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Intranasal dantrolene nanoparticles inhibit lipopolysaccharide-induced depression and anxiety behavior in mice

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Abstract: This study investigates the therapeutic effectiveness of intranasal dantrolene nanoparticles pretreatment to inhibit lipopolysaccharide (LPS)-induced pathological inflammation and synapse destruction and depressive and anxiety behavior in mice. Both wild-type (WT) B6SJLF1/J and 5XFAD adult mice (5-10 months old) were pretreated with intranasal dantrolene nanoparticles (dantrolene: 5mg/kg), daily, Monday to Friday, 5 days per week, for 4 weeks. Then, mice were treated with intraperitoneal injection of LPS (5mg/kg) for one time. Behavioral tests for depression, anxiety and side effects were performed 24 hours after a one-time LPS injection. Biomarkers for pyroptosis-related inflammation cytokines (IL-1β and IL-18) in blood and brains were measured using enzyme-linked immunosorbent assay (ELISA) and immunoblotting, respectively. The changes of primary proteins activation inflammatory pyroptosis (NLRP3: NLR family pyrin domain containing 3, Caspase-1, N-GSDMD: N terminal protein gasdermin D) and synapse proteins (PSD-95 and synpatin-1) in brains were measured using immunoblotting. Intranasal dantrolene nanoparticles robustly inhibited LPS-induced depression and anxiety behavior in both WT and 5XFAD mice, without obvious side effects. Intranasal dantrolene nanoparticles significantly inhibited LPS-induced pathological elevation of IL-1β and IL-18 in the blood and synapse loss in the brain. Intranasal dantrolene nanoparticles trended to inhibit LPS-induced elevation of IL1β and IL-18 and the pyroptosis activation proteins in the brain in both type of mice. In conclusion, intranasal dantrolene nanoparticles demonstrated neuroprotection against inflammation mediated depression and anxiety behaviors and should be studied furthermore as a future effective drug treatment of major depression disorder or anxiety psychiatric disorder, especially in AD patients.

Introduction

Three hundred million people worldwide suffer from major depressive disorder (MDD) (Herrman et al 2019). MDD is a chronic and recurrent disease affecting about 20% of the population and the leading cause of suicide (Rikhani et al 2023). MDD imposes a tremendous psychological burden, as well as significant social repercussions and contributions to other disabilities (Belge et al 2023). The expected direct and indirect costs of MDD are up to $6 trillion in the USA alone (Gururajan et al 2019). An estimated 4% of the global population currently experience an anxiety disorder. In 2019, 301 million people in the world had an anxiety disorder, with a lifetime prevalence of approximately 34 percent (Szuhany & Simon 2022). Anxiety disorders were approximately twice as prevalent among women, with overall age-specific rates remaining relatively stable or increasing across the lifespan (Somers et al 2006). In 2021, an estimated 5.8 million (9.4%) children and adolescents were impacted by anxiety (Bruton et al 2024). Depression and anxiety often co-exist and are the most common psychiatric diseases, which are chronic diseases with unclear mechanisms of pathology (Ait Tayeb et al 2023).

Classical treatment of depression or anxiety psychiatric disorders includes atypical antipsychotics that modulate dopamine and serotonin neurotransmission (Cowen 2024, Malena et al 2024). Pharmacotherapies for anxiety disorders also include drugs that interact with GABA neurotransmission systems (Nuss et al 2019). Unfortunately, antidepressants have significant limitations, including slow onset of action, high rates of nonresponse, and acute worsening of anxiety (Paganin et al 2022). Benzodiazepines are not recommended for long-term use in some anxiety disorders, due to concerns about their potential for abuse, tolerance, and withdrawal, and they are ineffective in some anxiety spectrum disorders (Robertson et al 2023). Treatment of MDD is prone to a high risk of resistance (up to 30% of patients are unresponsive to the first treatment) and relapse (up to 8%) (Ait Tayeb et al 2023, Belge et al 2023). Treatment-resistant anxiety with remission rates may be as low as 25–35%, and relapse rates post remission may be 30% after 10 years (Tabaac et al 2024). Thus, there is an urgent need to develop novel approaches to treat depression and anxiety, especially treatment-resistant depression, or anxiety, with minimal side effects or organ toxicity. Increasing studies indicated that glutamate neurotransmission plays a critical role in mood function and its imbalance may cause

psychiatric disorders (Sarawagi et al 2021). Glutamate is linked to the development of anxiety disorders through its regulation of neuropeptides, fear extinction, and stress response. Glutamate is also important in synaptic and neural plasticity related to psychiatric disorders. Ketamine, an N-methyl-d-aspartate (NMDA) glutamate receptor antagonist, was approved in 1970 by the Food and Drug Administration (FDA) for use in children and adults as an anesthetic. Recent studies indicate that ketamine is effective in treating MDD, especially treatment-resistant depression (TRD) (Costi 2023, Ren 2024). Ketamine is also effective in treating anxiety disorders, even those resistant to traditional treatments (Bruton et al 2024, Tully et al 2022). While traditional antidepressants can take weeks to months to have an effect, ketamine has rapid effects on mood and suicidality, with mood changes reported as early as the first 4 hours after treatment. As an antagonist of NMDAR, a primary glutamate receptor on the plasma membrane that causes Ca^{2+} influx into the cytosol from extracellular space, ketamine has become a safe and broadly effective drug to treat depression or anxiety disorders. This indicates that glutamate-mediated excitotoxicity and associated disruption of intracellular Ca^{2+} homeostasis play a key role in the pathology of depression and anxiety disorders.

Although molecular mechanisms are unclear, increasing evidence suggests that upstream disruption of intracellular Ca^{2+} homeostasis and associated downstream inflammation and synapse dysfunction play critical roles in MDD pathologies (Deutschenbaur et al 2016, Fries et al 2023, Garrosa-Jimenez et al 2021), as well as anxiety disorder (Valiati et al 2022, Wang et al 2024). The overactivation of ryanodine receptors (RyRs) in MDD and associated excessive Ca^{2+} release from endoplasmic reticulum (ER) results in depletion of ER and pathological elevation of cytosol and mitochondrial Ca^{2+} concentrations, detrimental to synapse function and cell survival (Cardon et al 2024, Garrosa-Jimenez et al 2021, Phillips et al 2019). RyRs have also been shown to increase anxious behavior (Phillips et al 2019). Upstream Ca^{2+} dysregulation results in mitochondria dysfunction (Song et al 2023), mitochondrial and cellular oxidative stress (Ait Tayeb et al 2023, Lindqvist et al 2017), activation of inflammasomes, and cell or neuron death by pyroptosis (Cao et al 2023, Li et al 2021a, Simon et al 2021, Zhou et al 2023). This results in the release of inflammatory cytokines (IL-1β and IL-18) and pathological inflammation-mediated cell death by pyroptosis (Kaufmann et al 2017, Li et al 2021a). Pathological cytokines, especially pyroptosis-related IL-18 play important roles in psychiatric disorders (Vogelzangs et al 2016, Zhang et al 2023b). Gut dysbiosis and associated inflammation also contribute to pathology in depression and anxiety behaviors (Anand et al 2022, Bhatt et al 2023). Thus, a drug that inhibits upstream Ca^{2+} dysregulation and then downstream pathological inflammation and programmed cell death by pyroptosis and synapse destruction is expected to treat MDD and anxiety disorder effectively.

