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Intranasal Lithium Chloride Nanoparticles Inhibit Inflammatory Pyroptosis in Brains and Ameliorate Memory Loss and Depression Behavior in 5xFAD mice

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Abstract

This study compares the changes in lithium concentrations in the brain and blood following the administration of intranasal or oral lithium chloride (LiCl) dissolved in either Ryanodex Formulation Vehicle (RFV) or water, as well as the therapeutic effectiveness and side effects of intranasal versus oral lithium chloride (LiCl) in RFV, and their mechanisms for inhibiting inflammation and pyroptosis in 5xFAD Alzheimer's Disease (AD) mice brains. In comparison to oral LiCl in RFV, intranasal LiCl in RFV decreased lithium blood concentrations but increased brain concentrations and duration, resulting in a significantly higher brain/blood lithium concentration ratio than intranasal LiCl in water or oral LiCl in RFV in young adult mice. Intranasal LiCl in RFV robustly protects both memory loss and depressive behavior in both young and old 5xFAD mice, with no side effects or thyroid/kidney toxicity. In fact, intranasal LiCl in RFV protects against age-dependent kidney function impairment in 5xFAD mice. This lithium mediated neuroprotection was associated with its potent effects on the inhibition of $InsP_3R$-1 Ca^{2+} channel receptor increase, ameliorating pathological inflammation and activation of the pyroptosis pathway, and the associated loss of synapse proteins. Intranasal LiCl in RFV could become an effective and potent inhibitor of pathological inflammation/pyroptosis in the CNS and treat both dementia and depression with no or minimal side effects/organ toxicity, particular in AD

Introduction

The importance of developing an effective treatment for AD, a devastating neurodegenerative disease and a major public health problem affecting 6 million patients in USA alone[1], cannot be overemphasized. The major AD pathology is the progressive dementia, which is the number one cause of dementia in aged population[1]. To be effective disease-modifying drugs for AD treatment, drugs should be capable targeting critical upstream pathology[2] and associated majority of the 25 proposed AD pathologies[3]. Drugs must target Sporadic AD (SAD, 95% of AD patients)[1], have increased therapeutic windows, and be well tolerated by AD patients without significant side effects/toxicity with chronic use[4].

In AD or many other neurodegenerative diseases, the concentration of the neurotransmitter glutamate in the synapse space can be as high as 100 mM[5], leading to Ca^{2+} dysregulation and excitotoxicity[6]. This can be further aggravated by excessive Ca^{2+} release from the primary intracellular Ca^{2+} store endoplasmic reticulum (ER) via over activation of inositol trisphosphate receptors (InsP3R)[7, 8]. This upstream Ca^{2+} dysregulation results in downstream multiple AD pathologies[9], including amyloid[10, 11] and tau[12, 13] pathologies, mitochondria dysfunction[14, 15], pathological reactive oxygen species (ROS) production and lipid peroxidation[16, 17], impairment of autophagy[18, 19], neuroinflammation[20-22], impaired neurogenesis[18, 23], disruption of synaptic structure and function[18, 24], and eventually cognitive dysfunction[25-27]. Importantly, an increasing number of studies suggest that downstream inflammation and associated cell/neuron death pyroptosis play critical role in AD pathology as etiology for both dementia[28, 29] and depression behavior[28, 30], and could be effective therapeutic target[31].

Three hundred million people worldwide suffer from major depressive disorder (MDD)[32]. MDD is a chronic and recurrent disease affecting about 20% of the population and is the leading cause of suicide[33]. The prevalence of MDD in patients of

Alzheimer's disease was particularly high, up to 40%[34]. The treatment of MDD is limited by the high risk of resistance (up to 30% of patients are unresponsive to the first treatment) and relapse (up to 8%)[35, 36], and the side effects/organ toxicity with chronic use[37]. It is important to develop new drugs for the effective treatment of MDD, with no or minimal side effects with chronic use.

Although molecular mechanisms for MDD pathology unclear, increasing evidence suggests that like the pathology for dementia, the upstream disruption of intracellular Ca^{2+} homeostasis and associated downstream multiple pathologies including mitochondria dysfunction, oxidative stress, pathological inflammation, pyroptosis and synapse dysfunction play critical role in MDD pathologies[36, 38-41]. The abnormally increased glutamate and associated excitotoxicity via overactivation of N-methyl-D-aspartate (NMDA) receptors (NMDAR) and overactivation of $InsP_3$ ($InsP_3R$) receptors and associated excessive Ca^{2+} release from endoplasmic reticulum (ER) results in pathological elevation of cytosol and mitochondrial Ca^{2+} concentrations, detrimental to cell survival[42-44]. The numbers and activity of InsP3R are also pathologically increased in AD[8, 45]. Like the pathology of dementia, the upstream Ca^{2+} dysregulation and associated multiple downstream pathologies including pyroptosis and pathological inflammation[41, 46], eventually lead to synapse and cell/neuron damage[47, 48], and subsequent depression behavior[49]. Thus, a drug that inhibits upstream critical Ca^{2+} dysregulation may be effective to treat MDD [38].

MDD has been considered a prodrome of dementia, and depression and dementia are two pathologies making a vicious cycle leading to symptoms in AD[50, 51]. No disease-modifying drugs are available to treat both depression and dementia in AD[52]. It is clearly and urgently important to develop new drugs for the effective treatment of both dementia and depression in AD patients, with no or minimal side effects from chronic use. Considering the critical roles of synapse dysfunction and cell or neuron damage in both depression and cognitive dysfunction, inhibition of upstream Ca^{2+} dysregulation and downstream pathological inflammation and pyroptosis is expected to treat both depression and cognitive dysfunction effectively, especially in AD.

Lithium, a first-line drug for depression and mania in bipolar disorder, is also neuroprotective against many neurodegenerative diseases[53, 54], including AD[55-57]. Although multiple mechanisms have been proposed for lithium treatment on depression behaviors and cognitive dysfunction in AD, the ability of lithium to ameliorate upstream Ca^{2+} dysregulation by inhibiting NMDAR[58] and suppressing $InsP_3R$[59], as well as addressing multiple downstream pathologies[9]—including oxidative stress, inflammation and pyroptosis, and synapse and neuronal destruction—is considered the primary mechanism by which lithium may alleviate both cognitive dysfunction and depression behaviors in AD and other neurodegenerative diseases[60-63]. However, major concerns or limitations for lithium in bipolar disorder patients is its narrow therapeutic window, proneness to side effects/toxicity, and intolerance in patients with chronic use [64-66]. As lithium neuroprotection is typically dose-dependent, the effective dose for neuroprotection in the CNS will likely cause intolerable side effects/toxicity. Thus, a new strategy to promote its CNS therapeutic effects, while minimizing its peripheral side effects and organ toxicity is crucial to promote lithium use for treatment of both dementia and depressive disorder, especially in AD or other neurodegenerative diseases.

In this study, we first compared the changes of lithium brain versus blood concentrations after administration of oral or intranasal LiCl in nanoparticles (RFV) or regular formation in water. Then, we investigated the therapeutic versus side effects and the potential mechanisms of intranasal LiCl in RFV to ameliorate both cognitive dysfunction and depression behavior in WT versus 5xFAD mice. Our results demonstrated that, compared to oral LiCl in RFV administration, intranasal lithium LiCl in RFV significantly increased brain but decreased blood lithium concentration and had a significantly increased lithium brain/blood concentration ratio. Consistently, Intranasal LiCl in RFV for 12 consecutive weeks abolished both cognitive dysfunction and depression behavior, as a disease-modifying drug, in both adult and aged 5xFAD mice, with no side effects or organ toxicity on the kidneys and thyroid. Furthermore, intranasal LiCl in RFV inhibited oxidative stress, pathological inflammation, programmed cell death by pyroptosis, and postsynaptic synapse protein loss in aged 5xFAD mice. This study suggests that intranasal LiCl in RFV is an effective treatment for both memory loss and depression behavior in AD, via inhibition of the pathological inflammation and pyroptosis and synapse loss, with no side effects or organ toxicity. This study will inspire future clinical studies investigating the therapeutic effectiveness versus side effects of intranasal LiCl in RFV for treatment of both MDD and dementia, especially in AD patients.

