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Comparing the effect of the novel ionic cocrystal of lithium salicylate proline (LISPRO) with lithium carbonate and lithium salicylate on memory and behavior in female APPswe/PS1dE9 Alzheimer's mice

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Abstract

Alzheimer's disease (AD) is characterized by progressive decline of cognition and associated neuropsychiatric signs including weight loss, anxiety, depression, agitation, and aggression, which is particularly pronounced in the female gender. Previously we have shown that a novel ionic cocrystal of lithium salicylate proline (LISPRO) is an improved lithium formulation compared to the carbonate or salicylate form of lithium in terms of safety and efficacy in reducing AD pathology in Alzheimer's mice. The current study is designed to compare the prophylactic effects of LISPRO, lithium carbonate (LC), and lithium salicylate (LS) on cognitive and non-cognitive impairments in female transgenic APPSWE/PS1dE9 AD mice. Female APPSWE/PS1dE9 mice at 4 months of age were orally treated with low-dose LISPRO, LS, or LC for 9 months at 2.25 mmol lithium/kg/day followed by determination of body weight, growth of internal organs, and cognitive and non-cognitive behavior. No significant differences in body or internal organ weight, anxiety or locomotor activity were found between lithium treated and untreated APPSWE/PS1dE9 cohorts. LISPRO, LC, and LS prevented spatial cognitive decline, as determined by Morris water maze and depression as determined by tail suspension test. In addition, LISPRO treatment was superior in preventing associative-memory decline determined by contextual fear conditioning and reducing irritability determined by touch escape test in comparison with LC and LS. In conclusion, low-dose LISPRO, LC, and LS treatment prevent spatial cognitive decline and depression-like

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CONFLICT OF INTEREST

The authors J.T. and R.D.S. are co-inventors on USF patents covering the use of LISPRO and lithium salicylate. All other authors report no conflicts of interest. All authors consented to publication of this article.

DATA ACCESSIBILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

behavior, while LISPRO prevented hippocampal dependent associative-memory decline and irritability in APPSWE/PS1dE9 mice.

Graphical Abstract

We compared the prophylactic effects of LISPRO (LP), lithium carbonate (LC), and lithium salicylate (LS) treatment on cognitive and non-cognitive impairments in female Alzheimer's disease transgenic mice. Low-dose LP, LC, and LS treatment prevented spatial cognitive decline and depression-like behavior, while LP alone prevented hippocampal dependent associative-memory decline and irritability.

Keywords

cognitive impairment; irritability; depression; Alzheimer's disease; transgenic mice

1. INTRODUCTION

Alzheimer's disease (AD) is characterized pathologically by the accumulation of neuritic plaques (Hardy & Allsop, 1991) and neurofibrillary tangles (Goedert, Spillantini, & Crowther, 1991) in the brain and phenotypically by progressive decline in cognitive function. As neuronal death correlates with memory and cognitive decline (Morrison & Hof, 2002), the degeneration of neural synapses especially in the hippocampus and cortical regions is also positively correlated with cognitive decline in patients with AD (Davies, Mann, Sumpter, & Yates, 1987; DeKosky & Scheff, 1990). It is widely believed that abnormal accumulation of plaques and tangles consisting of amyloid- β ($A\beta$) (Masters et al., 1985) and hyperphosphorylated tau (Grundke-Iqbal et al., 1986) proteins, respectively,

promotes neurodegeneration (Crews & Masliah, 2010) which may be involved in the development of cognitive and neuropsychiatric problems (Lyketsos et al., 2011). More recently, soluble oligomeric forms of A β and tau have been shown as the most toxic form of A β and tau aggregate to produce cognitive deficits in the absence of A β plaques or insoluble intraneuronal tangles (Gandy et al., 2010; Patterson et al., 2011). Many individuals with AD also experience emotional difficulties, such as depression, anxiety, apathy, irritation, agitation, and aggression (Lanctot et al., 2017). No single drug has been shown to be effective in improving both cognitive and non-cognitive problems.

Lithium salts were initially used in the treatment of gout (including “brain gout”) as early as 1859 and then recognized for its beneficial effects on mood by the late 1800’s (Shorter, 2009). Despite having a narrow therapeutic window that requires routine blood monitoring to avoid lithium toxicity and associated side effects, lithium salts are still considered even today as the gold standard by many clinicians for preventing the reoccurrence of mania and suicidality in bipolar patients (P. V. Nunes, Forlenza, & Gattaz, 2007; Sportiche et al., 2017). Based in part on evidence that lithium therapy may prevent cognitive decline in bipolar disorder, a growing body of studies using diverse preclinical and clinical models have provided evidence for potential disease modifying properties of lithium in treating neurodegenerative diseases, including AD (Forlenza, de Paula, Machado-Vieira, Diniz, & Gattaz, 2012). Overall, the available evidence suggest that the neuroprotective effects of lithium found in diverse preclinical models of neurodegeneration translate into therapeutic benefits in cognitive function and reduced biomarkers in clinical trials of mild cognitively impaired amnesic and AD patients at lower lithium doses than those typically used for mood stabilization (Forlenza, Aprahamian, de Paula, & Hajek, 2016). Other studies also suggest that long-term lithium treatment inhibits or slows down the core pathophysiologic features of AD in preclinical as well in human study (Fajardo, Fajardo, LeBlanc, & MacPherson, 2018; Zhang et al., 2011). Notably, in a recent review of 28 psychiatric medications, including antipsychotics, anticonvulsants and antidepressants, lithium had the most replicated evidence for neuroprotection in the widest range of neurodegenerative disease models (Lauterbach & Mendez, 2011). Despite these potential benefits of lithium pharmaceuticals, safety issues remain. Indeed, current Food and Drug Administration (FDA)-approved lithium pharmaceuticals have a narrow therapeutic window, producing a sharp peak in the serum blood levels which can cause adverse side effects. In addition, due to competition for sodium reuptake, high levels of lithium can accumulate in the kidney under certain conditions resulting in renal inflammation and toxicity (Shine, McKnight, Leaver, & Geddes, 2015).