The prevalence of psychiatric disorders in patients with Alzheimer's disease is particularly high, reaching up to 40% (Galts et al 2019). Depression and anxiety psychiatric disorders have been considered a prodrome of dementia, and they are two pathologies making a vicious cycle leading to symptoms in AD (Botto et al 2022, Hasan et al 2023). The Type 2 RyRs (RyRs-2) pathologically increased in the brains of AD patients (Bruno et al 2012). The number and activity of RyRs pathologically increase in different cell and animal models of AD (Chan et al 2000, Stutzmann et al 2006, Wang et al 2020b). Similar to the molecular mechanism of depression and anxiety, RyRs overactivation causes upstream Ca^{2+} dysregulation and downstream mitochondrial dysfunction (Walters & Usachev 2023), oxidative stresses (Gleitze et al 2021), initiation of pyroptosis pathway, and pathological inflammation (Yu et al 2021), eventually leading to synapse and cell or neuron damage (Briggs et al 2017). This leads to a high prevalence of depression and anxiety psychiatric disorders and cognitive dysfunction in AD (Garrosa-Jimenez et al 2021, Leonard 2001, Shi et al 2020, Singhal et al 2014). Thus, a drug that correct upstream critical Ca^{2+} dysregulation by inhibition of RyRs. Such as dantrolene, may be effective in treating both depression and anxiety psychiatric disorders in AD (Nakamura-Maruyama et al 2021, Oules et al 2012, Peng et al 2012, Shi et al 2020).

Dantrolene, a RyRs antagonist, is a US Food and Drug Administration-approved drug for the treatment of malignant hyperthermia, muscle spasms, and neuroleptic syndrome, with tolerable side effects and occasional liver toxicity at high doses (Ratto & Joyner 2020). Dantrolene, with its ability to inhibit the common upstream critical Ca^{2+} dysregulation, is neuroprotective against many neurodegenerative diseases, including cerebral ischemia (Nakayama et al 2002, Wei & Perry 1996), Huntington's disease (Chen et al 2011), spinocerebellar ataxia (Chen et al 2008), amyotrophic lateral sclerosis (Rothstein & Kuncl 1995), and seizures (Keshavarz et al 2016, Royero et al 2020). Dantrolene robustly inhibited up to 78% of NMDA-induced elevation of cytosolic Ca^{2+} concentration in cerebral cortical neurons (Frandsen & Schousboe 1992), suggesting it may have great potential to treat major depression disorder with a similar molecular mechanism of ketamine (Chen et al 2023). We and others have demonstrated that dantrolene abolishes or ameliorates cognitive dysfunction in multiple AD animal models (Chakroborty et al 2012, Oules et al 2012, Peng et al 2012, Shi et al 2020). Our recent studies pioneered a novel approach of administrating intranasal dantrolene nanoparticles in the Ryanodex formulation (a type of crystal nanoparticles) to increase its brain/blood concentration ratio, promote dantrolene's CNS therapeutic effects, and minimize its peripheral side effects/organ toxicity, especially in aged mice (Shi et al 2020, Vera et al 2024, Wang et al 2020a). Intranasal dantrolene nanoparticles abolished memory loss as a disease-modifying drug (Shi Y 2019). Importantly, chronic intranasal dantrolene nanoparticles administration had no significant side effects or organ toxicity in either amyloid (Shi et al 2020) or tau (Vera et al 2024) AD mice.

In this study, we investigated the therapeutic effectiveness and the potential mechanisms of intranasal dantrolene nanoparticles on depression and anxiety behavior associated with pathological inflammation in both wild-type and 5XFAD mice.

We induced inflammation by intraperitoneal injection of LPS. Our results demonstrated that intranasal dantrolene nanoparticles for 12 consecutive weeks robustly inhibited LPS-induced depression and anxiety behaviors in both WT and 5XFAD mice. These beneficial effects of intranasal dantrolene nanoparticles treatment were associated with its robust and significant inhibition of LPS-induced pathological elevation of pyroptosis related inflammation cytokine of IL-1β and IL-18 in blood and synapse proteins (PSD-95 and synapsin-1) in brains in both type of mice. This study provides proof of concept that intranasal dantrolene nanoparticles may be an effective treatment for depression and anxiety psychiatric disorders, potentially by inhibiting pathological inflammation and synapse destruction in CNS. This study will inspire future clinical studies investigating the therapeutic effectiveness versus side effects of intranasal dantrolene nanoparticles for the treatment of depression and anxiety psychiatric disorders, especially those that co-exist in AD patients.

Materials and Methods

Animals

All 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 mice (B6SJLF1/J) were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred. These 5XFAD transgenic mice over-express 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. Food and water were available in the cage. All mice were weaned at no later than one month of age and genetically identified by polymerase chain reaction (PCR) analysis before weaning. At that 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.

Experimental treatment groups

As demonstrated in Figure 1, age-matched male and female mice were randomly divided into experimental groups after being genotyped at around 1 month of age. The mice were pretreated with either intranasal dantrolene nanoparticles (IN Dan, 5mg/kg) or vehicle (Veh), once a day, 5X/week (Monday through Friday), in adult mice (5-10 months old). Control groups received no pretreatment. Fresh dantrolene nanoparticles were made every time before administration. Intranasal dantrolene nanoparticles stock solution was made at 5 mg/ml. The Ryanodex formulation vehicle was made fresh and contained all inactive ingredients in Ryanodex (Shi et al 2020, Vera et al 2023). At the end of the 4-week pretreatment period, mice were then treated with one-time intraperitoneal injection of lipopolysaccharide (LPS, 5 mg/kg), once. Mice in the control group, without pretreatment, were treated with LPS (LPS, 5mg/kg) once as well. The sham control group received no pretreatment or treatment. At 24 hours following the one-time LPS treatment, behavioral tests for depression or anxiety behaviors were performed on all mice, with the following order of least to most stressful tests: OPT, EPMT, TST, and FST (Fig. 1). Thereafter, all mice were euthanized, and the blood or brain were harvested for examination of inflammation and programmed cell death by pyroptosis and synapse destruction in blood and brains.

