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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 in the Ryanodex formulation (DNRF) pretreatment to inhibit lipopolysaccharide (LPS)-induced depressive and anxiety behavior in mice.
Both wild-type (WT) B6SJLF1/J and 5XFAD adult mice were pretreated with intranasal DNRF (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 and anxiety were performed 24 hours after a one-time LPS injection. Biomarkers for inflammation (IL-1β and IL-18) in blood were measured using enzyme-linked immunosorbent assay (ELISA). In both types of mice, intranasal DNRF significantly inhibited LPS-induced pathological elevation of IL-1β and IL-18 in the blood. Intranasal DNRF abolished LPS-induced depression and anxiety behaviors behavior in both WT and 5XFAD mice, without obvious side effects, which was associated with its significant inhibition of pathological elevation of pyroptosis related cytokines in blood.

Introduction

Three hundred million people worldwide suffer from major depressive disorder (MDD)[1]. MDD is a chronic and recurrent disease affecting about 20% of the population and the leading cause of suicide[2]. MDD imposes a tremendous psychological burden, as well as significant social repercussions and contributions to other disabilities[3]. The expected direct and indirect costs of MDD are up to $6 trillion in the USA alone[4]. 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[5]. Anxiety disorders were approximately twice as prevalent among women, with overall age-specific rates remaining relatively stable or increasing across the lifespan[6]. In 2021, an estimated 5.8 million (9.4%) children and adolescents were impacted by anxiety[7]. Depression and anxiety often co-exist and are the most common psychiatric diseases, which are chronic diseases with unclear mechanisms of pathology[8].

Classical treatment of depression or anxiety psychiatric disorders includes atypical antipsychotics that modulate dopamine and serotonin neurotransmission[9,10]. Pharmacotherapies for anxiety disorders also include drugs that interact with GABA neurotransmission systems[11]. Unfortunately, antidepressants have significant limitations, including slow onset of action, high rates of nonresponse, and they may worsen anxiety acutely[12]. 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[13]. 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%)[3,8]. Treatment-resistant anxiety with remission rates may be as low as 25–35%, and relapse rates post remission may be 30% after 10 years[14]. 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[15]. 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)[16,17]. Ketamine is also effective in treating anxiety disorders, even those resistant to traditional treatments[7,18]. 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. Ketamine has become a safe and broadly effective drug to treat depression or anxiety disorders. Ketamine is an antagonist of NMDAR, a primary glutamate receptor on the plasma membrane that causes Ca^{2+} influx into the cytosol from

extracellular space and has proven to be effective in treating both depression and anxiety. This indicates that glutamate-mediated excitotoxicity and associated disruption of intracellular Ca^{2+} homeostasis play an important 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[19-21], as well as anxiety disorder[22,23]. 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[21,24,25]. RyRs have also been shown to increase anxious behavior[24]. Upstream Ca^{2+} dysregulation results in mitochondria dysfunction[26], mitochondrial and cellular oxidative stress[8,27], activation of inflammasomes, and cell or neuron death by pyroptosis[28-31]. This results in the release of inflammatory cytokines (IL-1β and IL-18) and pathological inflammation-mediated cell death by pyroptosis[30,32]. Pathological cytokines, especially pyroptosis-related IL-18 play important roles in psychiatric disorders[33,34]. Gut dysbiosis and associated inflammation also contribute to pathology in depression and anxiety behaviors[35,36]. Thus, a drug that inhibits upstream Ca^{2+} dysregulation and then downstream pathological inflammation 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%[37]. 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[38,39]. The Type 2 RyRs (RyRs-2) pathologically increased in the brains of AD patients[40]. The number and activity of RyRs pathologically increase in different cell and animal models of AD[41-43]. Similar to the molecular mechanism of depression and anxiety, RyRs over-activation causes upstream Ca^{2+} dysregulation and downstream mitochondrial dysfunction[44], oxidative stresses[45], initiation of pyroptosis pathway, and pathological inflammation[46], eventually leading to synapse and cell or neuron damage[47,48]. This leads to a high prevalence of depression and anxiety psychiatric disorders and cognitive dysfunction in AD[21,49-51]. 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[49,52-54].

