not respond to it, particularly in bipolar depression.[14] There are several genetic mutations (for review, see Pickard[15]) and intracellular mechanisms that may explain lithium insensitivity, for example, decreased expression of lymphoid enhancer binding that leads to dysregulated Wnt/β-catenin signalling[16] or alterations in phosphodiesterase 11A expression that contributes to cyclic nucleotide signalling.[17] Another factor that blocks several effects of lithium in the cellular models is the lack of a serine protease, prolyl oligopeptidase (PREP, aka POP). In 1999, Williams et al showed that the lack of DpoA gene, encoding PREP, causes the loss of lithium response in *dictyostelium*.[18] After this finding, the authors showed that PREP deletion or inhibition blocks the effects of three different mood stabilizing drugs, lithium, valproic acid and carbamazepine.[19] Based on the lack of genetic association between DpoA gene coding PREP and GskA gene that codes GSK3 proteins in *dictyostelium*, it was concluded that PREP does not cause its lithium-related effects via GSK3b.[18] Williams et al found that PREP deletion elevated inositol 1,4,5-triphosphate (IP_3) levels in *dictyostelium*,[18] and similar results were seen in U343 cells after PREP silencing or inhibition.[20] As lithium is known to deplete intracellular IP_3 levels,[4] this was concluded to be the mechanism behind PREP-related the lithium resistance. In more detail, the PREP was shown to regulate to IP_3 turnover via multiple inositol polyphosphate phosphatase (Mipp1).[18,21]

Decreased PREP activity has been found from the plasma of bipolar disorder patients that were on lithium treatment,[22] although lithium does not directly inhibit PREP.[23] This suggests that the decreased PREP activity could be related to the pathophysiology of bipolar disorder. Additionally, PREP activity is reduced in the pituitary gland of the major depressive disorder patients.[24] Similar findings of the reduced PREP activity were done in the major depressive disorder patient plasma by Maes et al,[25,26] but these studies did not control the role of fibroblast activating protein that cleaves similar peptides as PREP in plasma,[22] and therefore, the results need to be interpreted with caution. However, to support these findings, PREP knock-out (PREPko) mouse shows various mood disturbances having reduced anxiety, impaired prepulse inhibition and elevated locomotor activity and aggressiveness.[27,28] A study looking for single nucleotide polymorphism (SNP) in PREP gene in bipolar disorder patients did not find any correlation,[29] but PREP very rarely shows any genetic association on diseases based on GWAS databases.[30] Moreover, the regulation of PREP gene or protein expression PREP or genes that PREP regulates is very poorly known, and therefore, the lack of genetic association between PREP and GSK3 in *dictyostelium* does not exclude the possibility that modifications of PREP could regulate, for example, GSK3b indirectly. Svarcbahs et al recently discovered that PREP negatively regulates protein phosphatase 2A (PP2A) via direct protein–protein interactions and that the lack of PREP or PREP inhibition elevates PP2A activity in cells and in vivo.[31] PP2A is one of the main phosphatases in cell, and GSK3b and Akt are substrates for PP2A.[32,33] On the basis of on this, we wanted to clarify if PREP could block the intracellular effects of lithium via PP2A. Our results show that PREP inhibition or deletion blocks the lithium-induced phosphorylation of GSK3b and Akt, and this effect is dependent on PP2A activity.

2 | MATERIALS AND METHODS

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[34]

2.1 | Reagents

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) if not otherwise specified. The PREP inhibitor, KYP-2047 (4-phenylbutanoyl-l-prolyl-2(S)-cyanopyrrolidine), was synthesized for us in the Division of Pharmaceutical Chemistry, University of Helsinki, as described in Jarho et al.[35]

2.2 | Cell cultures

Human embryonic kidney (HEK-293; RRID:CVCL_0045) cells and HEK-293 PREPko cells were cultured in full Eagle's medium (Dulbecco's modified Eagle medium [DMEM]; #D6429, Sigma) with an additional 10% (v/v) (20% with PREPko cells) foetal bovine serum (FBS; #16000-044, ThermoFisher Scientific), 1% (v/v) L-glutamine–penicillin–streptomycin solution