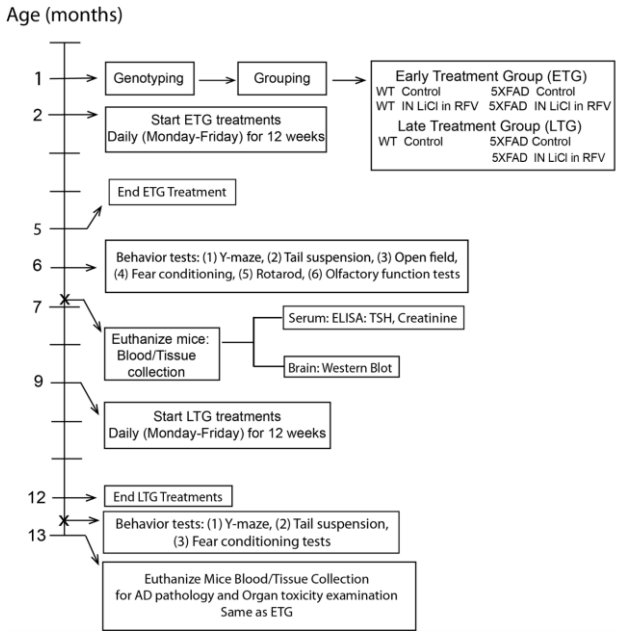

Figure 1: Experimental Designing. LiCl: Lithium Chloride, RFV: Ryanodex Formulation Vehicle, ELISA: Enzyme linked immunosorbent assay, TSH; Thyroid Stimulating Hormone.

Materials and Methods

Animals

All the procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. Four pairs of 5xFAD mice (B6SJL-Tg (APPSwFlL on, PSEN1*M146L*L286V) 6799Vas/Mmjax) and wild type (WT) mice (B6SJLF1/J) were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred. These 5xFAD transgenic mice overexpressed mutant human APP with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) Familial Alzheimer's Disease (FAD) mutations along with human PS1 harboring two FAD mutations, M146L and L286V. C57BL/6J mice were purchased from Jackson lab (Bar Harbor, Main, U.S.A.) and were used in lithium brain/blood concentration ratio studies. Food and water were available in the cage. All mice were weaned no later than one month of age and were genetically identified by polymerase chain reaction (PCR) analysis before weaning. At this time, mice were divided into different cages according to age and gender, with no more than five mice per cage. Both male and female mice were used in this study.

Lithium nanoparticles preparation and administration

LiCl (Sigma, Aldrich, Co, St. Louis, MO, U.S.A.) was dissolved in Ryanodex Formulation Vehicle (RFV: 125 mg mannitol, 25 mg polysorbate 80, 4mg povidone K12 in 5mL of sterile water and pH adjusted to 10.3) as in our previous publications[27, 67], or in water. For intranasal administration, the final concentration of LiCl was 3M/ml for intranasal administration and 0.6M/ml for oral administration. Intranasal administration of LiCl in RFV was performed following same protocol we described previously[27]. The mice were held and fixed by the scruff of their necks with one hand and with the other hand given a total of 1µL/gram or 5µL/gram of body weight of LiCl in RFV or vehicle. A mouse weighing 20 g would be given 20µL solution for intranasal and 100µL for oral administration, respectively. The LiCl dose for both intranasal and oral administration was 3 mM/Kg. The solution was slowly delivered directly into the mouse's nose. Care was taken to make sure that mice were minimally stressed, and that the solution stayed in the nasal cavity and did not enter the stomach or lungs.

Experimental treatment groups and drug administration

Lithium pharmacokinetics studies

C57BL/6J mice at 2 months of age were divided into three groups: 1). Intranasal administration of LiCl in RFV, 2). intranasal administration of LiCl in water or 3). Oral administration of LiCl in RFV. Brain versus plasma lithium concentrations were measured and brain/blood lithium concentration ratios were calculated at 30, 60 and 90 minutes after a single lithium administration, and compared among different groups.

Lithium brain versus blood concentrations measurements.

The lithium concentrations in brain tissue or in serum will be measured by a lithium detection the Lithium Assay Kit (Colorimetric) (Abcam: Car# ab235613, Boston, U.S.A.) according to the manufacturer's instruction. Briefly, brain tissues were homogenized in 0.5N Trifluoroacetic acid. Supernatant was collected after centrifugation at 10,000 x g for 5 minutes as sample preparation. The absorbance of all sample and standard curve wells was measured at both 540 nm and 630 nm in endpoint mode. The final lithium concentrations in sample will be calibrated and determined.

Lithium treatment experiment groups

As demonstrated in figure 1, age-matched male and female mice were randomly divided into experimental groups when they were genotyped around 1 month of age. The Early Treatment Groups (ETG) beginning treatment at 2 months of age and the late treatment group (LTG) beginning at 9 months of age include intranasal or oral LiCl in RFV and no treatment control. Mice were treated once a day, 5 times per week (Monday to Friday) for 12 weeks. The LiCl in RFV was made fresh and contained all inactive ingredients in Ryanodex[27, 67]. Fresh LiCl in RFV were made every time before administration. Intranasal LiCl in RFV stock solution was made at 10M/ml. All behavioral tests were performed after completion of 12 weeks of treatment at 5 or 12 months of age for the ETG or LTG, respectively. Mice were euthanized at 6 or 13 months of age after 1 month of behavior tests, and the brain and blood were harvested to determine various biomarkers for mechanisms study.

Behavioral Tests

Rotarod test

Motor function was examined for muscle weakness. The amount of time spent on the accelerating rotarod (IITC Series 8, Life Sciences, Woodland Hills, CA) was assessed for mice in the ETG at 6 months of age, as previously described[27]. Briefly, animals were acclimated to the testing room at least 1 hour before the test. Two 60-second training trials at a constant speed (9 rpm) were performed with a 30-minute interval. Then, three 120 second test trials were conducted at a gradually increasing speed (4–40 rpm) with a 60-minute interval between trials. The latency to fall from the rotarod was recorded automatically and analyzed.

Food buried test

Olfaction was assessed in ETG at 5 months of age as previously described with modifications[27, 67]. On the first day, cookies (Galletas La Moderna, S.A. de C.V.; one cookie for every two mice) were buried in the bedding of the home cages for 24 hours, and then the number of cookies consumed was recorded. The mice were placed on a fast starting on the second day at 4 pm and ending on the third day at 9 am. Water was freely available during this time. The buried food test was conducted on the third day from 9–11 am. They were acclimated to the testing room for at least 1 hour before the test. Mice were individually placed into a clean cage containing clean bedding with one cookie buried beneath the bedding in a corner. The latency for the animal to find the cookie (identified as catching the cookie with its front paws) was recorded manually. If the animal failed to find the cookie within 15 minutes, it would be placed back into its home cage.

A clean cage and bedding were used for each animal and investigators were blinded to the experimental conditions.

Fear conditioning test

Memory was assessed at 6 or 13 months of age for ETG or LTG, respectively. Both hippocampal-dependent and independent memory were assessed by the fear conditioning test, using the protocol previously described[27]. Each day, animals were acclimated to the testing room at least 1 hour before the test. On the first day, each mouse was placed in the test chamber and went through three condition stimulation parings with a 60-second interval between each cycle. A 30 second tone of 2000 Hz and 85 dB was used as the tone stimulation and a 2 second electrical foot shock of 0.7mA was used as the shock stimulation. The mice were removed from the chamber 30 seconds after the last stimulation. On the second day, the contextual fear conditioning test was first performed to measure the hippocampal-dependent memory. The mouse was placed in the same chamber for 4 minutes with no tone or shock and then removed from the chamber. Two hours later, the cued fear conditioning test was performed to measure hippocampal-independent memory. The mouse was placed in another chamber that was different in size and smell using different cleaning solutions. There was no tone or shock during the first 1 min. Later the mouse went through three cycles of the same tone with a 60-second interval between each cycle with the freezing time recorded. Animals were then removed from the chamber 60 seconds after the last tone. The ANY-maze controlled Fear Conditioning System consisted of a sound-attenuating chamber (Model: 46000-590, UGO Basile, Gemonio Italy) equipped with a video camera and ANY-maze software (V.4.99 Stoelting Co. Wood Dale, IL) which recorded the freezing time. The chamber was thoroughly cleaned between trials with a 75% alcohol solution on the first day in the training trials and on the second day in the contextual-fear conditioning test, and with water on the second day in the cued fear conditioning test. The investigator was blinded to the animal groups.

Y-maze test

Memory and learning were also assessed at 5- or 12-months age for the ETG or LTG, respectively. As described previously[68], memory was assessed using the Y-shaped maze with three grey, opaque plastic arms at a 120° angle from each other (Y-maze, NOLDUS Information tech Inc, Leesburg, VA, USA). Each day, animals were acclimated to the testing room at least 1 hour before the test. Every mouse was placed into the same arm of the maze and was allowed to freely explore the three arms. The number of arm entries and the number of triads were recorded to calculate the percentage of alternation. The whole course was recorded by the camera and the results were calculated manually. The investigator was blinded to the animal groups.