To improve lithium’s therapeutic and safety profile, we have recently employed a crystal engineering strategy to synthesize the first ionic cocrystals (ICCs) of lithium salts with organic anions. We were the first to assess the biological activity of any ICC and encouragingly found that the speciation did not affect established bioactivities of lithium, including inhibition of glycogen synthase kinase, BDNF production, inhibition of LPS-activated microglial NO production and differentiation of neural stem cells. Surprisingly, an ionic co-crystal of lithium salicylate and l-proline (LISPRO) exhibited modulated pharmacokinetics compared with lithium carbonate, with a smaller peak and more steady level of lithium in the blood and brain (Smith et al., 2013). In a subsequent study, we

determined the safety, pharmacokinetics, and therapeutic efficacy of LISPRO, in comparison with lithium carbonate and lithium salicylate in preclinical mouse models of AD (Habib et al., 2017). We found that 8- as well as 28-week oral treatment with LISPRO produced superior reduction of hippocampal pathology, including A β plaque deposition, tau hyperphosphorylation, neuroinflammation, and neurodegeneration, in two transgenic Alzheimer's-like mice models (Tg2576, 3XTg-AD). In cell culture studies, LISPRO reduced tau hyperphosphorylation, microglial inflammation and glycogen synthase kinase 3-beta activity and was superior to lithium carbonate in enhancing neuronal stem cell differentiation. Pharmacokinetic studies indicated that LISPRO treatment provided significantly higher brain lithium levels and more steady plasma lithium levels in both normal and transgenic Alzheimer's-like mice when compared to lithium carbonate. Moreover, a 2-week oral treatment of lithium carbonate in normal mice increased proinflammatory cyclooxygenase-2 expression in the kidneys, while the molar equivalent of lithium administered as LISPRO did not (Habib et al., 2017).

The purpose of this follow-up study was to compare the prophylactic effects of chronic oral treatment of LISPRO with lithium carbonate and lithium salicylate on behavioral impairment and various AD-associated neuropsychiatric symptoms, including depression, irritation, anxiety and impaired locomotion, as well as physical growth and development of internal organs in APP^{swe}/PS1^{dE9} double transgenic mice, the most widely used preclinical animal model in evaluating drug safety and efficacy for AD. Since AD shows sexual dimorphism, with female patients displaying greater prevalence, incidence and severity than males (Dye, Miller, Singer, & Levine, 2012; Koran, Wagener, & Hohman, 2017; Phung et al., 2010), we focused our study on female AD mice at a single age (12 mo of age). In addition, for consistency, we employed the same chronic dosing regimen of LISPRO that was used in our prior study (Habib et al., 2017).

2. METHODS AND MATERIALS

2.1. Animals and Treatment

Twelve-week-old (3 months of age) female transgenic (Tg) APP^{swe}/PS1^{dE9} mice [B6C3-Tg (APP^{swe}/PS1^{dE9})85Dbo/Mmjax; Stock #034829; n = 28] and wild-type (WT) non-transgenic control littermates (Non-Tg Ctrl, B6C3F1/J; n = 10) were directly obtained from Jackson Laboratory (Bar Harbor, ME), not bred, and housed 3–4 mouse per cage in a room with a 12 h light/12 h dark schedule (lights turn on at 6.00 in the morning). All mice had access to food and water ad libitum. APP^{swe}/PS1^{dE9} mice express human amyloid precursor protein (APP) with the K670N/M671L (Swedish) mutation and human presenilin 1 (PS1) with the deltaE9 mutation, both under control of the prion promoter. The Swedish mutation results in an increase in BACE1 cleavage of APP and the deltaE9 mutation increases γ -secretase activity. These mice show plaque deposits at 6 months of age, and increasing cognitive impairment at 12 months of age (Garcia-Alloza et al., 2006; Janus, Flores, Xu, & Borchelt, 2015). APP^{swe}/PS1^{dE9} mice were randomly assigned into four treatment groups, and starting at 4 months of age received normal chow without additional lithium (Tg-Ctrl, n = 8, Teklad 18% protein rodent diet, 2018, Harlan Laboratories, Madison, WI) or chow that contained lithium carbonate (LC, 0.05%, n = 6), lithium