(#15140122, ThermoFisher Scientific). Human neuroblastoma cells (SH-SY5Y) and SH-SY5Y PREPko cell lines were cultured with DMEM (DMEM-Glutamax; #31966021, ThermoFisher Scientific) containing 15% FBS (ThermoFisher Scientific) for wild-type (wt) and 30% FBS for SH-SY5Y PREPko cells, 1% non-essential amino acids (NEAAs; #11140050, ThermoFisher Scientific) and 50 μg/ml gentamycin (15 750-045, ThermoFisher Scientific). Higher FBS concentration is required for PREPko cell cultures due to the higher basal autophagy levels.[31,36] Generation of the PREPko cell cultures with CRISPR-cas9n plasmid is described earlier in Svarcbahs et al,[31,36] and the validation of the PREPko cell cultures is presented in the Figure S1.

2.3 | Cell treatments

Briefly, cells were plated on 6-well plates (400 000 cells per well) and allowed to attach overnight. For PREP inhibitor experiments, concentration of 1- and 10-μM KYP-2047 was used based on previous studies.[31,37,38] KYP-2047 was diluted to cell culture medium from 100-mM stock in 100% DMSO, and corresponding concentration of DMSO (0.01% or 0.001%) was used as a vehicle control. Okadaic acid (OA; O8010), a PP2A inhibitor, was diluted to DMSO as 10-μM stock and then diluted to 10 nM in cell medium for assays. Lithium chloride (LiCl; L9650) was dissolved in PBS as 10-M LiCl stock and diluted to cell medium in concentrations of 10- or 20-mM LiCl for assays. The concentration of the lithium was based on earlier PREP and lithium studies.[18]

2.4 | Western blot

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50-mM Tris HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150-mM NaCl) containing phosphatase inhibitor (#87786, ThermoFisher Scientific) and protease inhibitor cocktails (#78430, ThermoFisher Scientific). Samples were sonicated 3 × 1 s and centrifuged at 16 000 × *g* for 15 min. Protein concentration was measured from supernatant with bicinchoninic acid (BCA; #23225, ThermoFisher Scientific). Standard SDS-PAGE techniques were used, and 30 μg of sample was loaded to 12% gel (#4561044, Bio-Rad, Hercules, CA). Gels were transferred by Trans-Blot Turbo Transfer System (#1704150, Bio-Rad) onto Trans-Blot Turbo Midi PVDF (#1704157, Bio-Rad) or nitrocellulose (#1704159, Bio-Rad) membranes. Membranes were incubated at +4°C overnight in 5% skim milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T). A list and details of primary antibodies and respective concentrations are presented in Table 1. After overnight incubation, the membranes were washed and incubated in secondary antibody for 2 h in room temperature (1:2000 in TBS-T; HRP-conjugated goat-anti rabbit [#31463, ThermoFisher Scientific]). The images were captured using the ChemiDoc XRS+ (Bio-Rad). To verify that bands are in the linear range of the detection, increasing exposure time and automatic detection of saturated pixels in Image Lab software (version 6.01, Bio-Rad) was used.

Thereafter, the images were converted to 8-bit grayscale format, and the optical densities (ODs) of the bands were measured by ImageJ (histogram area analysis; version 1.48; National Institute of Health, Bethesda, MD). The OD obtained from each band was normalized against the corresponding vinculin band (or to beta-actin in Figure S1). For the negative control (NC) group, the OD values were averaged between the technical replicates in the same plate (in SH-SY5Y PREP-KO cell culture assays) and thereafter normalized to this value. For other treatments, OD value of the NC value was set to 100%. Treatment effects were then correlated to the control value. In the Western blot experiments, 1–2 replicates (technical replicates) of each treatment were used in the well plate with SH-SY5Y PREP-KO assays and one of each treatment with the PREP inhibitor and OA assays. Thereafter, we performed 4–6 individual experiments for each treatment (biological replicates) and immunoblots to have sufficient number of repeats. Samples were not reused to immunoblot same proteins.