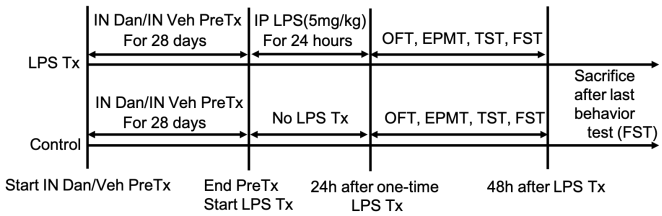

Figure 1. Experimental Design. Adult wild type (WT) and 5xFAD mice (5-10 months old) were pretreated with intranasal dantrolene nanoparticles in Ryanodex formulation (DNRF, 5mg/kg) or vehicle (Veh) for 4 weeks. Control group received no treatments. Mice were then treated with intraperitoneal (IP) injection of lipopolysaccharide (LPS, 5mg/kg) for one time. Depression and anxiety behavior tests were performed at 24 hours after one-time IP LPS injection. Behavioral tests in following orders: OFT, EPMT, TST, FST, designed to gradually increase stress levels. PreTX: Pretreatment, Tx: Treatment, IN: Intranasal, Dan: Dantrolene, OFT: Open Field Test, EPMT: Elevated Plus Maze Test, TST: Tail Suspension Test, FST: Forced Swimming Test

Intranasal dantrolene nanoparticle or vehicle administration

Dantrolene (Sigma, St Louis, MO) was dissolved in the 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), similar to our previous publications (Shi et al 2020, Wang et al 2020a). For intranasal administration, the final concentration of dantrolene was 5 mg/mL as we have previously described. Mice were held and fixed by the scruff of their necks with one hand. With the other hand, mice were given a total of 1μL/gram of body weight of dantrolene nanoparticles or RFV. Accordingly, a mouse weighing 20 g would be given 20μL of solution , which is equal to a dantrolene dose at dose at 5mg/kg. The solution was slowly delivered directly into the mouse's nose. Care was taken to ensure that mice were minimally stressed, and that the solution remained in the nasal cavity and did not enter the stomach or lungs.

Open field test (OFT)

The OFT was performed as previously described to evaluate anxiety behavior as described (Jones et al 2022). Each mouse was placed individually into the OFT apparatus (44 × 44 × 44 cm^3), facing the wall. The locomotor activity was recorded for six minutes with a camera above the apparatus under dim light. The anxiety behavior of each mouse was measured using the total distance traveled, center entries, immobility time, and time spent in the central zone. After each test, the apparatus was cleaned with 75% ethanol. Any-maze software (Stoelting USA) was used to analyze the collected data. The lesser the central zone distance and mean speed, the increased the anxiety behavior. The more immobile time, the more severe the anxiety behavior.

Elevated plus maze test (EPMT)

Anxiety was also assessed using EPMT, as described previously, although with some modification (Cho et al 2022). Mice were placed in the center of an elevated plus-maze (arms are 33 cm × 5 cm, with 25 cm tall walls on the closed arms) under dim lighting and their behavior was videotaped for 5 minutes. We used Any-maze software (Stoelting USA) to analyze the collected data. The time spent in the closed and open arms, as well as the number of explorations of open arms, was measured and recorded. The more time spent in closed arms, the more severe the anxiety behavior.

Forced swimming test (FST)

Depression behavior was assessed using the FST, as described previously with some modification (Ueno et al 2022). The mice from each group were settled in a crystal-clear glass tank of 25 cm height, 10 cm diameter, 15 cm water depth, and (23 ± 2) °C water temperature. The test's total period was 6 minutes, 2 minutes for adaptation, and the total immobility time was recorded in the next 4 minutes. Immobility occurred when the mice discontinued floundering on the surface of the water, instead appearing as floating in an equilibrium state. The greater the immobility time, the more severe the depression behavior.

Tail suspension test (TST)

Depression behavior was assessed using the tail suspension test described previously, with modifications (Can et al 2012). We use specially manufactured tail suspension boxes, made of cartons with the dimensions (42 cm height X 18 cm width X 30 cm depth). To prevent animals from observing or interacting with each other, each mouse was suspended within its three-walled rectangular box. Each day, animals were acclimated to the testing room for at least 1 hour before the test. Each mouse was suspended in the middle of the box. The width and depth of the box were sufficiently sized to prevent the mouse from contacting 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. depth), was used to suspend the tail of each mouse and positioned on the top of the box. At the bottom of each box, we placed a paper towel to collect feces or urine from the animals. A dark grey box, for albino animals, and a white colored box, for mice of other coat colors, were used. Before evaluation, the tail of the mouse was securely adhered to the suspension bar, which was able to withstand the mouse's weight. A video camera was placed in position and started recording the TST session. The total duration of the test was 6 minutes. The paper towel was replaced after each trial. After all sessions were finished, Any-maze software (Stoelting, USA) was used to analyze the collected data.

During the behavioral analysis, the mobility time of each mouse was measured and subtracted from 360 seconds, producing the immobility time. The investigator was blinded to the animal groups. The greater immobility time, the more severe the depressive behavior.

Euthanasia and tissue collection

Mice were euthanized after the completion of all behavioral tests. As described previously (Shi et al 2020), mice were deeply anesthetized with 2–4% isoflurane delivered through a nose cone, with the concentration was adjusted according to response to a toe pinch. The skin of each mouse was prepared, and an incision was made to open the chest and expose the heart. Blood was collected from the heart for the serum study using a syringe equipped with a 27G needle. The blood was centrifuged at 1400 rpm at 4◦C for 30 min, and the supernatant was collected and frozen at –80◦C. The mice were euthanized by trans-cardial perfusion and exsanguination was conducted with cold phosphate-buffered saline.

Measurements of serum concentration of IL-1β and IL-18

We measured the serum IL-1β and IL-18 cytokines using an ELISA kit, following company instructions, and as we described previously for measurement of S100β, with some modification (Joseph et al 2019, Liang et al 2010). All reagents and samples (the supernatant of the blood) were thawed to room temperature (18 - 25 °C) before use. It is recommended that all standards and samples be run at least in duplicate. Add 100 µL of each standard and sample into appropriate wells. Cover wells and incubate for 2.5 hours at room temperature or overnight at 4 °C with gentle shaking. Discard the solution and wash it 4 times with 1X Wash Solution. Wash by filling each well with Wash Buffer (300 µL) using a multi-channel Pipette or auto washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels. Add 100 µL of 1x prepared Detection Antibody to each well. Cover wells and incubate for 1 hour at room temperature with gentle shaking. Discard the solution. Repeat the wash procedure as in step 3. Add 100 µL of prepared Streptavidin solution to each well. Cover wells and incubate for 45 minutes at room temperature with gentle shaking. Discard the solution. Repeat the wash as in step 3. Add 100 µL of TMB One-Step Substrate Reagent (Item H) to each well. Cover wells and incubate for 30 minutes at room temperature in the dark with gentle shaking. Add 50 µL of Stop Solution (Item I) to each well. Read absorbance at 450 nm immediately. Mouse IL-1 beta ELISA Kit and Mouse IL18 ELISA Kit come from the company SIGMA(RAB0275-1KT/RAB08100-1KT).