salicylate (LS, 0.20%, n = 6) or lithium salicylate proline co-crystal (LISPRO, 0.35%, n = 8) for 9-months. Since mice of this age normally consume approximately 5g chow per day, this gave a dose of 83 mg/kg/day LC, 325 mg/kg/day LS, 583 mg/kg/day LISPRO and 2.25 mmol lithium/kg/day. This dosage was chosen based on our previous study (Habib et al., 2017) to give plasma lithium concentrations of 0.05–0.2 mmol/L, which fall in the lower range of clinical therapy for AD (Forlenza et al., 2011). Non-transgenic wild type (WT) littermates cohort (Non-Tg Ctrl, n = 10) were fed normal chow, 2018 Teklad protein rodent diet as a control. At the end of 8-months treatment (12 months of age) comprehensive cognitive and non-cognitive evaluations (Diagram 1) (Sawmiller et al., 2017) were conducted by individuals blind to genotype and treatment group. In general, behavioral testing was performed between 10.00 AM and 5.00 PM. All mice were assessed on the behavioral tests in the following sequence, morris water maze, fear conditioning, elevated plus maze, open field test, tail suspension test, and accelerated rotarod test (Diagram 1, left to right order). Two different tests were separated from each other by one or two days interval. All animal experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by USF Institutional Animal Care and Use Committee (IACUC reference number: IS000002240). At the end of behavioral testing, mice were humanely euthanized by an overdose of isoflurane.

2.2. Behavioral assays

2.2.1. Morris Water Maze—The Morris Water Maze (MWM) is used to investigate hippocampal spatial memory impairments in rodents. The apparatus consists of a circular tank (150 × 85 cm) filled with water (25 ± 2 °C). The circular tank was divided into four invisible quadrants (North, South, East, West) using ANY- Maze™ video recording software (Stoelting, Wood Dale, IL, USA). To increase the tracking sensitivity, white paint solution was mixed in the water to provide contrast to the dark color of the mouse and facilitate tracking of mouse movement. In the study reported here, a hidden platform was placed 1 cm beneath the water level in North East zone during the training period (4 trials) for two consecutive days, during which time individual mouse was placed in the tank and were allowed to find the platform. Over multiple trials wherein the mouse is placed in the tank at different locations, the mouse learns to find the hidden platform within 60 s by using distal spatial learning cues, here consisting of colored objects placed on the walls outside of the water tank but in view of the mouse. If the mouse failed to locate the hidden platform within 60 s, it was guided to the platform and allowed to stay there for 10 – 20 s. Spatial memory was evaluated by assessing the average latency time (in seconds) and path length of a mouse before reaching the hidden platform. Twenty-four hours following the end of the last trial each mouse was given a 60 s probe trial, which determines the ability of the mouse to retrieve learned information. During probe trial, the hidden platform is removed, and parameters such as escape latency (time to reach the previous platform position), time spent in the platform quadrant, crossings over the platform and total distance travelled were recorded using automated ANY-Maze™ video software.

2.2.2. Fear Conditioning—Fear Conditioning (FC) assesses the ability of rodents to learn and memorize the association between environmental cues (context) and aversive stimuli (cued). This test is used to investigate hippocampal- (contextual), as well as

amygdala-dependent (cued) memory functions, as indicated by the freezing response (complete absence of movement except respiration, i.e., motionless position for at least two consecutive seconds). The mouse learns rapidly in an environment where the aversive stimulus consists of conditioned (white tone) and unconditioned stimuli (shock). The experimental set-up consists of a transparent Plexiglas box with a photo beam detection system and an electric shock generator wired to a grid in the floor on the box. On Day 1 a mouse was placed in the chamber and allowed to explore freely for 6 min during which baseline freezing time (conditioning) was observed using automated video recording software. After 3 min, an auditory stimulus (80 dB tone) was given for 30 s followed by a 0.35 mA mild foot shock for 2s. Two minutes later another conditioned-unconditioned pairing was given. The mouse was then removed from the chamber and returned to the home cage. At Days 2, 7, and 21, each mouse was placed back to the chamber for 6 min without any shock and the freezing time was recorded (contextual response). Cued test is conducted on the next week of the contextual test. Mice were placed into a different testing chamber with dissimilar properties, providing a new context that is unrelated to the conditioning chamber. Similarly, the mouse was placed in the chamber for 6 min after changing contextual cues with a few drops of vanilla extract, the auditory stimulus (80 dB tone) was delivered during the last 3 min, and freezing time was recorded (cued response).

2.2.3. Elevated Plus Maze—Elevated Plus Maze (EPM) is used extensively with the open field test to assess anxiety-like behavior. The apparatus is elevated 50 cm above the ground and consists of four arms of 60×20 cm at 90° , two open and two closed with elevated walls of 40 cm, and a central square of 10×10 cm dimension. In this study, each mouse was placed in the center of the maze facing an open arm and the video tracking software is used to record arm entries for 5 min in standard room-lighting conditions. The entry in any arm is counted when all four legs of the mouse are crossed the entrance line of the arm. Mice that stay in the closed arms of the EPM are thought to be exhibiting anxiety-like behavior, whereas those frequently exploring open arms are considered to be exhibiting less anxiety behavior.

2.2.4. Open Field test—The Open Field (OF) test is used to evaluate anxiety-like behavior by placing the mouse in a cubic ($1\text{m} \times 1\text{m} \times 1\text{m}$) box with walls to prevent the escape of the mouse. The open field is comprised of peripheral and central areas marked with a grid and square crossings. Video tracking software (ANY-Maze[®]) is used to separate the open field into peripheral and central areas of the grid. The mouse is placed in the center of the open field and allowed to freely move for 10 min under in standard room-lighting conditions without any background noise and odors, while tracking total distance travelled and times spent in the open and periphery using video tracking software. The entry in the field is counted when all four legs of the mouse are crossed the line. The amount of time spent or number of entries into central area is indicative of reduced level of anxiety, while more time spent in the periphery indicates higher anxiety level.