TABLE 1 Details of primary antibodies

Antibody	Species	Product code and manufacturer	Dilution used
GSK3b	Rabbit	ab32391, Abcam	1:1000
pGSK3b	Rabbit	ab75814, Abcam	1:1000
Akt	Rabbit	ab8805, Abcam	1:1000
pAkt	Rabbit	ab8933, Abcam	1:500
Vinculin (loading control)	Rabbit	ab129002, Abcam	1:10 000
Beta-actin (loading control, Figure S1)	Rabbit	ab8227, Abcam	1:2500

2.5 | Data and statistical analyses

Data are expressed as mean values ± standard error of the mean (mean ± SEM), and NC average was set as 100% on each assay to reduce variability between repeats. When having two variables, two-way ANOVA followed by Sidak's post hoc comparison was used if ANOVA test gave statistical significance ($P < 0.05$). With one variable, one-way ANOVA was followed by Tukey's post hoc comparison if ANOVA test gave statistical significance ($P < 0.05$). In all cases, P values of <0.05 were considered to be significant. Statistical analysis was performed using PRISM GraphPad statistical software (version 6.07, GraphPad Software, Inc., San Diego, CA).

3 | RESULTS

At first, we wanted to assess the effect of lithium on GSK3b phosphorylation in PREPko cells because the lack of PREP was connected to lithium resistance in earlier studies. In the assays, we used 10 and 20 mM of LiCl as these concentrations have been used to assess the impact of lithium on the intracellular kinases in the HEK-293[39–41] and SH-SY5Y cell cultures.[42–44] However, it needs to be noted that the concentrations are significantly higher than the therapeutic plasma concentration of lithium in patient use (0.8–2 mM).[45] Phosphorylation of GSK3b at Ser9 (pGSK3b) is well-characterized effect of lithium, and both in the wt HEK-293 and SH-SY5Y cells, 24-h incubation with 20-mM lithium significantly elevated the levels of pGSK3b. However, this was not seen in PREP-KO cells (Figure 1B, F; SH-SY5Y cells: $F_{2,28} = 4.134$; $P = 0.0267$ wt vs. PREP-KO cells; HEK-293 cells: $F_{2,28} = 4.537$; $P = 0.0254$ wt vs. PREP-KO cells; $P < 0.05$ 20-mM LiCl compared with NC in wt cells; two-way ANOVA with Sidak's multiple comparison test); 24 h was selected as the time point since 4-h incubation with 10- or 20-mM lithium did not alter the levels of pGSK3b (Figure S2).

We also studied the changes in Akt phosphorylation (pAkt), as it has been indicated as one of the main targets for lithium in the cell. Similar to pGSK3b, 20-mM lithium significantly elevated the phosphorylation of Akt in both wt cell cultures but not in PREP-KO cells (Figure 1D,G; SH-SY5Y cells: $F_{2,26} = 11.03$; $P = 0.0003$ wt vs. PREP-KO cells; HEK-293 cells: $F_{2,26} = 13.49$; $P = 0.006$ wt vs. PREP-KO cells; $P < 0.05$ 20-mM LiCl compared with NC in wt cells; two-way ANOVA with Sidak's multiple comparison test). The levels of total GSK3b or Akt were not significantly changed by the treatments (Figure 1A,C,E,G).

PREP inhibition by a small-molecular inhibitor has been shown to block the effects of lithium and other mood stabilizers.[19] On the basis of this, we assessed if PREP inhibition by KYP-2047 has similar effect as PREP deletion on lithium-induced phosphorylation of GSK3b and Akt. The results showed that simultaneous incubation of SH-SY5Y and HEK-293 cells with 20-mM lithium and 1- or 10-μM KYP-2047 blocked the lithium-induced phosphorylation of GSK3b (Figure 2B,F; SHSY: $F = 6.216$, $P = 0.0018$; $P < 0.01$ 20-mM LiCl vs. 20-mM LiCl + 10-μM KYP-2047. HEK-293: $F = 10.02$, $P = 0.0001$; $P < 0.001$ 20-mM LiCl vs. 20-mM LiCl + 1- and 10-μM KYP-2047; one-way ANOVA with Tukey's multiple comparison test). Similar to PREP-KO cells, pAkt was also significantly reduced by 1- and 10-μM KYP-2047 when incubated with LiCl (Figure 2D,H; SHSY: $F = 4.159$, $P = 0.0158$; $P < 0.05$ 20-mM LiCl vs. 20-mM LiCl + 1- and 10-μM KYP-2047. HEK-293: $F = 4.635$, $P = 0.0062$; $P < 0.001$ 20-mM LiCl vs. 10-μM KYP-2047; $P < 0.01$ 20-mM LiCl vs. 1-μM KYP-2047; one-way ANOVA with Tukey's multiple comparison test). LiCl and KYP-2047 caused fluctuation in the protein levels of unphosphorylated GSK3b and Akt, but the changes were not significant (Figure 2A,C,E,G).