Immunoblotting (Western Blot)

As we described previously (Wang et al 2020b), 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 (20µg/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,

then the PVDF membrane was incubated with primary antibodies overnight at 4 °C including NLRP3, cleaved caspase-1, GSDMD-NT, IL-1β, IL-18, PSD95, Synapsin-1, GAPDH (Table 1). Then, the membranes were incubated with a secondary antibody including anti-Mouse IgG1 HRP-linked; Anti-rabbit IgG, HRP-linked that was conjugated to horseradish peroxidase and 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, protein bands 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), by two persons blinded to treatments and averaged for each mouse.

Statistical Analysis

All data were 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 one-way ANOVA with Tukey's multiple comparison test or two-way ANOVA with Turkey's multiple comparison test (MCT). The N values in each group represent the number of mice. P<0.05 was considered statistically significant.

Results

Intranasal dantrolene nanoparticle pretreatment significantly inhibited LPS-induced depression behaviors in both WT and 5xFAD mice

The tail suspension test and the forced swimming test are commonly used to evaluate depression behaviors in mice and to test drug's efficacy versus side effects (Amada et al 2023, Liu et al 2022, Wang et al 2024). Compared to wild type control, 5XFAD mice have shown to increase depression behavior (Fig. 2A). One time IP injection of LPS (5mg/kg) significantly increased immobile time in FST (Fig. 2A) and in TST (Fig. 2B), indicating the depression behaviors, in both WT and 5XFAD adult mice (Fig. 2). Four weeks pretreatment with intranasal dantrolene nanoparticles (5mg/kg) but not its RFV significantly inhibited the increased immobile time in FST and TST in both WT and 5XFAD mice (Fig. 2), indicating dantrolene capability to ameliorate LPS-induced depression behaviors.

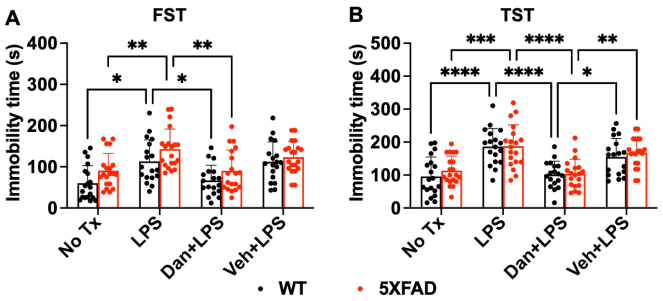

Figure 2. Intranasal dantrolene nanoparticles inhibited lipopolysaccharide (LPS)-induced depression behavior significantly in adult mice. Wild type and 5xFAD adult mice (5-10 months old) were pretreated with intranasal dantrolene nanoparticles (dantrolene, 5mg/kg) or vehicle (Veh) daily, Monday to Friday, for continuous 4 weeks. Mice were then treated with one-time IP injection of LPS (5 mg/kg. Forced swimming test (FST, **A**), Tail Suspension test (TST, **B**) were performed at 24 hours after the one-time IP LPS injection. The more immobility time, the severe depression behavior for both tests. Mice in control group (No Tx) received no treatments. Means±SD, N=18-20 mice. Two-Way ANOVA followed by Tukey post hoc test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Intranasal dantrolene nanoparticle pretreatment significantly inhibited LPS-induced anxiety behaviors in both WT and 5XFAD mice

The elevated plus maze test (EPMT) and open field test (OPT) are commonly used to evaluate anxiety behaviors in mice and test drug efficacy to treat anxiety behaviors(Pentkowski et al 2021, Varkonyi et al 2022, Wang et al 2024). Compared to wild-type control mice, 5XFAD mice did not demonstrate increased anxiety behavior by both EPMT and OPT. However, compared to control mice without treatment, one-time LPS treatment significantly increased the time mice spent in closed arm in EPMT (Fig. 3A) and immobility time in OPT (Fig. 3B), indicating the LPS-induced anxiety behavior in both WT and 5XFAD mice. Four weeks of pretreatment of intranasal dantrolene nanoparticles but not corresponding vehicle control significantly inhibited LPS-induced anxiety behavior determined by both EPMT and OFT in both wild-type and 5XFAD mice (Fig. 3).

In the open field test, the less central zone distance mice traveled and less moving speed, the more severe the anxiety behavior (supplemental figure 1). Compared to WT mice, 5XFAD mice traveled a lesser central zone distance than the wild-type group (supplemental figure 1 A), but the same speed (supplemental figure 1B) in the OPT. One-time LPS treatment did not affect mice travel distance (supplemental figure 1A) but significantly decreased moving speed (supplemental figure 1B) in both WT and 5XFAD mice. Intranasal dantrolene or vehicle treatment did not affect the travel distance or moving speed in OPT in both types of mice (Supplemental Figure 1).

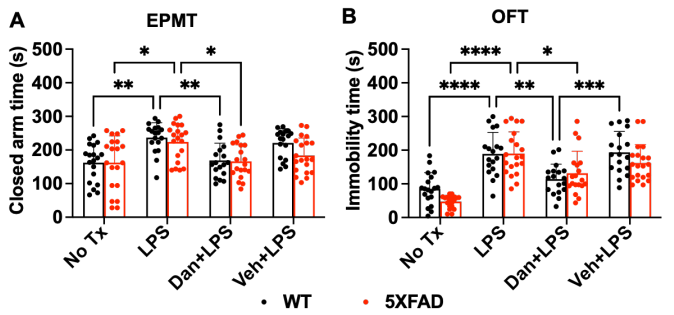

Figure 3. Intranasal dantrolene nanoparticles inhibited lipopolysaccharide (LPS)-induced anxiety behavior in adult mice. Wild type and 5xFAD adult mice (5-10 months old) were pretreated with intranasal dantrolene (dantrolene, 5mg/kg) or vehicle (Veh) control daily, Monday to Friday, for continuous 4 weeks. Mice were then treated with one-time intraparietal (IP) injection of LPS (5 mg/kg). Elevated plus maze test (EPMT, **A**) and open field test (OFT, **B**) was performed at 24 hours after one-time IP LPS injection. Mice in control (No Tx) group received no treatments. The more time in the closed arm, the greater anxiety behavior the mice had (**A**). The more immobility time, the more anxiety behavior for OFT (**B**). Means±SD, N=18-20 mice. Two-Way ANOVA followed by Tukey post hoc test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Intranasal dantrolene nanoparticles significantly inhibited LPS-induced pathological elevation of pyroptosis-related inflammation cytokines in blood.