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[55]. Dantrolene, with its ability to inhibit the common upstream critical Ca^{2+} dysregulation, is neuroprotective against many neurodegenerative diseases, including cerebral ischemia[56,57], Huntington's disease[58], spinocerebellar ataxia[59], amyotrophic lateral sclerosis[60], and seizures[61,62]. We and others have demonstrated that dantrolene abolishes or ameliorates cognitive dysfunction in multiple AD animal models[49,53,54,63]. Our recent studies pioneered a novel approach of administrating intranasal dantrolene nanoparticles in the Ryanodex formulation (DNRF, 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[49,64,65]. Intranasal DNRF abolished memory loss as a disease-modifying drug66. Importantly, chronic intranasal DNRF administration had no significant side effects or organ toxicity in either amyloid[49] or tau[65] AD mice.

In this study, we investigated the therapeutic effectiveness and the potential mechanisms of intranasal DNRF on depression and anxiety behavior associated with pathological inflammation in both wild-type and 5XFAD mice. We induce inflammation by intraperitoneal injection of LPS. Our results demonstrated that intranasal DNRF for 12 consecutive weeks robustly abolished LPS-induced depression and anxiety behaviors, as a disease-modifying drug, in both WT and 5XFAD mice. These beneficial effects of intranasal DNRF treatment were associated with its inhibition of LPS-induced pathological elevation of pyroptosis related inflammation cytokine of IL-1β and IL-18 in blood. This study provides proof of concept that intranasal DNRF are effective treatments for depression and anxiety psychiatric disorders, potentially by inhibiting pathological inflammation. This study will inspire future clinical studies investigating the therapeutic effectiveness versus side effects of intranasal DNRF 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 DNRF (IN Dan, 5mg/kg) or vehicle (Veh), once a day, 5X/week (Monday through Friday), at either 5 or 10 months old. Control groups received no

pretreatment. Fresh DNRF were made every time before administration. Intranasal DNRF stock solution was made at 5 mg/ml. The Ryanodex formulation vehicle was made fresh and contained all inactive ingredients in Ryanodex[49,67]. At the end of the 4-week pretreatment period, mice were then treated with an 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 the pyroptosis-related inflammatory cytokines in blood.

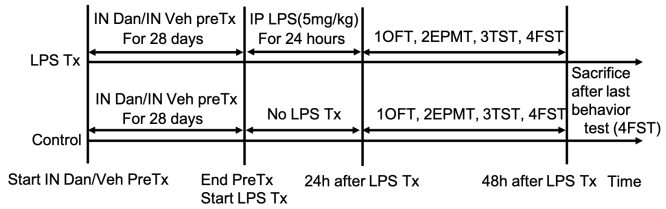

Figure 1. Experimental Design. Adult Wild type (WT) and 5xFAD mice (5-10 months old) were pretreated with intranasal dantrolene nanoparticles in Ryanodex formulation (DNRF, 5mg/kg) or vehicle (Veh) for 4 weeks. Control group received no treatments. Mice were then treated with intraperitoneal (IP) injection of lipopolysaccharide (LPS, 5mg/kg) for one time for 24 hours. Depression and anxiety behavior tests were performed at 24 hours after one-time IP LPS injection. 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[49,64]. 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 DNRF or RFV. Accordingly, a mouse weighing 20 g would be given 20µL of solution. 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[68]. Each mouse was placed individually into the OFT apparatus (44 × 44 × 44 cm3), 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[69]. 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 forced swimming test (FST), as described previously with some modification[70]. 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[71]. 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[49], 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 transcardial 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[72,73]. 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).

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 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[23,74,75]. 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 DNRF (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.