Finally, we wanted to test if the impact of PREP modifications on LiCl effects is dependent on PP2A activation. To study this, the cells were incubated with a PP2A inhibitor, OA (10 nM), together with 20-mM LiCl and 20-mM LiCl + 10-μM KYP-2047 in HEK-293 cells. As expected, PP2A inhibition by OA elevated significantly pGSK3b and pAkt levels (Figure 3B,D; pGSK3b: $F = 4.365$, $P = 0.0228$; $P < 0.05$, NC vs. 10-nM OA; pAkt: $F = 3.386$, $P = 0.0362$; $P < 0.05$, NC vs. 10-nM OA one-way ANOVA with Tukey's multiple comparison test). When LiCl was added with OA, the levels of pGSK3b and pAkt were not significantly altered but slight decrease in phosphorylation levels were seen (Figure 3B,D); 10-μM KYP-2047 did not have effect on the phosphorylation of GSK3b or Akt in the presence of the OA, suggesting that PREP inhibition effect is dependent on PP2A activity (Figure 3B,D; no significance compared with 10-nM OA or 20-mM LiCl + 10-nM OA, -way ANOVA with Tukey's multiple comparison test). The levels of total GSK3b or Akt were not significantly altered by OA or other treatments (Figure 3A,C).

4 | DISCUSSION

In the late 1990s and early 2000s, the lack of PREP was found to block the lithium response in *dictyostelium* by regulating the IP_3 turnover by Mipp1.[18,19] Later, the same effect was shown by PREP inhibitors in rat primary neurons and in U343 cells, and the impact of PREP on GSK3b was excluded based on genetic association.[18–20]