IP injection of LPS (5mg/kg) for one time significantly increased blood concentrations of IL-1β (Fig. 4A) and IL-18 (Fig. 4B), which could be inhibited significantly by intranasal dantrolene nanoparticles but not RFV control pretreatment for 4 weeks in both WT and 5XFAD mice (Fig 4). In both types of mice brains, intranasal dantrolene nanoparticles trended to inhibit LPS-induced elevation of IL-1 and IL-18 proteins in brains (supplemental figure 2).

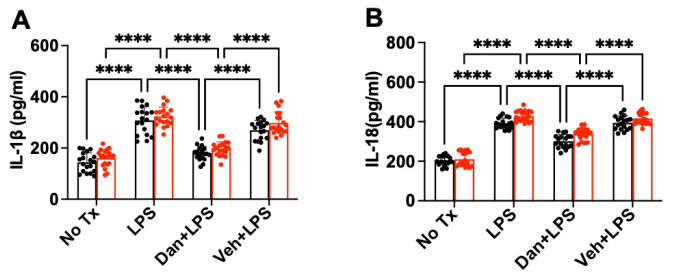

Figure 4. Intranasal Dantrolene significantly Inhibited lipopolysaccharide (LPS)-induced pathological elevation of IL-18 and IL-1β cytokines in the blood. Wild type and 5xFAD adult mice (5-10 months old) were pretreated with intranasal dantrolene nanoparticles (dantrolene, 5mg/kg) or vehicle (Veh) control daily, Monday to Friday, for continuous 4 weeks. Mice were then treated with one-time IP injection of LPS (5 mg/kg). Mice in control (No Tx) group received no treatments. IL1β (**A**) and IL18 (**B**) in blood were measured using ELISA assay kit. Means±SD, N=18-20 mice (**A, B**), Two-Way ANOVA followed by Tukey post hoc test. ****P<0.0001.

Intranasal dantrolene nanoparticles significantly inhibited LPS-induced synapse protein loss in mice brains.

LPS significantly decreased the PSD-95 (Fig. 5, A, C) and synapsin-1 (Fig. 5, B, D) proteins in both WT and 5XFAD mice. Intranasal dantrolene nanoparticles but not vehicle control pretreatment for 4 weeks significantly inhibited LPS-induced decrease of PSD-95 and synapsin-1 synapse proteins in both WT and 5XFAD mice (Fig 5).

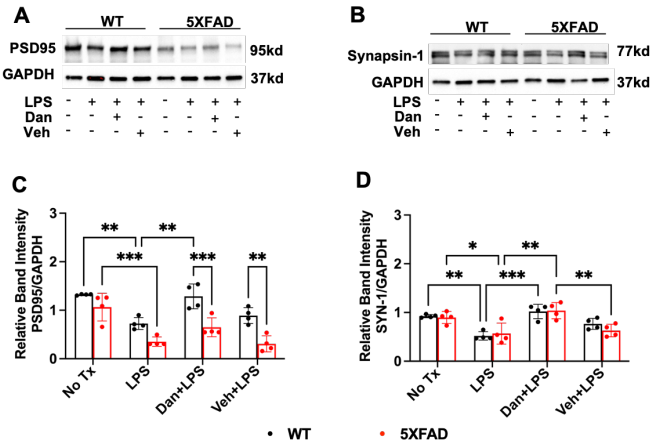

Figure 5. Intranasal dantrolene nanoparticles significantly inhibited lipopolysaccharide-induced synapse protein loss in brains. Wild type and 5xFAD adult mice (5-10 months old) were pretreated with intranasal dantrolene nanoparticles (dantrolene, 5mg/kg) or vehicle (Veh) daily, Monday to Friday, for continuous 4 weeks. Mice were then treated with one-time IP injection of LPS (5 mg/kg). Mice in control (No Tx) group received no treatments. Western blot measured changes of synapse proteins of PSD-95 (**A, C**) and Synapsin-1 (**B, D**) in the whole brains. GAPDH as loading control. Means±SD, N=4 different mice, Two-Way ANOVA followed by Tukey post hoc test. *P<0.05, **P<0.01, ***P<0.001.

Intranasal dantrolene nanoparticles trended to inhibit LPS-induced programmed cell death by pyroptosis in both WT and 5XFAD mice brains.

The proteins levels in mice of pyroptosis pathway activation (NLRP3, caspase-1 and N terminal GSDMD) in 5XFAD mice trended to be increased in comparison to those in WT mice, which were further increased by one-time LPS treatment (Supplemental Fig. 3). 4 weeks of intranasal dantrolene nanoparticles trended to inhibited LPS-induced elevation of pyroptosis pathway activation proteins in both types of mice (Supplemental figure 3).

Discussion

There is an urgent need for new therapeutic interventions to prevent or treat MDD (Belge et al 2023, Brady et al 2023) and anxiety (Szuhany & Simon 2022) psychiatric disorders, especially those drug-resistant and recurrent depression and anxiety (Jha & Mathew 2023, Szuhany & Simon 2022, Tabaac et al 2024). This study has demonstrated that intranasal dantrolene nanoparticles are capable of ameliorating depression and anxiety behaviors in both WT and 5XFAD mice, associated with its ability to inhibit pathological elevation of pyroptosis related cytokines (IL-1β and IL-18) in blood and synapse destruction in brains. Considering that upstream Ca^{2+} dysregulation plays an important role in the pathology of depression and anxiety, evidenced by the effectiveness of ketamine and esketamine in treating depression and anxiety resistant to traditional drug treatments (Paganin et al 2022, Tabaac et al 2024), a new class of drugs, such as dantrolene, could be developed with a mechanism to correct the

disruption of upstream Ca^{2+} dysregulation and associated pathological inflammation in psychiatric disorders.

Increasing studies suggests that inflammation is an important pathology causing MDD and anxiety and could be a target for effective treatments (Chin Fatt et al 2023, Guo et al 2023, Han et al 2024, Su et al 2024). Intracellular Ca^{2+} dysregulation has been proposed as the upstream cause of downstream mitochondria dysfunction, oxidative stress, and inflammation in MDD(Arioz et al 2019, Garrosa-Jimenez et al 2021, Resende et al 2020) and anxiety (Zhang et al 2023b, Zhou et al 2024). Inhibition of upstream Ca^{2+} dysregulation through inhibiting over-activation of RyRs has been shown to suppress inflammation in Gulf War illness characterized by depression and dementia (Phillips et al 2019). Although dantrolene has been shown to inhibit pathological inflammation with increased cytokines in diabetes (Clark et al 2017, Johnson et al 2004), sepsis (Fischer et al 2001, Hotchkiss & Karl 1994) and in COVID-19 infection(Al-Kuraishy et al 2024, Alkazmi et al 2023), its effects to inhibit Ca^{2+} dysregulation and associated inflammation for the treatment of MDD and anxiety has not yet been investigated until now and studied herewith.