Tail suspension test

Depression behavior was assessed 5 or 12 months of age for the ETG or LTG respectively using tail suspension test described previously with modifications[69]. We used specially manufactured tail suspension boxes, made of carton with the dimensions (42 cm height X 18 cm width X 30 cm depth). To prevent animals from observing or interacting each other, each mouse was suspended within its own three-walled rectangular box. Each day, animals were acclimated to the testing room at least 1 h before the test. Each mouse is suspended in the middle of the box and the width and depth were sufficiently sized so that the mouse cannot contact the walls. In this setting, the approximate distance between the mouse's nose and the apparatus floor was 20-25 cm. A plastic suspension bar (50 cm. height X 40 cm. width40 cm. depth), used to suspend the tail of each mouse, was positioned on the top of the box. The bottom of each box had paper towel that collected feces or urine from the animals. We used a dark grey box for albino animals and a white colored box for mice of other coat colors. We securely adhered both the mouse's tail and the suspension bar to the box so that it was strong enough to carry the weight of the mouse being evaluated. A video camera was placed in position. After suspending the mouse on the suspension bar, the TST session was recorded. The total duration of the test was 6 minutes. The paper towel was replaced after each trial. After all sessions were finished, the videos were analyzed manually. During the behavioral analysis, the time that each mouse spent as mobile was measured and was subtracted from 360s as the immobility time. The immobile time reflects the level of depression behavior. The investigator was blinded to the animal groups.

Euthanasia and tissue collection

Mice of the ETGs and LTGs were euthanized at 6 or 13 months of age respectively after all the behavior tests were finished. As we described previously[27], animals were deeply anesthetized with 2–4% isoflurane delivered through a nose cone, and the concentration was adjusted according to the animals' response to a toe pinch. The animal's skin was prepped, and an incision made to open the chest and expose the heart. Blood was collected for the serum study from the heart using a syringe equipped with a 27G needle, without heparin. The blood was centrifuged at 1400 rpm at 4°C for 30 minutes, the supernatant collected and frozen at –80◦C. The animals were euthanized by trans cardial perfusion and exsanguination with cold phosphate-buffered saline. The thyroid gland, kidney, and the brain were dissected. The brain was stored in –80°C for western blot.

Immunoblotting

Total brain tissues were extracted by homogenization using cold RIPA buffer (#9806S, Cell Technology, USA) supplemented with protease inhibitor cocktails (P8340 Roche). The brain homogenates were rocked at 4°C for 90 minutes and then centrifuged at 14,000 rpm (Brushless Microcentrifuge, Denville 260D) for 20 minutes at 4°C to remove cell debris. After collecting the supernatant, the protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL 61101 USA). Briefly, equal amounts of protein (50µg/lane) were loaded onto 4-20% gel electrophoresis of mini-protein TGX precast (Cat. #4561094, BIO-RAD) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, MERK Millipore, Ireland) using wet electrotransfer system (BIO-RAD, USA). Following the transfer membrane blocking

with 5% BSA (Sigma-Aldrich) for 1 hour, the PVDF membrane was incubated with primary antibodies overnight at 4°C including InsP3R1, MDA-modified protein, NLRP3, Human procaspase-1/cleaved caspase-1 p20, GSDMD, cleaved GSDMD, IL-1β, IL-18, IL-6, TNF-α, PSD95, and GAPDH (Details antibodies information in Supplementary table 1). Afterward, the membranes were incubated with secondary antibodies, including HRP-linked anti-mouse IgG1 and anti-rabbit IgG that was conjugated to horseradish peroxidase, then washed with Tris-buffered saline containing 0.2% Tween-20 (TBST). Following that, TBST was used for washing the membranes three time for 10 minutes. After incubation with secondary antibodies, proteins band were visualized using ECL Western Blotting Detection Reagents (Cytiva, amersham, UK) and quantified for band intensity using ImageJ software (National Institutes of health, Bethesda, MD, USA).

Serum TSH (Thyroid Stimulating Hormone)

Serum TSH, a hormone that indicates the thyroid gland function, was measured using a TSH Colorimetric Assay Kit (CHM01J578, Thomas scientific, Inc, Swedesboro, NJ, USA) according to the manufacturer's instructions. We measured the serum thyroid stimulating hormone for the 5xFAD mice in the ETGs and all mice in the LTGs. Briefly, 10µl serum was diluted in a total 50µl with 40µl of sample diluent and was incubated for 30 minutes at 37°C. After five washes with washing buffer, 50 µl of HRP-conjugate reagent was added. Following 30 minutes of incubation at 37°C and another five washes, the reaction was stopped with a stop solution. The optical density (OD) at 450 nm was measured within 15 min. At the same time, a standard curve was generated, using TSH concentrations of 0, 50,100, 200, 400, 600 and 900 pg/ml. The sample concentration was calculated by using the linear regression equation of the standard curve.

Serum creatinine (Cr)

Serum creatinine, an indicator of kidney function, was measured using a Creatinine Colorimetric Assay Kit (ab65340, Abcam, Inc, Waltham, MA, USA) according to the manufacturer's instructions. We measured the serum creatinine for the 5xFAD mice in the ETGs and all mice in the LTGs. Briefly, 50µl serum was diluted in a total 100µl reaction mixture, including 42µl Cr Assay Buffer, 4µl Creatinase, 2µl Cr Enzyme Mix, and 2µl Creatinine Probe. A standard curve was generated, using creatinine concentrations of 0, 2, 4, 6, 8, and 10 nmol/well. The optical density (OD) at 570 nm was measured at 60 minutes after incubating the reaction at 37°C. The creatinine concentration was measured in a linear range of the standard curve. The trendline equation (Sa) was calculated from the standard curve data. The creatinine concentration was calculated using the formulation: creatinine concentration =Sa/50 nmol/µl.

Statistics

All data was represented as mean ± standard deviations (Means±SD). Statistical analyses were employed with GraphPad Prism (Version 9.3.1, CA, USA). Comparisons of more than two groups were conducted by two-way ANOVA followed by Tukey's multiple comparison test. P-values were considered

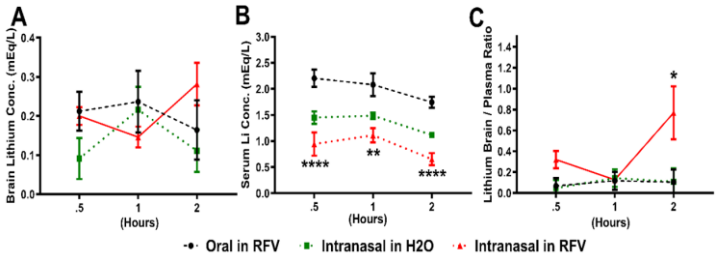

Figure 2: Intranasal lithium chloride in Ryanodex Formulation Vehicle (RFV) increased its duration in the brain while significantly decreasing blood lithium concentrations compared to oral administration, resulting in an increased brain/blood lithium concentration ratio. Wild type (WT) mice at 2 months of age were given LiCl (3 mM/kg) dissolved in Ryanodex formulation vehicle (RFV) intranasally (red), orally (black), or intranasally in water (green). Brain **(A)** vs. serum **(B)** lithium concentrations (conc) were measured and brain/blood concentration ratio were calculated (**C**), at 30, 60 and 120 minutes after drug administration. Means±SD, N=6, Two-way ANOVA followed by Tukey post hoc tests. *, **, **** P<0.05, P<0.01, P<0.0001, compared to oral administration (black).