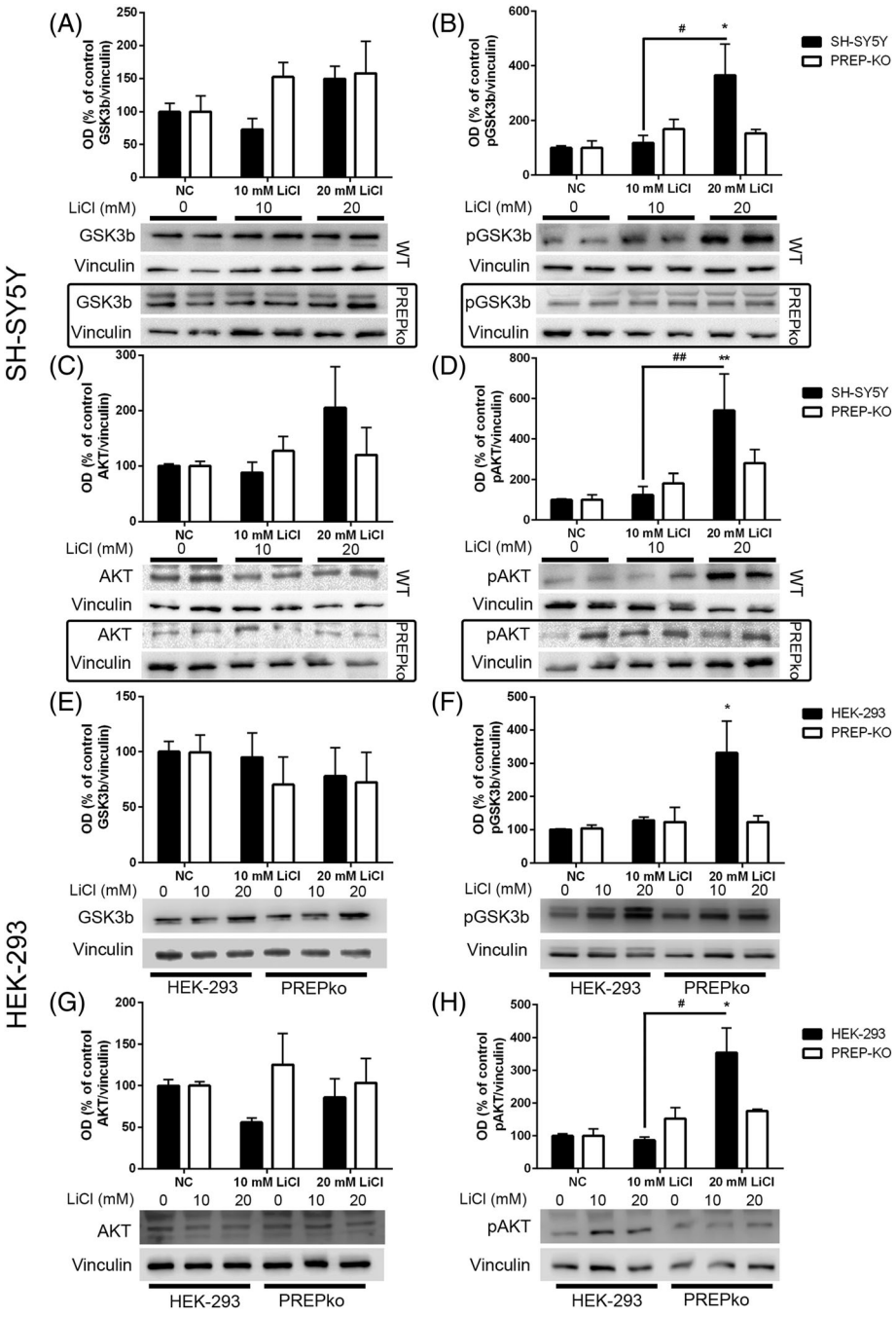

FIGURE 1 Lithium does not induce GSK3b or Akt phosphorylation in prolyl oligopeptidase (PREP) knock-out (PREPko) SH-SY5Y or HEK-293 cells. Total protein levels of GSK3b or Akt were not altered by 24-h incubation of 10- or 20-mM lithium chloride (LiCl) in SH-SY5Y cells (A,C) or in HEK-293 cells (E,G). Phosphorylated GSK3b (pGSK3b) was significantly elevated with 20-mM LiCl in SH-SY5Y and HEK-293 wild-type cells (B,F), and similar effect was also seen in phosphorylated Akt (pAkt; D, H). In both PREP-KO cell cultures, significant effects by LiCl on pGSK3b or pAkt were not seen (B,F). *$P < 0.05$ 20-mM LiCl versus negative control (NC); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; two-way ANOVA with Sidak's multiple comparison test

However, in the current study, we show that the lack of PREP or PREP inhibition blocks the lithium-induced phosphorylation of GSK3b and Akt, and this effect is PP2A dependent.

After its discovery in 1971,[46] PREP has been studied particularly in the context of neuropeptide catabolism and neurodegenerative diseases, and this was also the rationale to develop PREP enzyme inhibitors to combat memory and cognitive deficits, for example, in Alzheimer's disease (for review, see Garcia-Horsman et al[47]). However, the effect of PREP and its inhibition on neuropeptide levels in vivo remained unclear,[48,49] and a phase II clinical trial testing PREP inhibitor S-17092 as a memory enhancer failed.[50] Therefore, the finding that PREP could regulate IP_3 levels in the cells was opening new avenues for PREP studies in early 2000s.[18,19] Lithium regulates IP_3 levels by blocking the inositol monophosphatase (IMPase) and inositol polyphosphate 1-phosphatase (IPPase).[4,51] This leads to decreased inositol levels and eventually to reduced IP_3 production in the phosphoinositide synthesis. Based on this, it is interesting how PREP deletion or inhibition can elevate IP_3 levels via reduced turnover of IP_3 to IP_5, if the whole IP cascade is already depleted after lithium exposure. The effect of