Several animal models to establish depression and anxiety behaviors have been suggested based on the proposed neurobiology mechanisms, including altered neurotransmission, HPA axis abnormalities involved in chronic stress, inflammation, reduced neuroplasticity, and network dysfunction (Dean & Keshavan 2017, Sapolsky 2016, Villas Boas et al 2019). Induction of inflammation in animals has been increasingly used to establish depression and anxiety animal models, especially using LPS to induce inflammation, depression, and anxiety behaviors to test drug treatment efficacy (Arioz et al 2019, Li et al 2021b, Li et al 2021c, Mahdirejei et al 2023, Vogelzangs et al 2016, Zhuo et al 2022). The common approach of LPS administration is intraperitoneal injection because of its easiness and feasibility. The dose and duration of IP injection of LPS to establish depression or anxiety behaviors has been varied but appears to be dose dependent. The high dose of LPS at 5 mg/kg, administered once, seems to be dependable in inducing inflammation and establishing depression behavior (Arioz et al 2019). Our study confirmed the efficiency of a one-time administration of IP LPS (5 mg/kg) to induce inflammation and depression and anxiety behavior in both WT and 5XFAD adult mice. Accordingly, the LPS-induced inflammation-mediated depression behaviors are adequate to evaluate the therapeutic effects of dantrolene to treat depression. Our results demonstrated robust inhibition of LPS-induced inflammation and associated depression and anxiety behaviors by intranasal dantrolene nanoparticles pretreatment in adult WT and 5XFAD mice. Consistent with the protective effects of dantrolene against memory loss in various AD animal models (Chakroborty et al 2012, Oules et al 2012, Peng et al 2012, Shi et al 2020), intranasal dantrolene nanoparticles also significantly inhibited depression and anxiety behaviors, and associated inflammation, suggesting that pathological inflammation play a critical role in cognitive dysfunction and psychiatric disorders in AD. Drugs that target inflammation pathology should be developed to treat both memory loss and psychiatric disorders in AD patients.

Although we have hypothesized that LPS will induce severe inflammation, resulting in worsened depression and anxiety behavior in 5XFAD mice, this appears not to be true in our study. Likely, the inflammation in 5XFAD mice is already maximized, meaning that LPS could not induce further inflammation and worsen depression and anxiety behaviors compared to wild-type control mice. Nevertheless, intranasal dantrolene nanoparticles significantly inhibited LPS-induced pathological elevation of pyroptosis related inflammation cytokines (IL-1β and IL-18) in blood, and ameliorated both depression and anxiety behavior in both WT and 5XFAD mice, suggesting that intranasal dantrolene nanoparticles could be developed as an effective drug to treat depression and anxiety in a patient with either only those psychiatric disorders or co-existing with AD.

Intranasal administration of drugs, especially in a nanoparticle formulation, significantly promotes drugs that bypass the blood-brain barrier (BBB) and penetrate the CNS, with reduced peripheral toxicity (Fonseca et al 2021, Hanson & Frey 2008, Maher et al 2023). Our recent research work indicated that intranasal dantrolene nanoparticles achieved higher therapeutic efficacy in the brain compared to oral and subcutaneous forms administration, with minimal or no side effects after chronic use (Shi et al 2020, Vera et al 2024, Wang et al 2020a). Intranasal dantrolene nanoparticles significantly ameliorated memory loss in adult 5XFAD mice as a disease-modifying drug(Shi et al 2020). Increasing studies suggest ryanodine receptor overactivation and associated Ca^{2+} dysregulation is an upstream critical pathology leading to multiple downstream pathologies including mitochondria dysfunction (Garbincius & Elrod 2022), oxidative stresses (Gleitze et al 2023), pathological inflammation (Triantafilou et al 2013), and neuron damage by pyroptosis (Wongpun et al 2023). Eventually, these pathologies result in depression and/or anxiety psychiatric disorders (Guo et al 2023, Li et al 2021a, Wan et al 2022). This study demonstrated that inhibition of RyRs overactivation by dantrolene inhibited pathological inflammation and synapse proteins destruction and ameliorates depression and anxiety behavior robustly. This study further strengthens the indication that upstream RyRs overactivation and Ca^{2+} dysregulation and associated downstream pathological inflammation and synapse destruction play important roles in depression and anxiety psychiatric disorders. Like esketamine, the intranasal dantrolene nanoparticles could become an effective disease-modifying drug treatment for both depression and anxiety psychiatric disorders and need to be investigated in future clinical studies.

Since depression and anxiety psychiatric disorders are chronic diseases, they require long-term drug treatments. Accordingly, proposed drugs must limit side effects or organ toxicity with chronic treatment. The major advantage of using intranasal dantrolene nanoparticles, in comparison to

commonly used oral or intravenous approaches, is that it significantly increases the brain/blood concentration ratio of dantrolene (Shi et al 2020, Wang et al 2020a), especially in aged mice (Vera et al 2024). This promotes its CNS therapeutic effects, while minimizing its side effects or organ toxicity (Shi et al 2020). Our previous study demonstrated no side effects on nose structure and smell function (Jiang et al 2022, Shi et al 2020), liver structure (Shi et al 2020) and function, or muscle function (Peng et al 2012, Shi et al 2020, Vera et al 2023) after up to 10 months of chronic treatment in adult 5XFAD mice (Shi et al 2020), suggesting that intranasal dantrolene nanoparticles are safe to be used chronically in animals. Chronic use of dantrolene in patients' needs to be investigated further.

This study has the following limitations: 1). The N values for immunoblotting of pyroptosis activation proteins and related cytokines proteins are relatively low. Intranasal dantrolene nanoparticles only trended to inhibit LPS-induced elevation of these proteins. 2). We were unable to measure the changes of cytosol versus mitochondria Ca^{2+} levels in the brain tissue due to technological challenges. However, dantrolene has been shown to inhibit LPS or AD gene mutation-induced overactivation of RyRs and associated Ca^{2+} dysregulation in different cell culture models (Hasko et al 1998, Zhang et al 2023a). 3). We did not measure the contents of reactive oxygen species (ROS) concentrations in brains which is usually the upstream pathology to activate NLRP3 inflammasome (Abais et al 2015). 4). We did not measure other biomarkers demonstrating effects of intranasal dantrolene nanoparticles on programed cell death by pyroptosis in brains. 5). We did not study the dose-response of dantrolene to treat depression and anxiety behaviors. Physiological Ca^{2+} release from ER is important for many physiological functions in cells, which means over-inhibition of Ca^{2+} release from the ER may be harmful to cells, as we described previously. The adequate dose of intranasal dantrolene nanoparticles to treat psychiatric disorders must be investigated thoroughly before its clinical use in patients.