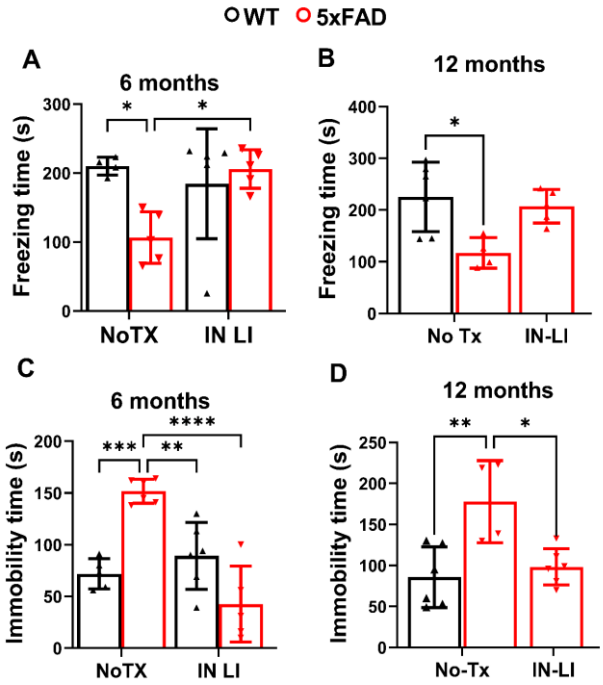

Figure 3: Intranasal LiCl in Ryanodex formulation vehicle (RFV) inhibited memory loss and depressive behavior in 5xFAD mice. Wild type (WT) or 5xFAD mice at 2 or 9 months of age were treated with intranasal LiCl in RFV (INLI 3 mM/Kg), daily, Monday to Friday, for 12 consecutive weeks. Cognitive function was determined by fear conditioning at 6 (**A**, hippocampus-dependent) or 12 (**B**, hippocampus-independent) months of age. Depressive behavior was determined by tail suspension test at 6 **(C)** or 12 **(D)** months of age. Data represents Means±SD from 4-6 mice and were analyzed using the 2-way ANOVA followed by Dunnett's post-hoc test **(A, C)** or one-way ANOVA **(B, D)** followed by Tukey's post-hoc test. *, **, ***, ****, P<0.05, P<0.01, P<0.001, P<0.0001, respectively.

statistically significant based on asterisk including *, **, ***, ****, P<0.05, P<0.01, P<0.001, P<0.0001, respectively.

Results

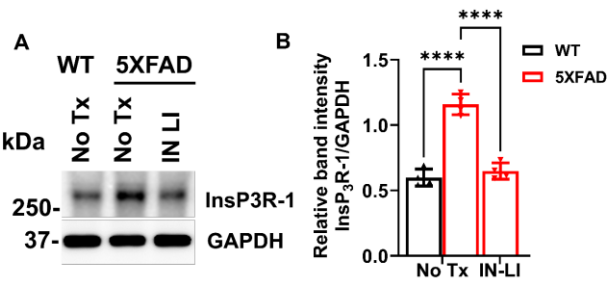

Figure 4: Intranasal LiCl in RFV inhibited the pathological increase of type 1 InsP3R (InsP3R-1) proteins in aged 5xFAD mice brains. Wild type (WT) or 5xFAD mice at 9-month-old were treated with intranasal LiCl in RFV (INLI, 3 mM/kg), 5 days/week for 3 consecutive months. Mice brains harvested at 13 months old. Immunoblot determined the expression levels of $InsP_3R$-1 (**A**) and statistical analysis of relative band intensity of InsP3R-1/GAPDH (**B**). Data are Means±SD from 4 separate mice in each treatment group (N=4) and were analyzed using the two-way ANOVA followed by Tukey's multiple comparison test. ****, P<0.0001

Intranasal LiCl in RFV significantly increases the brain/blood lithium concentrations ratio

The brain lithium concentrations at 120, but not at 30 or 60 minutes after intranasal LiCl in RFV administration were significantly higher than after both oral administration of LiCl in RFV or intranasal LiCl in water (Fig. 2A). On the other hand, the lithium blood concentrations at 30, 60 and 120 minutes after intranasal LiCl in RFV administration were generally lower than those observed after intranasal LiCl in water or oral lithium LiCl in RFV administration. Specifically, they were statistically significantly lower compared to concentrations following oral LiCl in RFV administration (Fig. 2B). Subsequently, the brain/blood lithium concentration ratio at 120 minutes after intranasal administration of LiCl in RFV was dramatically and significantly higher than after either oral LiCl in RFV or intranasal LiCl in water, exceeding by more than four times (Fig. 2C).

Intranasal LiCl in RFV inhibits both memory loss and depression behavior in both young adult and aged 5xFAD mice

The freezing time in fear condition test indicates the level of memory. The longer the freezing time the better memory. The contextual fear conditioning (CFC; hippocampus-dependent) test demonstrated significant memory loss in young 6 months of age 5xFAD, which could be abolished by 12 weeks of intranasal LiCl in RFV treatment (Fig. 3A). In old adult mice at 12 months of age in LTG group, the hippocampus-independent FC-cued test indicated a significant memory loss in aged mice and could be corrected by intranasal LiCl in RFV treatments for 12 weeks in old adult mice (Fig. 3B). However, the cognitive function determined by the Y-maze did not detect a memory loss, nor did the intranasal or oral LiCl in RFV affect cognitive function in 5xFAD mice at either 6 (Supplemental figure 1A) or

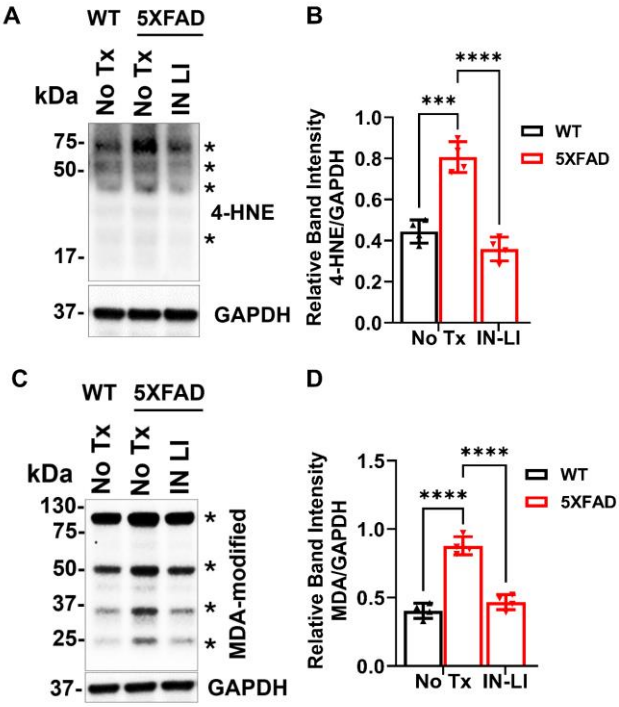

Figure 5. Intranasal LiCl in Ryanodex formulation vehicle (RFV) inhibited oxidative stress in aged 5xFAD mice brains. Wild type (WT) or 5xFAD mice at 9-month-old were treated with intranasal LiCl in RFV (INLI, 3 mM/kg), 5 days/week for 3 consecutive months. Mice brains harvested at 13 months old. Immunoblot determined the lipid peroxidation product 4HNE (**A, B**) or MDA modified proteins (**C, D**). Data are Means±SD from 4 separate mice in each treatment group (N=4) and were analyzed using the two-way ANOVA followed by Tukey's multiple comparison test. ***,****, P<0.001, P<0.0001

12 (Supplemental figure 1B) months of age. In the tail suspension test to measure levels of depression behavior, immobility time represented the level of depression behavior. The more immobility time, the more severe the depression behavior. The immobility times in 5xFAD mice were significantly higher than in WT control mice in both young adult (Fig. 3C) and old adult groups (Fig. 3D). However, intranasal LiCl in RFV treatment for 12 consecutive weeks abolished the increased immobility time in both young (Fig. 3C) and old (Fig. 3D) 5xFAD mice, returning it to wild-type levels. Overall, these results demonstrate that intranasal LiCl in RFV can abolish both memory loss and depression behavior in both young and old adult 5xFAD mice.

Intranasal LiCl in RFV inhibits the pathological increase of type 1 $InsP_3R$ (InsP3R-1) in aged 5xFAD mice