2.2.5. Tail Suspension test—Depression-related behaviors and antidepressant activity of drugs can be studied in the mouse using tail suspension test. In this test, a mouse is suspended above a surface by anchoring the tail with tape and is observed for its efforts to

escape for 300 s. The duration of periods of attempts to escape (agitation) and immobility is recorded with a stopwatch.

2.2.6. Touch Escape test—The Touch Escape test is a non-cognitive behavior test used to analyze the irritability of the animal in response to touch as summarized by SHIRPA (Smithkline Beecham, Harwell, Imperial College, Royal London Hospital, Phenotype assessment) (Rogers et al., 1999). To determine Touch Escape, each mouse was taken from the home cage and placed on a table. The evaluator brushed the sides and back of the mouse from behind starting from the upper front of the thorax then slowly moving caudally to its tail while evaluating its irritability. A Touch Escape score from 0 to 2 was determined with the following scale: “0” included freezing, or no progression or regression of movement from the evaluator’s hand, score “1” comprised of startle responses like agitation, small jump, jerk, twitching followed by a small amount of progression away from the evaluator, score “2” included jumping or quick progressive movements away from the evaluator and/or biting.

2.2.7. Accelerated Rotarod test—Briefly, mice were placed on a rotating rod beam, which underwent an acceleration from 4 to 40 rotations per minute (RPM) over 5 min for three consecutive trials separated by a 60 min interval, and latencies to fall as well as maximal speed before falling were determined. The amount of time a mouse remains on the accelerating rotating beam is an index of motor coordination.

2.2.8. Statistical Analysis—All data are presented as mean $\pm$ SEM. We used one-way ANOVA to determine effect of treatment on organ weights and behavior and two-way ANOVA to determine effect of age and treatment on BW, after elimination of outliers (values greater than 3 interquartile range). Alpha was set at 0.05 for all analyses. When treatment effects were significant, Tukey’s honest significant difference *post hoc* analyses was performed for pair-wise comparisons. Effect size (η^2) and power were also determined for each analysis. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), release 24.0 (IBM, Armonk, NY).

3. RESULTS

3.1. LISPRO did not affect body weight in female APP_{SWE}/PS1dE9 mice

Among the long-term adverse effects, lithium therapy is associated with weight gain in a subset of bipolar patients through an unknown mechanism. But contradictory results also exist regarding effects of lithium treatment on weight gain or loss in both rodents and humans (Praharaj, 2016). To investigate whether LISPRO, LS, and LC treatment had any effect on weight gain in APP_{SWE}/PS1dE9 mice, body weights of mice were measured at the beginning (4 months age) as well as at the end (13 months of age) of the treatment. Nontransgenic WT control mice weighed significantly more than untreated transgenic APP_{SWE}/PS1dE9 cohort both before and after the treatment period (Fig. 1), presumably due to a significantly greater deposition of fat tissue in nontransgenic WT compared with transgenic APP_{SWE}/PS1dE9 mice. However, no statistically significant difference in body

weight was found between untreated transgenic APP_{SWE}/PS1dE9 mice receiving normal chow and those receiving LISPRO, LS, or LC treatment for 9 months

3.2 LISPRO did not adversely affect the growth of internal organs in female APP_{SWE}/PS1dE9 mice

Lithium treatment has been shown to influence the functions of endocrine hormones regulating growth and development of internal organs in mice (Giusti, Amorim, Guerra, & Portes, 2012). Therefore, we wanted to examine if long-term lithium treatment would adversely affect the growth of internal organs in female transgenic APP_{SWE}/PS1dE9 AD mice. To our knowledge, no such data is available on the long-term effect of LISPRO, LC, and LS treatments on organ development in these mice. At sacrifice, post-hoc analysis showed statistically significant decrease in weights of liver (Fig. 2b, left panel) and kidneys (Fig. 2b, center panel) in untreated transgenic APP_{SWE}/PS1dE9 cohort compared with non-transgenic WT control mice. However, the weights of brain, heart, lung and spleen were not significantly different between untreated transgenic APP_{SWE}/PS1dE9 and nontransgenic WT control (Fig 2a). In addition, lithium treatment did not significantly alter the weight of any organ in transgenic mice, indicating no adverse effect of lithium treatment on growth of these organs.