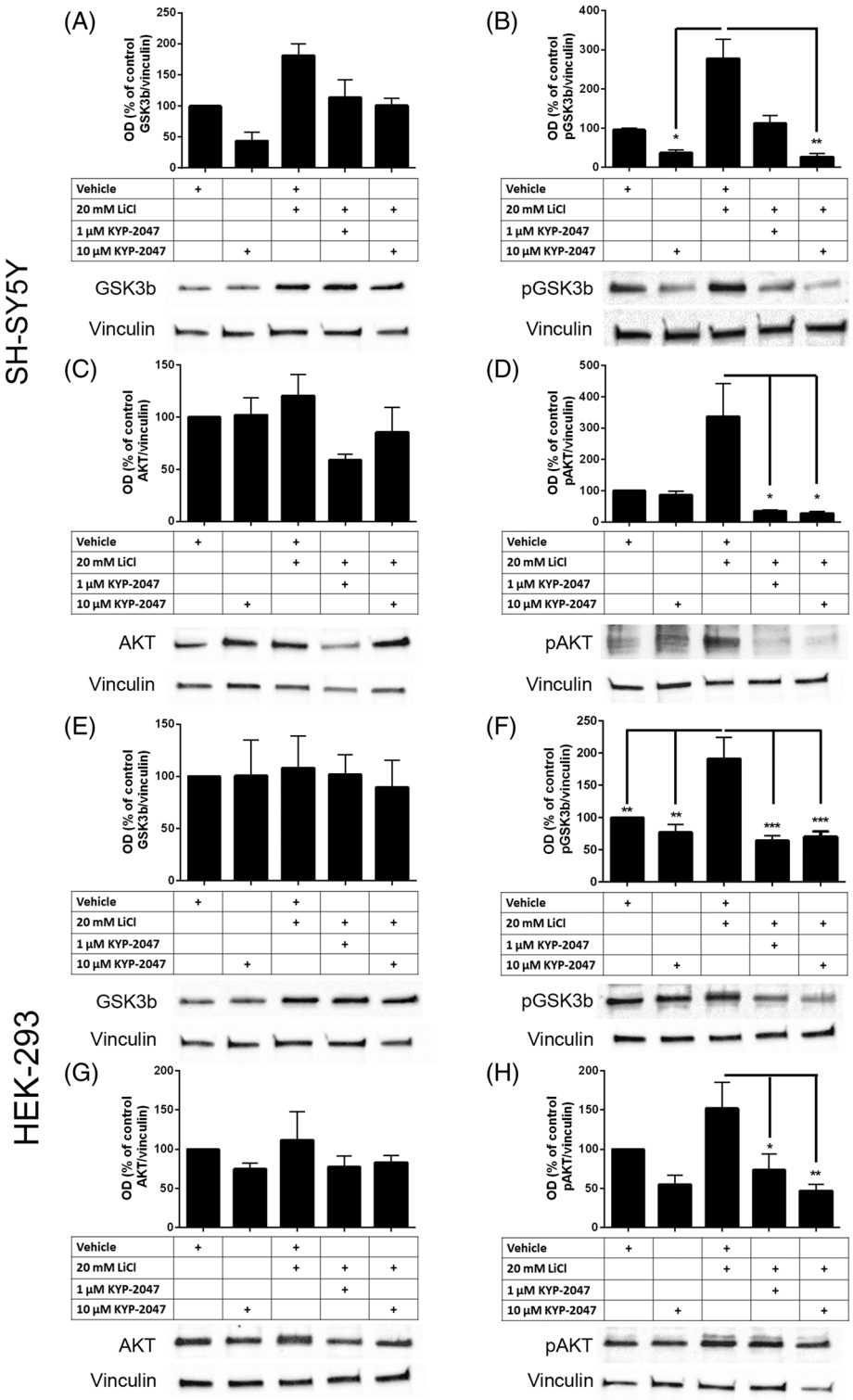

FIGURE 2 Prolyl oligopeptidase (PREP) inhibition by KYP-2047 blocks the impact of lithium on GSK3b and Akt phosphorylation; 1- and 10-μM KYP-2047 blocked the effect of 20-mM lithium chloride (LiCl) on GSK3b (pGSK3b; 1 μM was not significant in SH-SY5Y cells) and Akt phosphorylation (pAkt) in both cell cultures (B,D,F,H). No significant changes were observed in total protein levels by any treatment (A,C,E,G). *P < 0.05; **P < 0.01; ***P < 0.001; one-way ANOVA with Tukey's post-test

PREP inhibition on cellular IP_3 levels seems to be also cell line dependent since Schulz et al showed that PREP inhibition elevated IP_3 levels only in U343 cells but not in the SH-SY5Y or in LN405 cell cultures.[20] Additionally, Jalkanen et al[52] did not see changes in the IP_3 levels in rat brain after 4-day PREP inhibition, but the changes in IP_3 levels in vivo are difficult to measure due the rapid degradation of IP_3. Therefore, it is possible that there are also other causes behind the elevated IP_3 levels after PREP modifications. Nevertheless, it is interesting how the impact of PREP on other intracellular targets of lithium, such as GSK3b, was eliminated in the early phase of the studies because a double mutant combining *gskA* and DpoA mutations showed no genetic interaction.[18]