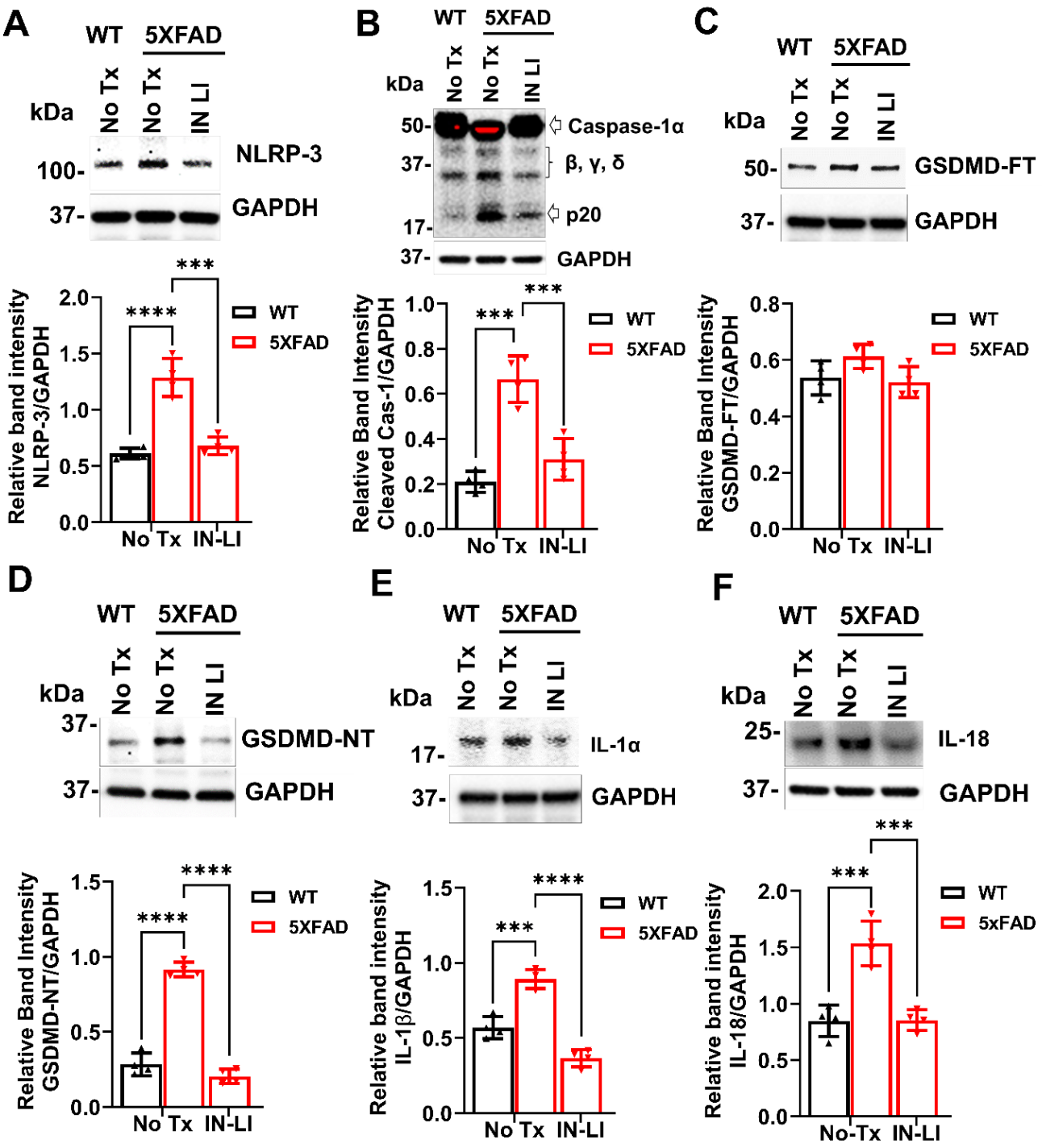

Figure 6. Intranasal LiCl in Ryanodex formulation vehicle (RFV) inhibited pathological activation of pyroptosis in 5xFAD mice brains. Wild type (WT) or 5xFAD mice at 9-month-old were treated with intranasal LiCl in RFV (INLI, 3 mM/kg), 5 days/week for 3 consecutive months. Mice brains harvested at 13 months old. NLRP3: NLR family pyrin domain containing 3, GSDMD-NT: N terminal Gasdermin D, GSDMD-FL: full length GSDMD Gasdermin D, IL-1β & 18: Interleukin 1β & 18. **A-F**. Representing Western Blot, and Statistical analysis. Data are Means±SD from 4 separate mice brains (N=4) and were analyzed using two-way ANOVA for each protein followed by Tukey's multiple comparison test. ***, ****, P<0.001, P<0.0001, respectively.

As an important AD pathology, InsP3R-1 activity is significantly increased in AD cells[8] or animal models[45]. Therefore, we determined the effects of intranasal LiCl in RFV treatment on changes of InsP3R-1 protein levels using

immunoblotting. Intranasal LiCl in RFV treatments for 12 weeks abolished the pathological increase of InsP3R-1 protein in aged 5xFAD mice brains (Fig. 4). These results suggested that intranasal LiCl in RFV treatment abolish pathological elevation of InsP3R-1 protein, the critical Ca^{2+} channels that lead to abnormal and excessive Ca^{2+} release from the ER, thus inhibiting an upstream critical Ca^{2+} dysregulation core pathology and subsequently ameliorate or abolish both memory loss and depression behavior in AD.

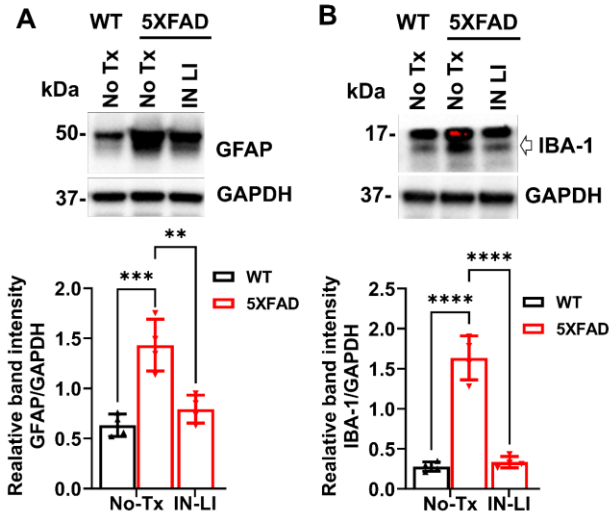

Figure 7. Intranasal LiCl in Ryanodex formulation vehicle (RFV) inhibited astrogliosis/microgliosis in aged 5xFAD mice brains. Wild type (WT) or 5xFAD mice at 9-month-old were treated with intranasal LiCl in RFV (INLI, 3 mM/kg), 5 days/week for 3 consecutive months. Mice brains harvested at 13 months old. Immunoblot was used to determine protein biomarkers of astrogliosis (GFAP) (**A**) and microgliosis (IBA-1) (**B**), neurotoxic. Data are Means±SD from 4 separate mice brains (N=4) and analyzed using two-way ANOVA followed by Tukey's multiple comparison test. **, ***, **** P<0.01, P<0.001, P<0.0001, respectively.

Intranasal LiCl in RFV suppresses the oxidative stress in 5xFAD mice

The pathological elevation of oxidative stress plays a crucial role in pathologies of cognitive dysfunction and depression behavior in AD[70], which is an initiation factor for pathological inflammation and pyroptosis[56, 71]. In fact, oxidative stress is a key feature in the early stages of AD and mild cognitive impairment (MCI)[72]. It has been reported that oxidized biomolecule by-products, produced in the neuronal cells due to the overproduction of MDA-modified proteins, lead to increased ROS in AD brains[73, 74]. Protein-bound 4-hydroxynonenal (HNE) is also widely used oxidative stress markers[62, 75]. Therefore, we measured protein oxidation by determining oxidized biomolecule by-products, malondialdehyde (MDA) modified proteins, and the protein bound HNE. Both protein-bound 4-HNE and MDA-modified proteins were significantly increased in 5xFAD mice, in

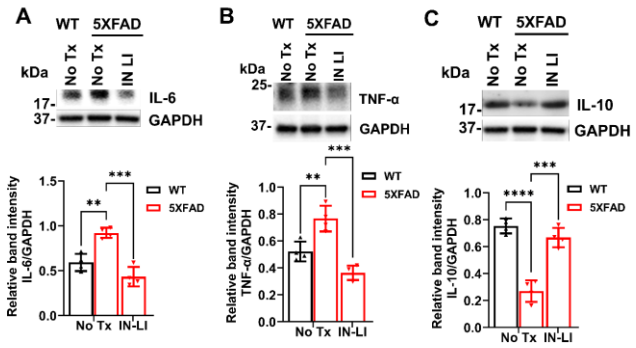

Figure 8. Intranasal LiCl in Ryanodex formulation vehicle (RFV) inhibited the pathological increase of neurotoxic cytokines and increase neuroprotective cytokine in aged 5xFAD mice brains. Wild type (WT) or 5xFAD mice at 9-month-old were treated with intranasal LiCl in RFV (INLI, 3 mM/kg), 5 days/week for 3 consecutive months. Mice brains harvested at 13 months old. Immunoblot was used to determine protein biomarkers of neurotoxic (TNF-α, IL-6) (**A, B**) or neuroprotective (IL-10) cytokines (**C**). Data are Means±SD from 4 separate mice brains (N=4) and analyzed using two-way ANOVA followed by Tukey's multiple comparison test. **, ***, **** P<0.01, P<0.001, P<0.0001, respectively

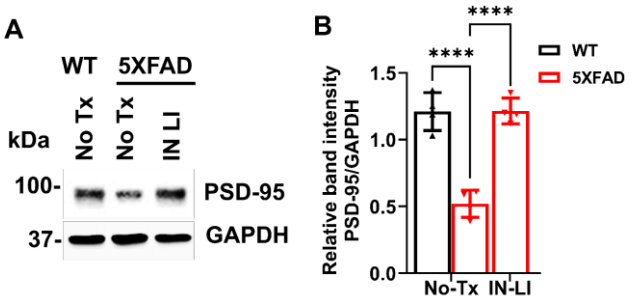

Figure 9: Intranasal LiCl in Ryanodex formulation vehicle (RFV) inhibited synapse protein loss in aged 5xFAD mice brains. Wild type (WT) or 5xFAD mice at 9-month-old were treated with intranasal LiCl in RFV (INLI, 3 mM/kg), 5 days/week for 3 consecutive months. Mice brains harvested at 13 months old. Immunoblot was used to determine the expression levels of PSD-95 (**A**) and Statistical analysis (**B**) . Data are Means±SD from 4 separate mice brains (N=4) and analyzed using two-way ANOVA followed by Tukey's multiple comparison test ****, P<0.0001

comparison to the WT control. Intranasal LiCl in RFV abolished the pathologically increased protein-bound 4-HNE (Fig. 5 A, B) and MDA-modified proteins (Fig. 5 C, D).