3.3 Improvement of spatial memory deficits in female APP_{SWE}/PS1dE9 mice by LISPRO treatment

Within the medial temporal lobes, the hippocampus has been considered to be critical for spatial memory function and is therefore the focus of this study. In our previous study, we found that LISPRO treatment prevents the progression of amyloid deposition in the hippocampus and cortex in Tg2576 and 3xTg-AD mice models (Habib et al., 2017). Due to abundant amyloid deposition in the cortex and hippocampus by twelve months of age, the transgenic APP_{SWE}/PS1dE9 mouse model of AD also shows spatial and stress-induced memory deficits. To examine whether long-term LISPRO treatment ameliorates spatial learning and memory deficits in this AD model, the MWM test was conducted after 8-months of treatment, when the mice were approximately 12 months of age. All groups showed a gradual decrease in escape latency to find the hidden platform in second and third trials of training (Trial 1 vs. Trial 2 and Trial 2 vs. Trial 3: $p < 0.05$; in all five groups; Fig. 3a), indicating the learning ability of all mice throughout the training period. At probe trial, untreated transgenic APP_{SWE}/PS1dE9 mice, as well as APP_{SWE}/PS1dE9 mice treated with LC, showed longer escape latencies compared to non-transgenic WT control mice, confirming an AD-associated learning and memory deficit as determined by the MWM test (Fig. 3b). However, the time to find the target platform in transgenic APP_{SWE}/PS1dE9 mice treated with LS or LISPRO was not significantly different than WT control mice, suggesting that LS and LISPRO ameliorates spatial memory deficits. Untreated transgenic mice also spent significantly less time in the target quadrant compared with nontransgenic WT control mice, but time in target quadrant of transgenic mice treated with LC, LS or LISPRO was not significantly different from that in non-transgenic mice (Fig. 3c). Thus, long-term treatment with LISPRO, LC or LS protects against memory loss in transgenic APP_{SWE}/PS1dE9 mice. Swimming distance and speed was not significantly different between untreated and lithium treated transgenic and WT control mice (Fig. 3d & e).

3.4 LISPRO treatment ameliorated contextual associative memory deficits in female APP_{SWE}/PS1dE9 mice

FC test is used to examine associative learning and memory in rodent models of AD by measuring their freezing behavior. APP_{SWE}/PS1dE9 mice show impaired contextual FC memory at 3 months of age, whereas cued FC is found to be normal. To examine the effect of LISPRO treatment on hippocampal- and amygdala-dependent memory, we examined both contextual and cued fear conditioning in female APP_{SWE}/PS1dE9 mice at 12 months of age after 8 months of treatment. At day 1, the baseline freezing responses were recorded where mice were exposed to conditioned stimulus (mild foot shock) paired with an unconditioned stimulus (audio tone). Total baseline freezing time was not significantly different between non-transgenic WT and untreated or lithium treated transgenic APP_{SWE}/PS1dE9 mice (Fig. 4a). The contextual freezing response was then recorded without tone at 1, 7, and 21 days after conditioning. Freezing times were not significantly different between lithium treated and untreated transgenic APP_{SWE}/PS1dE9 mice after 1 and 7 days of conditioning ($p > 0.05$ in all case; data not shown). However, untreated transgenic APP_{SWE}/PS1dE9 mice, as well as transgenic mice treated with LC or LS, exhibited significantly less freezing to the context compared with nontransgenic WT control mice after 21 days of conditioning (Fig. 4b). More importantly, freezing times of transgenic mice treated with LISPRO were not significantly different from non-transgenic control mice at this time point. In addition, we also investigated cued FC, consisting of an audio tone without any foot shock application at days 1, 7, and 21 days after conditioning training. No statistically significant differences in freezing time were observed among treatment groups for cued FC at any day after training. Taken together, these results indicated that LISPRO improves contextual memory deficits with no change in cued FC, suggesting prevention of hippocampal with maintenance of amygdala-dependent cognitive impairment in female APP_{SWE}/PS1dE9 mice.

3.5 LISPRO did not affect anxiety-like behavior in female APP_{SWE}/PS1dE9 mice

It is estimated that more than eighty percent of dementia patients show non-cognitive psychological changes including apathy, depression, anxiety, agitation, and irritability during the progression of AD (Lyketsos et al., 2002). Thus, in this study, we examined anxiety by the open field and elevated plus maze tests. APP_{SWE}/PS1dE9 transgenic mice did not exhibit enhanced anxiety compared with WT mice as determined by the open field test, reflected as reduced time in center, center entries or distance traveled in center or enhanced time in periphery or distance traveled in periphery (Fig 5a – e). The transgenic mice also did not exhibit impaired locomotor activity, reflected as altered total time mobile (Fig 5f). In addition, we did not observe any alteration of anxiety as determined by elevated plus maze test, since transgenic mice exhibited decreased time spent on both open and closed arms, with no change in open and closed arms entries (Fig 5g – j). In addition, LC, LS or LISPRO treatment did not alter times spent in closed or open arms or closed or open entries compared with untreated transgenic mice, but the decreased time spent in closed arms observed in transgenic mice was reversed by LS and LISPRO treatment. Overall, these results suggest that LISPRO, LC, or LS have little or no effect on anxiety-like behavior in female APP_{SWE}/PS1dE9 mice.

3.6 LISPRO did not alter motor function in female APP_{SWE}/PS1dE9 mice

Mild tremor and weakness is one of the common side effects for patients receiving chronic lithium treatment (Gitlin, 2016). Therefore, we determined whether or not long-term LISPRO treatment changes motor coordination and performance in transgenic APP_{SWE}/PS1dE9 mice, as determined using the accelerated rotarod test. All mice showed learning of motor functions as indicated by longer time spent on the rod and higher speed at fall over the course of three consecutive trials (Trial 1 vs. Trial 2: $p < 0.05$, within each cohort; Trial 2 vs. Trial 3: $p < 0.05$, within each cohort except LS, Fig. 6c & d). There was no significant difference in time spent on the rod or speed at fall (RPM) between nontransgenic WT and untreated and LC-, LS- or LISPRO-treated transgenic APP_{SWE}/PS1dE9 mice (Fig 6a & b).