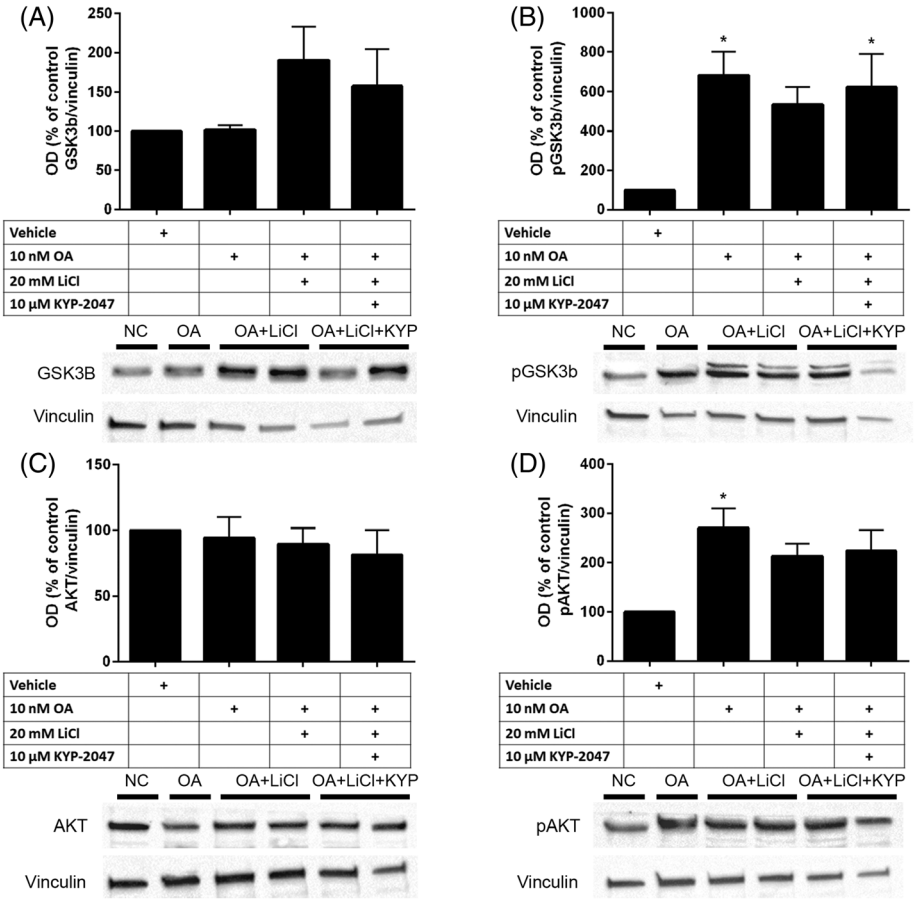

FIGURE 3 Inhibition of protein phosphatase 2A (PP2A) by okadaic acid (OA) blocks the effect of prolyl oligopeptidase (PREP) inhibition on Akt and GSK3b phosphorylation; 10-nM OA significantly elevated the levels of phosphorylated GSK3b (B; pGSK3b) and Akt (D; pAkt); 20-mM lithium chloride (LiCl) did not have additional effect on OA-induced increase of GSK3b and Akt phosphorylation, and 10-μM KYP-2047 could not decrease the phosphorylation levels. Total proteins were not significantly altered (A, C). *$P < 0.05$ compared with vehicle; one-way ANOVA with Tukey's post-test