Intranasal LiCl in RFV inhibits pathological activation of pyroptosis pathway in 5xFAD mice brains.

Recent studies have shown that Aβ plaque deposition and oxidative stress can result in activation of the NLRP3

inflammasome complex, which leads to pathological inflammation[76, 77] and programmed cell death by pyroptosis[78]. We measured the protein levels of primary regulators of pyroptosis, including NLR family pyrin domain containing 3 (NLRP3), caspase-1, Gasdermin D full-length (GSDMD-FT) and N-terminal Gasdermin D (N-GSDMD) in old adult WT versus 5xFAD mice. The core inflammasome complex protein NLRP3 was significantly increased in 13-month-old 5xFAD mice brain compared with the WT control, which was be robustly inhibited by the intranasal LiCl in RFV treatment for 12 consecutive weeks (Fig. 6A). Intranasal LiCl in RFV treatment robustly inhibited pathological elevation of cleaved caspase-1 (Fig. 6B), not change full length (Fig. 6C) but inhibited elevation of N-terminal Gasdermin D (N-GSDMD, Fig. 6D) Consistently, intranasal LiCl in RFV robustly inhibited the pathologically increased IL-1β (Fig. 6E) and IL-18 (Fig. 6F) in 5xFAD mice brain, two critical cytokines contributing activation of pyroptosis[78]. Overall, these results suggest that intranasal LiCl in RFV significantly inhibited increase of pyroptosis pathway activation proteins in aged 5xFAD mice brains.

Intranasal LiCl in RFV inhibits the pathological elevation of neurotoxic cytokines and inhibited astrogliosis and microgliosis in 5xFAD mice

Both astrogliosis and microgliosis contribute to pathological inflammation[79-81]. GFAP, a biomarker protein for astrogliosis, was significantly increased in 5xFAD mice brain compared to WT controls, but this increase was robustly inhibited by intranasal LiCl in RFV treatments(Fig. 7A). Similarly, the biomarker protein for microgliosis, IBA-1, was significantly elevated in 5xFAD mice compared to WT controls , and this increase was also abolished by intranasal LiCl in RFV (Fig. 7B). Pathological elevation of cytotoxic cytokines plays a critical role on programmed death by pyroptosis[78, 82]. We have determined changes of critical neurotoxic (IL-6, TNF-α) and neuroprotective (IL-10) cytokines in the brains of aged 5xFAD mice, both with or without intranasal LiCl in RFV treatments. Cytotoxic cytokines IL-6 (Fig. 8A) and TNF-α (Fig. 8B) were pathologically elevated, while the cytoprotective cytokine (IL-10) was abnormally decreased (Fig. 8C). These imbalances were corrected by intranasal LiCl in RFV treatments. Altogether, our results indicate that intranasal LiCl in RFV inhibits the pathological inflammation in aged 5xFAD mice.

Intranasal LiCl in RFV inhibited the decrease of postsynaptic synapse proteins in 5xFAD mice

Normal synapse structure plays an important role in synapse function, while their destruction contributes to cognitive dysfunction and depression behavior[83, 84]. Levels of the postsynaptic synapse protein PSD95 were significantly decreased in 13-month-old, aged 5xFAD mice compared to WT controls. This decrease was reversed by intranasal LiCl in RFV treatments for 12 weeks from 9 to 12 months of age (Fig. 9).

Chronic administration of intranasal LiCl in RFV did not cause thyroid or kidney toxicity or side effects

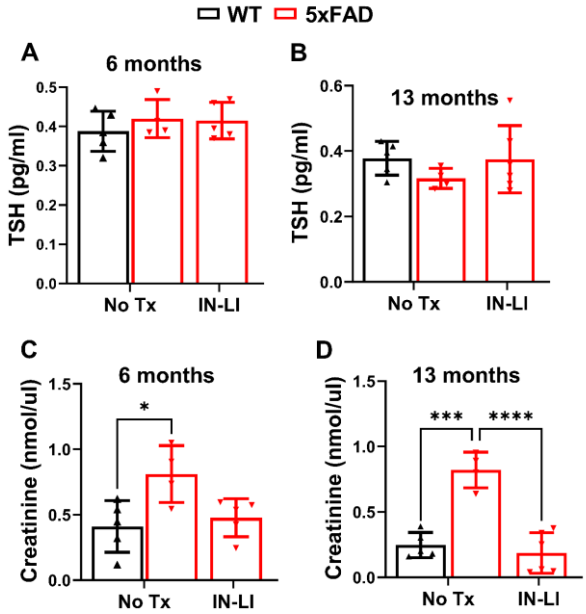

Figure 10. Intranasal LiCl in RFV protected kidney dysfunction in 5XFAD mice. Wild type (WT) or 5xFAD mice at 2 or 9 months old were treated with intranasal LiCl in RFV (INLI 3 mM/Kg), 5days/week, for 3 consecutive months. Mice bloods were harvested at the age of 6 (**A, C**) and 13 (**B, D**) months old. Blood biomarkers for function of thyroid (**A, B**) (TSH: thyroid stimulating hormone), kidney (**C, D**) (Creatinine) was determined at indicated ages. Data are Means±SD from blood of 4-6 separate mice (N=4-6) and analyzed using two-way ANOVA followed by Tukey's multiple comparison test. *, ***, **** represents P<0.05, P<0.001, P<0.0001, respectively.

Chronic use of lithium, especially at a high dose, is prone to cause thyroid or kidney dysfunction[64, 85]. We used various ELISA kits to measure the biomarkers reflecting function of thyroid and kidney. Intranasal LiCl in RFV treatment for 12 consecutive weeks in either ETG group (from 2 to 5 months of age) or LTG group (from 9 to 12 months of age), did not cause significant changes in blood biomarkers for thyroid (Fig. 10 A and B), but inhibited kidney dysfunctions in both young (6 months of age) and old (13 months of age) adult 5xFAD mice (Fig. 10 C and D). Intranasal LiCl in RFV for 12 weeks in the ETG did not cause significant changes of motor or nose smell function determined by the rotarod (Supplemental figure 2A) and food buried test (Supplemental Fig. 2B), respectively. No significant changes in body weight were detected during intranasal LiCl in RFV treatment from 9 to 12 months of age in the LTG group (Supplemental Fig. 2, C and D). These results suggest that chronic intranasal LiCl in RFV treatment is safe without significant organ side effects or organ toxicity.

Discussion

This study has demonstrated that a novel approach involving intranasal LiCl in RFV administration significantly increased the brain/blood concentration ratio. Intranasal LiCl

in RFV robustly protected against the pathological increase of $InsP_3R$ Ca^{2+} channel protein levels, oxidative stress, pathological inflammation, and programmed cell death by pyroptosis, with no toxic effects on the thyroid and with protective effects kidney function with chronic use.