3.7 LISPRO prevented depression-like behavior in female APP_{SWE}/PS1dE9 mice

The prevalence of depression among AD patients has been reported to be 10 to 50% (Park et al., 2007). Therefore, we assessed whether 12-months old APP_{SWE}/PS1dE9 mice with advanced state of disease progression show depressive-like behavior and whether LISPRO treatment could reverse this effect as assessed by tail suspension test. Mice were individually suspended by the tail from a horizontal stand and immobility time was recorded as an index of depressive-like behavior (Fig. 7a). While transgenic APP_{SWE}/PS1dE9 mice exhibited increased immobility time compared with non-transgenic WT control mice, lithium treatment with LC, LS, or LISPRO reversed this increase in immobility time. No statistically significant difference in immobility time was found between LISPRO, LC, or LS treatment. These data suggest that lithium treatment as LISPRO or carbonate and salicylate salt reduces depression as a secondary effect in the treatment of AD.

3.8 LISPRO reduced irritability in female APP_{SWE}/PS1dE9 mice

The prevalence of irritability among AD patients was reported from 13–23% in clinical studies. In addition, transgenic APP_{SWE}/PS1dE9 mice show an increased hypersensitivity to touch indicative of enhanced irritability (Filali, Lalonde, & Rivest, 2009). Therefore, we examined whether LISPRO treatment reduces irritability in transgenic APP_{SWE}/PS1dE9 mice as determined by the touch escape test. While transgenic APP_{SWE}/PS1dE9 mice showed higher irritability compared to non-transgenic WT control mice, as shown by its irritability score, LISPRO but not LC or LS treatment reduced irritability compared to untreated transgenic APP_{SWE}/PS1dE9 mice (Fig 7b).

4. DISCUSSION

A growing body of both translational and preclinical evidence suggests that lithium may potentially have significant disease modifying therapeutic benefits for patients with mild cognitive impairment or early stage AD (Forlenza, De-Paula, & Diniz, 2014; Forlenza et al., 2011). Despite the therapeutic potential for lithium in the treatment of AD neuropathology and cognitive impairment, lithium formulations currently available exhibit a narrow therapeutic window with an increased risk of side effects and noncompliance in elderly populations (Nilsson & Axelsson, 1989). With the goal of improving lithium's therapeutic profile, we have been investigating the safety, pharmacokinetics, and therapeutic efficacy of LISPRO, a novel ionic co-crystal of lithium salicylate and L-proline (Smith et al., 2013),

compared with lithium carbonate and lithium salicylate across a host of preclinical models of AD (Habib et al., 2017). Employing the same chronic dosage regimen found to reduce AD-like neuropathology in our previous study, the major findings of the present study was that chronic 8-month oral treatment with LISPRO ameliorated spatial (Fig. 3b) and contextual memory deficits (Fig. 4b) typically found in female APP_{SWF}/PS1dE9 mice at 12 months of age, without adversely effecting body or internal organ development (Fig. 1 & 2), anxiety (Fig. 5) or motor function (Fig. 6). Similar to our previous study which found LISPRO to have a superior therapeutic profile for several neuropathological endpoints in cellular and animal models, LISPRO exhibited superior improvement in associative memory function assessed by contextual fear conditioning (Fig. 4b), and amelioration of irritability (Fig. 7b), compared with molar equivalent doses of lithium carbonate and lithium salicylate, in female APP_{SWF}/PS1dE9 mice. All three lithium formulations significantly improved spatial memory deficits (Fig. 3b & c) and depressive-like behavioral symptoms measured by tail suspension test (Fig. 7a). This improved spatial and associative memory function could be mediated through restoration of pre- and post-synaptic protein functions, as shown in our previous study (Habib et al., 2017). A further electrophysiological study is necessary to reveal the protective synaptic functions of LISPRO treatment. In addition, future studies should determine the effects of LISPRO in male AD mouse models. Although female patients display greater prevalence, incidence and severity of AD than males (Dye et al., 2012; Koran et al., 2017; Phung et al., 2010), the increasing prevalence, incidence and severity of AD in male patients should not be overlooked.

Our study confirms previous studies showing a beneficial effect of lithium in animal models of AD and AD patients, while also highlighting the potential benefit of LISPRO. Although a few studies reported no effect of lithium chloride on A β levels (Caccamo, Oddo, Tran, & LaFerla, 2007; Feyt et al., 2005) or even neurodegeneration induced by tau suppression (Lei et al., 2017), the overriding evidence indicates that lithium treatment effectively reduces extracellular β -amyloid (Habib et al., 2017; Trujillo-Estrada et al., 2013) and tau hyperphosphorylation in cell culture and animal models of AD (Nery et al., 2014). Likewise, intracerebroventricular injection of A β in zebrafish embryo impairs cognition which was successfully reversed by lithium administration (Nery et al., 2014). Lithium has been shown to ameliorate neuropathology and restore hippocampal neurogenesis in Ts65Dn mice, a transgenic model of Down syndrome, in which chromosome 21 with its APP gene is inherited in triplicate (Bianchi, Ciani, Contestabile, Guidi, & Bartesaghi, 2010). Gelfo et al. (2017) recently investigated the potential beneficial effects of a 30- day oral lithium treatment (0.24% lithium in diet) in preventing neuropathology and cognitive dysfunction following basal forebrain cholinergic neurodegeneration in rats (Gelfo et al., 2017). Using a rat hippocampal neuron model, Dwivedi *et al.* (2015) reported that lithium-induced neuroprotection is associated with epigenetic modification of brain-derived neurotrophic factor (BDNF) gene promoter and altered expression of apoptotic-regulatory proteins (Dwivedi & Zhang, 2015).