Conclusion

This study demonstrated that intranasal dantrolene nanoparticles significantly ameliorated inflammation mediated depression and anxiety behaviors in adult WT and 5XFAD mice. This neuroprotective effect against depression and anxiety behaviors was associated with its robust inhibition of pathological elevation of pyroptosis related inflammation cytokines in blood and the synapse destruction in brains. This study may inspire future studies to repurpose intranasal dantrolene nanoparticles in treating depression and anxiety psychiatric disorders, especially in AD patients.

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Author contributions

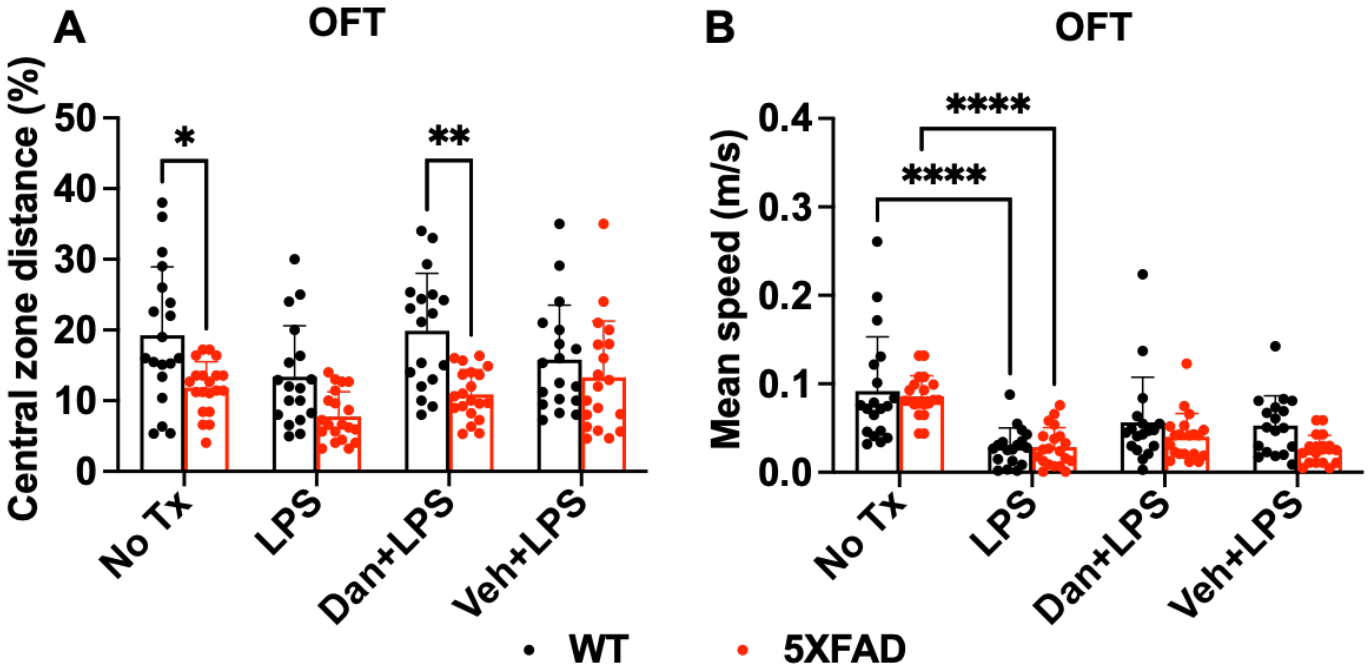
H.W. conceived and designed the study. J.L., Y.L, P.B. J.G, L.S.L, Y.Y., G.L, H.W. conducted experiments, acquired and the data, J.L., Y.L, P.B. J.G, L.S.L, Y.Y., G.L, H.W. analyzed data and contributed to the manuscript preparation. H.W. wrote the manuscript. All the authors reviewed and approved the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

Competing interest statement

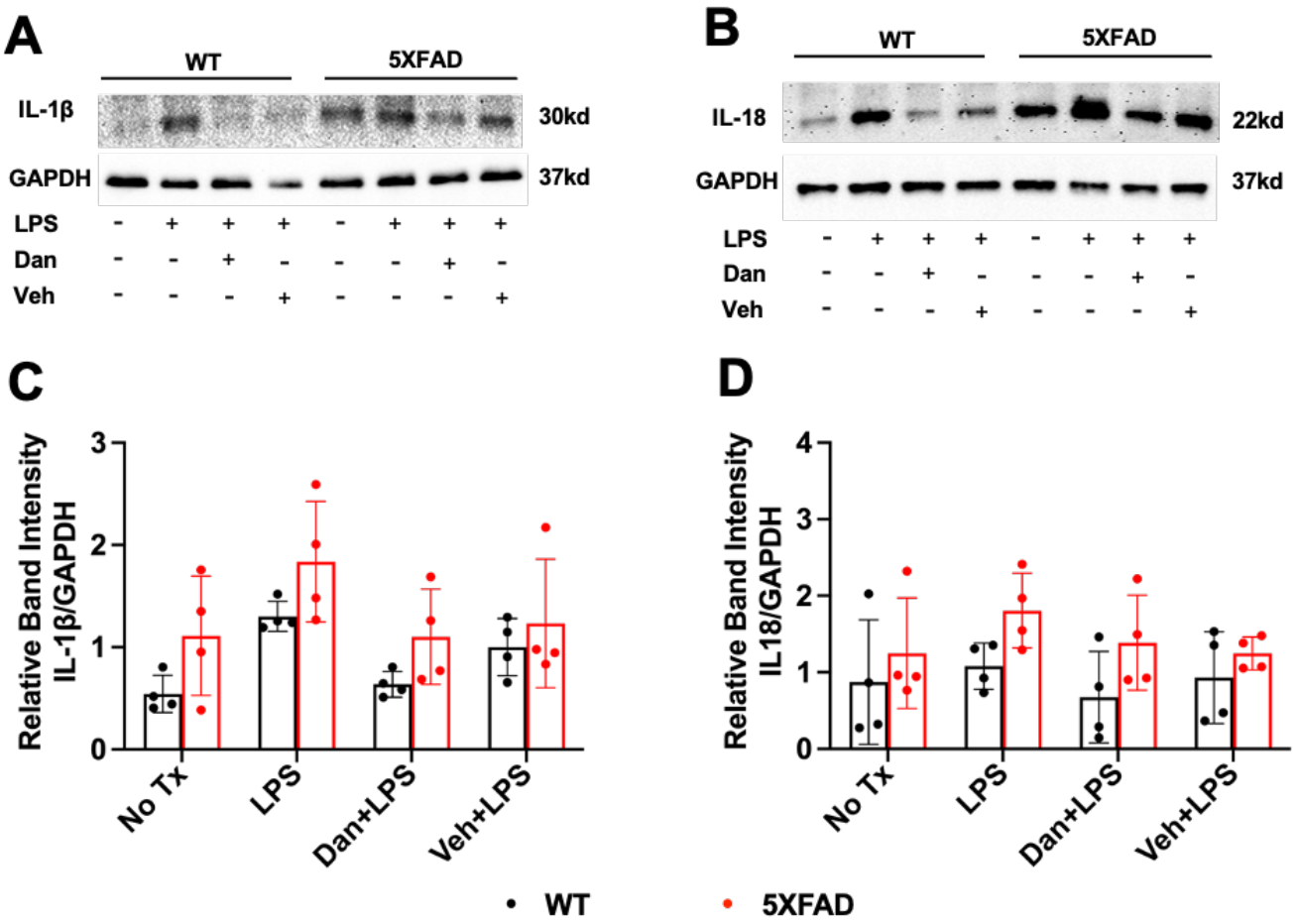
Drs. Huafeng Wei and Ge Liang are listed as inventors of patent applications entitled “Intranasal Administration of Dantrolene for Treatment of Alzheimer’s Disease” in multiple countries by The Trustees of the University of Pennsylvania.