Although lithium has been demonstrated to have some neuroprotective efficacy in some preclinical AD cell or animal models and even in some AD patients[86, 87], its notorious narrow therapeutic window and being prone to side effects or organ toxicity may limit its potential future use for treatment of AD patients, especially under the need of chronic use[66]. Intranasal administration of drugs promote their penetration and entrance into the CNS compared to the peripheral blood system, increases the duration on the nasal surface, and maintains a high level of concentrations in CNS[88]. Also, intranasal administration of drugs in nanoparticle formulation further promotes penetration into the CNS and has been increasingly used for treatment of CNS disorders[89, 90]. We have demonstrated that dantrolene dissolved in RFV, a nanoparticle suspension, significantly increased the dantrolene brain/blood concentration ratio[27, 67], and inhibited AD pathologies and abolished memory loss in 5xFAD mice as a disease-modifying drug, without side effects or organ toxicity after up to 10 months of treatment [27]. Similarly in this study, our novel approach of intranasal administration of LiCl in RFV significantly increased the lithium brain/blood concentration ratio at 120 minutes after administration, compared to either intranasal LiCl in water or oral LiCl in RFV. A higher brain/blood lithium concentration ratio indicates a promotion of lithium's therapeutic effects in the CNS, with a lower risk of peripheral system side effects or organ toxicity. Therefore, a lower dose of lithium nanoparticles via intranasal administration can be used to achieve same therapeutic effects rather than by the commonly-used oral lithium administration in RFV or water. The particle structure of LiCl or other forms of lithium (lithium carbonate, lithium orotate etc.) dissolved in RFV should be further studied to optimize its brain/blood lithium concentration ratio for a strong therapeutic efficacy in the CNS with minimal side effects or organ toxicity in peripheral system.

Although dementia is the primary symptom in AD patients, the high incidence of major depressive disorder may deteriorate dementia and even form a vicious cycle to worsen each other. Thus, a drug capable of effectively treating both dementia and depression behavior would promote high therapy efficacy in AD patients. Lithium is a first-line drug to treat depression and mania in bipolar disorder, but its use to treat depression in AD is less well studied. The efficacy of lithium to treat cognitive dysfunction is not consistently approved in AD patients, with controversy. Considering both the necessity for prolonged treatment duration and the narrow therapeutic window of lithium, the chronic use of high-dose lithium for treatment of AD may face limitations. With the new approach of intranasal lithium in RFV administration, this study demonstrates a robust protection against both cognitive dysfunction and depression behavior. Given the need of chronic treatments of most neurodegenerative diseases, especially AD, achieving higher brain/blood concentration ratios through intranasal lithium chloride administration compared to the oral approach provides a better opportunity to minimize the lithium dose. This approach aims to keep the dose as low as possible, reducing the risk of thyroid and kidney toxicity and other side effects with chronic lithium. The therapeutic efficacy and benefit of intranasal administration of various lithium formulations in RFV should be further studied in different AD animal models and eventually evaluated in AD patients, as well as in patients with psychiatric disorders, such as bipolar disorder.

Accumulating studies suggest that inflammation plays a significant role in AD pathology and contributes to both cognitive dysfunction and depression behavior. Overactivation of NMDA glutamate receptors (NMDAR) on plasma membranes and RyRs on ER membranes in AD collectively contribute to the upper stream pathological increase in cytosolic and mitochondrial Ca^{2+} concentrations. This increase leads to downstream mitochondrial dysfunction, oxidative stress, and subsequent activation of the NRLP3 inflammasome complex and caspase-1. This cascade also leads to an elevation of cytokines IL-1β and IL-18, cleavage of NSDMD into an ion channel N-terminal to release cytotoxic cytokines, and ultimately, programmed cell death by pyroptosis[9]. Although lithium has been demonstrated to be neuroprotective by inhibition of inflammation, its effects and mechanisms on inflammation associated pyroptosis has not been well investigated. This study demonstrates that intranasal LiCl nanoparticles significantly inhibits upstream Ca^{2+} dysregulation by ameliorating the pathological increase of $InsP_3R$-1 and abolishes downstream oxidative stress, pathological inflammation, pyroptosis, and synapse destruction in 5xFAD mice brains. In consistence, intranasal LiCl in RFV effectively treats both memory loss and depression behavior by its potent inhibition of inflammatory pyroptosis and synapse proteins loss.

Calcium dysregulation, along with associated inflammation and pyroptosis, also plays critical role in pathologies of neurodegenerative diseases, such as stroke, Parkinson disease, Huntington disease, seizure, and Amyotrophic Lateral Sclerosis (ALS) [91-96]. These neurodegenerative diseases often are chronic diseases and need prolonged treatments. The novel approach involving intranasal lithium nanoparticle administration is expected to promote its therapeutic effects in the CNS while minimizing peripheral side effects and organ toxicity, especially under prolonged treatment for various psychiatric disorders[66]. Thus, we propose that intranasal LiCl in RFV may provide neuroprotection not only in AD, but other commonly seen neurodegenerative diseases and psychiatric disorders.

Lithium is a first-line drug to treat bipolar disorder, but it is limited by a narrow therapeutic window and is prone to causing thyroid or kidney toxicity with chronic use[64]. Our study is the first to demonstrate that blood creatinine, a kidney

function biomarker, was significantly increased in 5xFAD mice compared to the WT control, especially in aged 5XFAD mice, indicating a kidney dysfunction. Blood creatinine levels are associated with the blood biomarkers for neurodegeneration, including neurofilament light chain(NFL)[97], amyloid protein[98], and phosphorylated tau[97], as well as the risk of dementia[99, 100]. An important finding in this study is that both intranasal and oral lithium in RFV, especially via the intranasal approach, abolished the abnormally increased blood creatinine level, suggesting that lithium can protect kidney dysfunction in AD, and function as another mechanism to ameliorate dementia. As GSDMD, a critical pyroptosis player, and inflammation play important role contributing to kidney damage and dysfunction[101, 102], the potent inhibition of pyroptosis and inflammation by lithium may explain its protective effects on kidney function in this study. Notably, kidney dysfunction is one of the organ toxicities associated with chronic and high-dose lithium treatment for bipolar disorder, and the blood lithium concentration in this study is relatively low. This result suggests that a low concentration of lithium may be cytoprotective while a high concentration of lithium can be cytotoxic. It is important to promote the use of low doses of lithium for chronic treatment of AD, neurodegenerative diseases, or psychiatric disorders to minimize side effects and organ toxicity. Intranasal lithium nanoparticles, with their potential to reduce side effects and organ toxicity compared to commonly used oral administration, warrant further investigation

This study has the following limitations: 1). The sample size in each experimental group is relatively small. However, lithium demonstrated significantly statistically therapeutic effectiveness, suggesting its robust potency of neuroprotection in this AD animal model. 2). We were unable to measure the changes of cytosol versus mitochondria Ca^{2+} levels in the brain tissue, but with lithium inhibition on the increase of InsP3R protein levels, the study indirectly suggests lithium inhibits upstream Ca^{2+} dysregulation. 3). We did not measure the contents of reactive oxygen species (ROS) concentrations in brains directly but used the changes of oxidized lipid membrane proteins as indirect indication of oxidative stress. 4). We only examined the inflammation biomarkers and pyroptosis pathway activation proteins in old, but not young-adult brains. As 5xFAD mice at 6 months of age already developed full AD pathologies and cognitive dysfunction, effectiveness of lithium to ameliorate memory loss and depression behavior suggest that intranasal lithium chloride nanoparticles can be considered a disease-modifying drug in AD. We predict that the lithium will have similar neuroprotective effects against inflammation and pyroptosis and associated synapse destruction in young-adult 5xFAD mice. Additionally, we did not administer intranasal lithium nanoparticle vehicle treatment in older adult mice because the treatment did not significantly alter memory or depression behavior in young-adult WT mice.

Conclusion

In conclusion, this study suggests that chronic intranasal LiCl in RFV significantly ameliorates both cognitive dysfunction and depression behavior in 5xFAD mice, without affecting thyroid, smell, and motor function, and while also protecting kidney function. Lithium neuroprotection in 5xFAD mice was associated with its robust inhibition of pathologically increased $InsP_3Rs$ protein levels, oxidative stress on lipid membrane proteins, pathological inflammation, cell death by pyroptosis and synaptic protein loss. This study will inspire future research work investigating neuroprotective versus side effects of intranasal lithium in RFV in AD and other neurodegenerative diseases or psychiatric disorders.