In an initial open label feasibility and tolerability clinical trial in mild to moderate AD patients, no significant effects of lithium on cognition were observed after 1 year of treatment (Macdonald et al., 2008). In another single blind multi-center trial in mild to moderate AD patients, no significant effects on cognition were observed after 10 weeks

(Hampel et al., 2009). However, in these early clinical studies, the dosage was titrated to reach a target serum lithium level based on the prophylaxis range (0.5–0.8 mmol/L) typically used for the maintenance of bipolar disorder. In a more recent double-blind placebo controlled disease modification trial, subjects with mild cognitive impairment (MCI) who received lower levels of lithium (0.25–0.5 mmol/L) had significantly lower conversion rates to AD than those receiving placebo (Forlenza et al., 2011). Moreover, those who had received lithium had significantly lower CSF phospho-tau levels as well as better compliance and performance on the cognitive subscale of the Alzheimer's disease Assessment Scale (Forlenza et al., 2011). In another double-blind, placebo control trial, microdoses (300 µg) of lithium daily for 15 months prevented cognitive decline in AD patients as measured by the mini-mental state examination (MMSE) compared to placebo, with the benefits starting after 6 months and persisting until the end of trial (M. A. Nunes, Viel, & Buck, 2013). In addition, chronic lithium treatment has been also shown to increase the serum levels of BDNF in patients with early AD (Leyhe et al., 2009), which could be responsible for cognitive enhancement (Gold, Herrmann, & Lanctot, 2011).

Secondary symptoms such as agitation and psychosis are common in AD and are associated with increased patient distress, increased caregiver burden, more rapid cognitive decline, greater risk of institutionalization and mortality, and increased health care costs. Unfortunately, currently available medications used often off-label to target these secondary symptoms have been reported to have long term adverse effects. For example, the FDA issued a black box warning in 2005 that antipsychotics significantly increased all-cause mortality in dementia (Koponen et al., 2017; Seo, Song, Park, Ha, & Shin, 2017). Moreover, valproic acid, a mood stabilizer used often off-label to treat agitation across diverse patient populations was found to significantly accelerate brain atrophy as determined by magnetic resonance imaging as well as cognitive impairment following 1-year treatment in AD patients (Fleisher et al., 2011). Therefore, the ability of chronic treatment with LISPRO to safely reduce AD-like neuropathology and cognitive deficits but also depressive like behavior and irritability in APP_{SW}/PS1dE9 mice has intriguing implications if these preclinical findings translate into clinically meaningful therapeutic benefits.

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SIGNIFICANCE STATEMENT

Previously we have shown that a novel ionic cocrystal of lithium containing lithium salicylate and proline (LISPRO) is superior to lithium carbonate (LC) and lithium salicylate (LS) in reducing AD pathology in an AD mouse model. The current study was designed to compare the therapeutic effects of LISPRO, LC and LS on cognitive and psychiatric impairments in female AD mice (APPSWE/PS1dE9). Overall, low-dose LISPRO, LC, and LS prevented spatial cognitive decline and depression but LISPRO was superior in reducing associative-memory decline and irritability in comparison with LC and LS.

Diagram 1. Outline of study design.

Female APP_{SWE}/PS1dE9 mice at 4 months of age were fed with either regular chow (Tg-Ctrl, n = 8) or chow that contained lithium carbonate (LC, 0.05% equivalent to 83 mg/kg/day, n = 6), lithium salicylate (LS, 0.20% equivalent to 325 mg/kg/day, n = 6) or LISPRO (LP, 0.35% equivalent to 583 mg/kg/day, n = 8) for 9 months. In addition, aged- matched non-transgenic background control mice (B6C3F1/J, Non-Tg Ctrl, n = 10) were fed regular chow for 9 months as control. Each treatment group was subject to a battery of behavioral tests at 12 months of age and mice were sacrificed at 13 months of age.

Figure 1. Lithium treatment did not affect body weight gain in female APP_{SWE}/PS1dE9 mice. Female transgenic APP_{SWE}/PS1dE9 mice were fed with either regular chow (Tg-Ctrl) or chow that contained lithium carbonate (LC), lithium salicylate (LS) or LISPRO (LP), as described for Diagram 1. In addition, age-matched non-transgenic WT mice (B6C3F1/J, Non-Tg Ctrl) were fed normal chow as control. Each treatment group were subjected to body weight measurement at the beginning of treatment (4 months) and at the time of sacrifice (13 months). Transgenic APP_{SWE}/PS1dE9 mice had significant less body weight than nontransgenic mice at 3 and 13 months and both transgenic and nontransgenic mice gained weight over the course of treatment. However, lithium treatment (LC, LS, or LP) for nine months had no effect on weight gain in transgenic APP_{SWE}/PS1dE9 mice. Data shown as scatter plot as well as means $\pm$ SEM. Asterisk indicates $p < 0.05$.

Figure 2. LISPRO treatments did not affect growth of internal organs in APP_{SW}E/PS1dE9 mice. The weight of brain, heart, lung (a), liver, kidney(s), and spleen (b) were determined at time of sacrifice in female APP_{SW}E/PS1dE9 cohort fed normal chow (Tg-Ctrl) or chow containing LC, LS, or LP as well as aged-matched non-transgenic WT control cohort fed normal chow (Non- Tg Ctrl), as described for Diagram 1. Transgenic mice showed less liver and kidney weight than nontransgenic mice, but no significant differences in the weight of any internal organ were observed after 9-months treatment with LP, LC, or LS compared with untreated APP_{SW}E/PS1dE9 mice. Asterisk indicates p<0.05 compared with nontransgenic mice.