Table 1

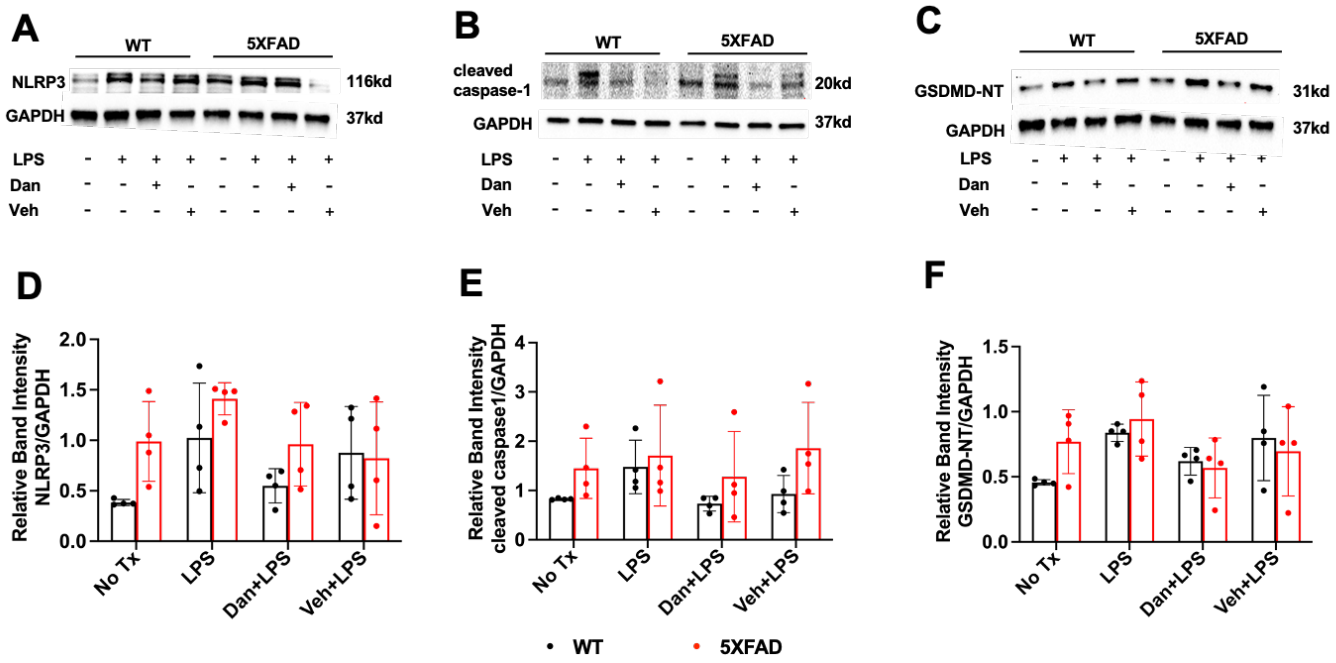
	Cat. and Manufacture	Dilution
Primary antibody		
Anti- NLRP3	68102-1-Ig; Proteintech	1:2000
Anti-Human procaspase-1/ cleaved caspase-1 p20	#2225S, Cell signaling Technology,USA	1:1000
Anti- Cleaved GSDMD	#36425S; Cell signaling Technology, USA	1:1000
Anti-IL-1β	31202S, Cell signaling Technology, USA	1:1000
Anti-IL-18	10663-1-AP; Proteintech	1:3000
Anti-PSD95	#2507; Cell signaling Technology, USA	1:1000
Anti-Synapsin-1	#5297; 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



Supplemental figure 1. Effects of intranasal dantrolene nanoparticles on lipopolysaccharide (LPS)-induced anxiety behavior in adult mice. Wild type (WT) and 5xFAD adult mice (5-10 months old) were pretreated with intranasal dantrolene nanoparticles (dantrolene, 5mg/kg) or vehicle (Veh) control daily, Monday to Friday, for continuous 4 weeks. Mice were then treated with one-time IP injection of LPS (5 mg/kg). Mice in control (No Tx) group received no treatments. Open field test (OFT) was performed at 24 hours after one-time IP LPS injection. The less central zone distance or less mean speed, the more anxiety behavior for OFT. Means±SD, N=18-20 mice. Two-Way ANOVA followed by Tukey post hoc test. *P<0.05, **P<0.01, ****P<0.0001.



Supplemental figure 2. Intranasal Dantrolene trended to inhibit lipopolysaccharide (LPS)-induced pathological elevation of IL-18 and IL-1β cytokines in the brain. Wild type and 5xFAD adult mice (5-10 months old) were pretreated with intranasal dantrolene nanoparticles (dantrolene, 5mg/kg) or vehicle (Veh) control daily, Monday to Friday, for continuous 4 weeks. Mice were then treated with one-time IP injection of LPS (5 mg/kg). Mice in control (No Tx) group received no treatments. IL1β (**A, C**) and IL18 (**B, D**) proteins levels in the brain were measured using western blot. GAPDH as loading control. Means±SD, N=4 different mice (**C, D**), Two-Way ANOVA followed by Tukey post hoc test.



Supplemental Figure 3. Intranasal dantrolene nanoparticles trended to inhibit lipopolysaccharide (LPS)-induced programed cell death by pyroptosis in the brains. Wild type (WT) and 5xFAD adult mice (5-10 months old) were pretreated with intranasal dantrolene nanoparticles (dantrolene, 5mg/kg) or vehicle (Veh) daily, Monday to Friday, for continuous 4 weeks. Mice were then treated with one-time IP injection of LPS (5 mg/kg). Mice in control (No Tx) group received no treatments. Representative western blot (**A, B, C**) and statistical analysis (**D, E, F**) determined the protein levels of critical regulatory proteins of pyroptosis pathway in the brain. GAPDH as loading control. Means±SD, N=4 different mice. Two-Way ANOVA followed by Tukey post hoc test.