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Funding Declaration

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Author contributions

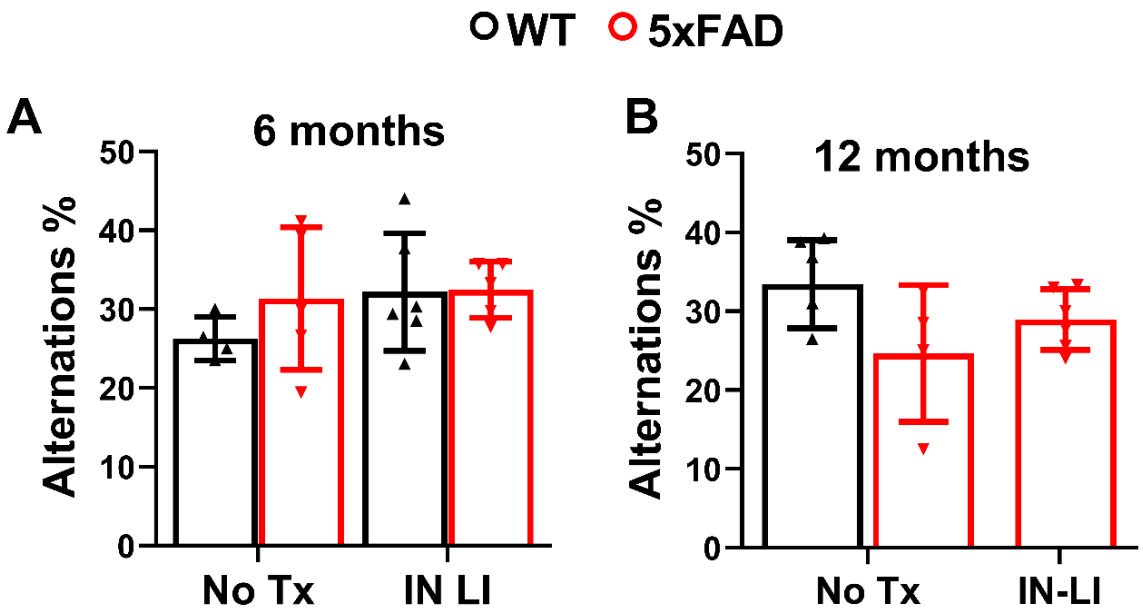
H.W. conceived and designed the study. P.B, W.Z, G.L, B.J, R.V, R.C, K.K, L.S.L, Y.W, J.L, H.W. conducted experiments and acquired the data, P.B, W.Z, G.L, B.J, R.V, R.C, K.K, L.S.L, and H.W. analyzed data. P.B, W.Z, G.L, R.C, K.K, L.S.L and H.W contributed to the manuscript preparation. All the authors reviewed and approved the final manuscript.

Conflict of Interest

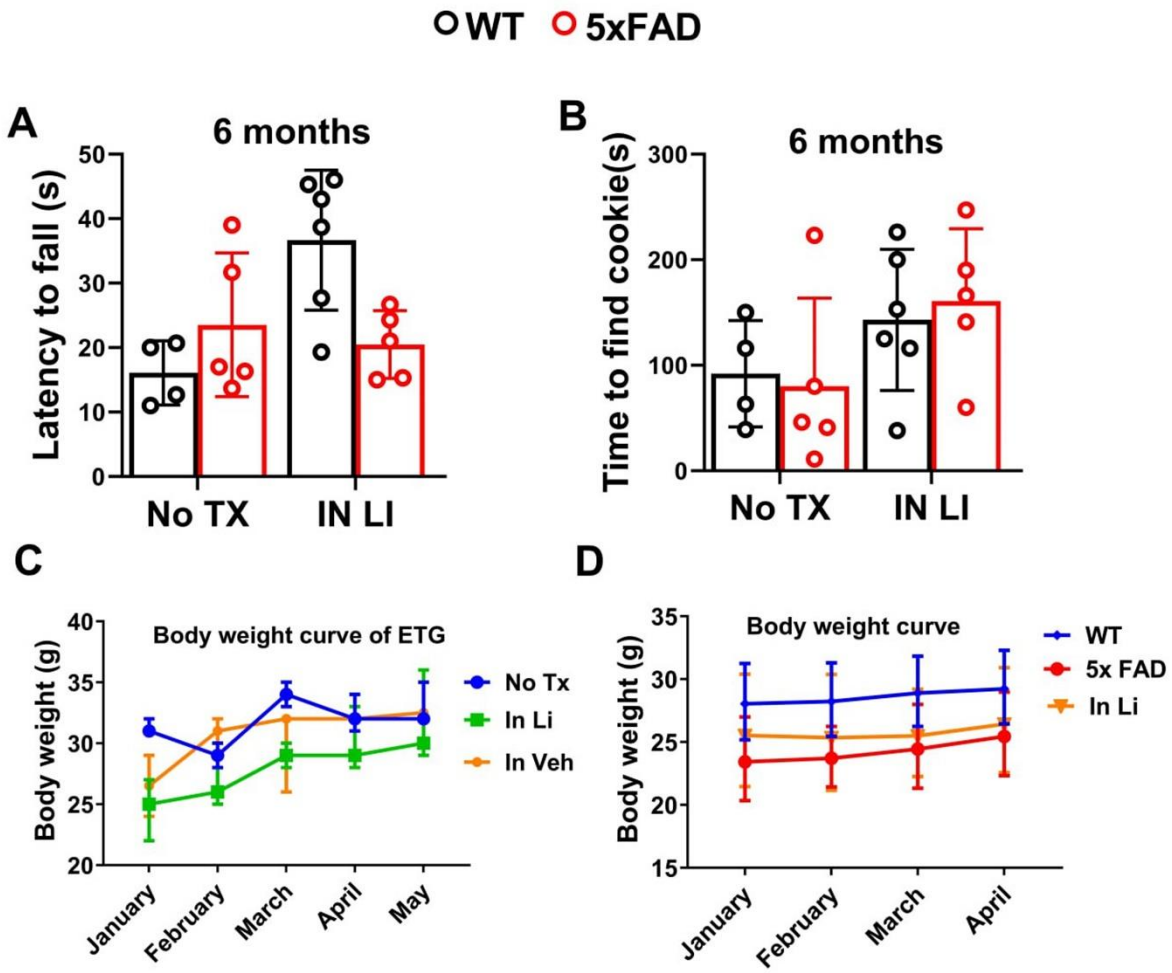
No authors have conflict of interest.

Data Availability

Data is available upon request.



Supplemental Figure 1. Effect of intranasal LiCl in Ryanodex Formulation Vehicle (RFV) on cognitive function in 5XFAD mice. Wild type (WT) or 5xFAD mice were treated with intranasal (IN) lithium chloride (IN LI, 3 mM/kg) dissolved in Ryanodex Formulation Vehicle (RFV), daily, Monday to Friday for 12 consecutive weeks. Y-maze was performed at the age of 6 (**A**) and 12 months (**B**) after completion of treatments. Data represents Means±SD from 4-6 mice (N=-6) and was analyzed using the two- way followed by Tukey's multiple comparison test.



Supplemental figure 2: Effects of chronic intranasal LiCl in Ryanodex Formulation Vehicle (RFV) on motor and smell functions and body weight. Wild type (WT) or 5xFAD mice were treated with intranasal (IN) LiCl in RFV (IN LI, 3 mM/kg) nanoparticles, daily, Monday to Friday for 12 consecutive weeks from 2 to 5 months old. Motor (**A**) and olfactory (**B**) functions were determined by rotarod, and food buried tests respectively at 6 months of age. Body weights were determined monthly from initiation of IN LiCl in RFV at 2 months old **(C)** until the end of all behavioral tests at 12 months old (**D**). Data is Means±SD from 4-6 mice.

Table 1: Antibodies applied in immunoblotting

	Cat. and Manufacture		**Dilution**
Primary antibody			
Anti- RYR2	19765-1-AP; Proteintech		1:1000
Anti- InsP3R1	# PA1-901, Invitrogen		1:1000
Anti-MDA-modified protein	MA5-27560, Invitrogen		1:1000
Anti-4 Hydroxynonenal	(ab46545), Abcam		1:1000
Anti- NLRP3	68102-1-Ig; Proteintech		1:2000
Anti-Human procaspase-1/ cleaved caspase-1 p20	#2225S, Cell signaling Technology,USA		1:1000
Anti-GSDMD	ab155233; Abcam		1:1000
Anti- Cleaved GSDMD	#36425S; Cell signaling Technology, USA		1:1000
Anti-IL-1β	31202S, Cell signaling Technology, USA		1:1000
Anti-IL-18	10663-1-AP; Proteintech		1:3000
Anti-IL-6	# M620; Invitrogen		1:1000
Anti-TNF-α	60291-1-Ig; Proteintech		1:2000
Anti-GFAP	#3670; Cell signaling Technology, USA		1:1000
Anti-Iba1	# 17198; Cell signaling Technology, USA		1:1000
Anti-IL-10	#12163S, Cell signaling Technology, USA		1:1000
Anti-PSD95	#2507; Cell signaling Technology, USA		1:1000
GAPDH	MA5–15738, Thermo Fisher Scientific		1:10,000
Secondary antibody			
Anti-Mouse IgG1; HRP - linked	# PA1-74421, Thermo Fisher Scientific		1:10,000
Anti-rabbit IgG, HRP-linked	#7074; Cell signaling Technology, USA		1:1000