Figure 3. Spatial memory deficits in female APP_{SWE}/PS1dE9 mice are improved by chronic LISPRO treatment.

Female APP_{SWE}/PS1dE9 and non-transgenic WT control mice were fed normal chow or chow containing LC, LS, or LP as described for Diagram 1 followed by MWM testing. Latency to find the hidden platform was determined over the course of four trials during the training period (a). During the training period, the untreated transgenic APP_{SWE}/PS1dE9 cohort (Tg-Ctrl) took significantly more time to find the platform compared with the non-transgenic control mice (Non-Tg Ctrl), indicating a deficit in spatial learning. All three lithium treatments (LC, LS, and LP) significantly reduced spatial learning deficit as indicated by reduced latency, compared with the previous trial within the same cohort. Latency to find platform target was determined during the probe trial of MWM, performed 24 h after the last training trial (b). Transgenic APP_{SWE}/PS1dE9 cohort took more time to find the target platform than the non-transgenic WT cohort (* $p < 0.01$) and LS and LP treatment reversed this learning deficit. Time spent in target quadrant was determined for probe trial (c). Untreated transgenic APP_{SWE}/PS1dE9 cohort spent significantly less time in target quadrant compared with non-transgenic WT cohort, but no significant differences were found in time spent in target quadrant for lithium treated transgenic compared with non-transgenic WT cohort. In addition, no statistically significant differences were found in total swimming distance travelled (d) and mean swimming speed (e) during the probe trials. Swim tracking plot during probe trial for representative mouse in each cohort is displayed (f). Asterisk indicates $p < 0.05$ compared with nontransgenic mice.

Figure 4. LISPRO improves associative-learning and memory on contextual fear conditioning in female APP_{SWE}/PS1dE9 mice.

Female transgenic APP_{SWE}/PS1dE9 and non-transgenic WT control cohort were fed with normal chow or chow containing LC, LS, or LP as described for Diagram 1, followed by contextual and cued fear conditioning testing. No statistically significant differences for freezing time were found during the conditioning training between non-transgenic WT control (Non-Tg Ctrl), transgenic APP_{SWE}/PS1dE9 mice untreated (Tg-Ctrl) and transgenic mice treated with LC, LS, or LP (a). In addition, no statistically significant difference in contextual freezing times were found among transgenic APP_{SWE}/PS1dE9 mice untreated or treated with LC, LS, or LP at 1 or 7 days after conditioning (data not shown). However, contextual freezing time after 21 days conditioning was significantly reduced in untreated transgenic or transgenic cohort treated with LC or LS but not LP compared with non-transgenic cohort (b). No statistically significant difference in cued freezing time was found among nontransgenic WT control and transgenic APP_{SWE}/PS1dE9 mice untreated or treated with LC, LS, or LP in the same time period recorded. Asterisk indicates $p < 0.05$ compared with nontransgenic mice.

Figure 5. LISPRO does not alter anxiety-like behavior or locomotor activity in female APP_{SWE}/PS1dE9 mice.

Time spent in center (a) or periphery (b), number of center entries (c), distance traveled in center (d) or periphery (e) and total mobile time in open field test (f) was not significantly

different between female non-transgenic WT control (Non-Tg Ctrl) and APP_{SWE}/PS1dE9 mice (Tg-Ctrl) untreated or treated with LP, LC or LS. In addition, no significant difference was found for number of open (i) and closed arm entries in elevated plus maze (j). Times spent in open (g) and closed arms (h) were significantly decreased for transgenic compared with non-transgenic mice, but LC, LS and LP treatment did not alter time spent in open arms and LC treatment did not alter time spent in closed arms for transgenic mice. However, time spent in closed arms for LS and LP treated transgenic mice was not significantly different from that in non-transgenic control mice. Asterisk indicates $p < 0.05$ compared with nontransgenic mice.

Figure 6. LISPRO does not alter motor coordination as determined by rotarod test.

Latency to fall (a) and RMP at fall (b) throughout three consecutive trails of accelerated rotarod test were not significantly different between female non-transgenic WT control and APP_{SWF}/PS1dE9 mice untreated (Tg-Ctrl) or treated with LC, LS or LP (Non-Tg Ctrl). All cohorts exhibited enhanced motor function learning as determined by increased latency to fall (c) and RMP at fall (d) over three consecutive trials. Asterisk indicated $p < 0.05$ compared with trial 1 or 2.

Figure 7. LISPPO reduces depressive-like behavior and irritability in female APP_{SWE}/PS1dE9 mice.

Female transgenic APP_{SWE}/PS1dE9 mice (Tg-Ctrl) exhibited depressive- like behavior compared with nontransgenic WT control mice (Non-Tg Ctrl) as shown by increased immobility time in tail suspension test, which was reduced by LC, LS, and LP treatment (a). In addition, transgenic APP_{SWE}/PS1dE9 mice exhibited increased irritability compared to nontransgenic WT control mice as shown by increased irritability score in touch escape test, which was reversed by LP treatment (b). Asterisk indicates $p < 0.05$ compared with non-transgenic control or untreated transgenic mice.