Later, the connection between PREP, IP_3 and lithium has been studied only a little, but in 2005, Sarkar et al showed that the PREP inhibition by Z-pro-prolinal can block the lithium-induced autophagy. The positive effect of lithium on autophagy was related to decreased IP_3 levels in the cell in this study, and simultaneous incubation of cells with lithium and PREP inhibitor blocked the IP_3 depletion[53] as shown in the studies by Williams et al IP_3 has several pathways to regulate autophagy. Elevated IP_3 initiates Ca^{2+} release from the endoplasmic reticulum that leads to the decreased autophagic flux by reducing the maturation of autophagosomes.[54] Moreover, the IP_3 regulates mTOR-dependent autophagy by inhibiting 5′adenosine monophosphate-activated protein kinase (AMPK).[54] Another connection is between IP_3 receptor and Bcl2–Beclin1 complex, where IP_3 receptor forms an interaction with nutrient-deprivation autophagy factor-1 (NAF-1) and thus regulates Bcl2.[55] Bcl2 blocks autophagy when interacting with Beclin1, and IP_3 receptor–NAF1 complex can enhance the interaction between Bcl2 and Beclin1 in endoplasmic reticulum[55] where PREP is also located.[56] Interestingly, other studies have shown that the PREP inhibition or deletion induces autophagy, and this occurs via PP2A-DAPK1-Beclin1 pathway.[31,36–38,57–60] Sarkar et al did not show the impact of PREP inhibition on autophagy alone in their study[53] so it appears that the negative impact of PREP inhibition on autophagy is related to lithium-induced changes but not to PREP inhibition alone. It is possible that when lithium decreases IP_3 levels, PREP inhibition does not have its normal impact on Bcl2 and Beclin1 phosphorylation, but this would require more detailed studies. The major shortcoming of the current study was that we could not verify the effects of PREP modifications on IP_3 levels in our models. Widely used radioligand assay for IP_3 levels has been discontinued, and available enzyme-linked immunosorbent assays for IP_3 were not reliable in our hands.

PP2A dysfunction has been widely studied in the context of cancer (for review, see Wlodarchak and Xing[61]) and in Alzheimer's disease (for review, see Clark and Ohlmeyer[62]) but not in the mental disorders. There are studies reporting that the mutations in the B-subunit of PP2A are involved with the intellectual disability[63,64] and with schizophrenia.[65] Moreover, SNPs and risk haplotypes for bipolar disorder were found in *PPP2R2C* gene that encodes the brain-specific PP2A-Bγ regulatory subunit.[66] Mutation in the PP2A-Bγ altered the function of

KCNQ2 potassium channel, leading to decreased lithium response in cells and to reduced neuronal excitability. Lithium has been shown to inhibit PP2A in several preclinical studies, promoting the role of PP2A on intracellular signalling cascades that are regulated by lithium treatment.[67,68] Additionally, taking in the account that wide range of PP2A target kinases is related, for example, to pathophysiology of bipolar disorder, such as GSK3b[5] and Akt,[12] the role of PP2A in mental disorders should be studied in more detail. It is also interesting that lowered PREP activity has been connected with bipolar disorder[22] and major depressive disorder,[24] and in both diseases, lithium is used as a therapy. PREP deletion or inhibition elevates PP2A activity[31] that then again has an effect on GSK3b and pAkt as shown in the current study. Based on this, it would be interesting to investigate if lowered PREP activity in the mood disorders contributes to elevated PP2A activity and altered intracellular signalling.

Although the elevated PP2A activation in PREPko cells and after PREP inhibition explains the lack of effects by lithium on GSK3b and pAkt, studies by Williams et al[18,19] have shown that the PREP deletion and inhibition increases IP_3 levels in *dictyostelium*, and Schulz et al showed the same in U343 cells after PREP silencing and inhibition.[20] Interestingly, none of these studies report the changes in the intracellular Ca^{2+} levels although the release of Ca^{2+} from endoplasmic reticulum is the main effect of IP_3 signalling.[69] In the study by Rostami et al, PREP inhibition on intracellular Ca^{2+} levels was studied,[57] but the Ca^{2+} levels were measured after 48-h PREP inhibition that is too long timeframe for rapid Ca^{2+} alterations caused by IP_3. PP2A inhibition decreases the IP_3 levels in the rabbit platelets[70] and human erythrocytes,[71] and PP2A has a negative regulatory effect on IP_3 signalling receptor complex.[72,73] Therefore, it is possible that the effects of PREP modifications on IP_3 levels are mediated by PP2A activity, but this should be studied in more detail. Therefore, it is possible that part of the PREP-related effects on lithium come also via IP_3 pathway where PP2A may have a role and in part via PP2A-dependent GSK3b and Akt pathway.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Additional supporting information may be found online in the Supporting Information section at the end of this article.

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