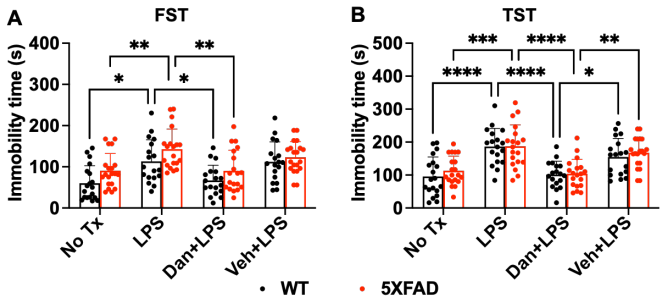

Figure 2. Intranasal dantrolene nanoparticles inhibited lipopolysaccharide (LPS)-induced depression behavior significantly in adult mice. Wild type and 5xFAD adult mice (5-10 months old) were pretreated with intranasal dantrolene nanoparticles in Ryanodex formulation (DNRF, 5mg/kg) or vehicle (Veh) daily, 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[23,76,77]. 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 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).

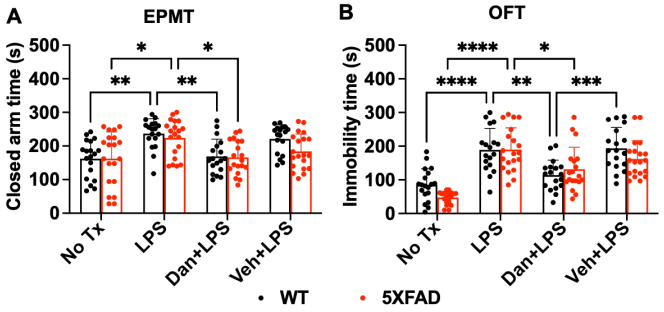

Figure 3. Intranasal dantrolene nanoparticles inhibited lipopolysaccharide (LPS)-induced anxiety behavior in adult mice. Wild type and 5xFAD adult mice (5-10 months old) were pretreated with intranasal dantrolene in Ryanodex formulation (DNRF, 5mg/kg) or vehicle (Veh) control daily, Monday to Friday, 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 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 DNRF but not RFV control pretreatment for 4 weeks in both WT and 5xFAD mice (Fig 4).

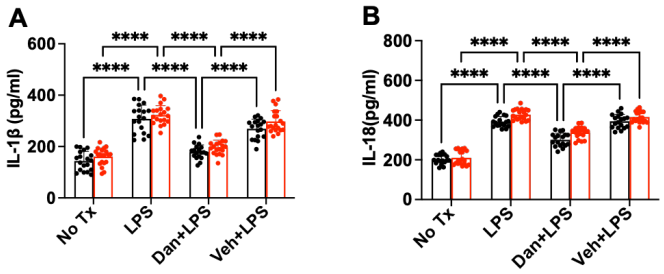

Figure 4. Intranasal Dantrolene Inhibited lipopolysaccharide (LPS)-induced pathological elevation of IL-18 and IL-1β cytokines in the blood. Wild type and 5xFAD adult mice (5-10 months old) were pretreated with intranasal dantrolene nanoparticles in Ryanodex formulation (DNRF, 5mg/kg) or vehicle (Veh) 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<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Discussion

There is an urgent need for new therapeutic interventions to prevent or treat MDD[3,78] and anxiety[5] psychiatric disorders, especially those drug-resistant and recurrent depression and anxiety[5,14,79]. This study has demonstrated that intranasal DNRF 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. 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[12,14], 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[80-83]. Intracellular Ca^{2+} dysregulation has been proposed as the upstream cause of downstream mitochondria dysfunction, oxidative stress, and inflammation in MDD[21,84,85] and anxiety[34,86]. 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[24]. Although dantrolene has been shown to inhibit pathological inflammation with increased cytokines in diabetes[87,88], sepsis[89,90] and in COVID-19 infection[91,92], 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[93-95]. 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[33,84,96-99]. 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 behavior84. 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 DNRF pretreatment in adult WT and 5XFAD mice. Consistent with the protective effects of dantrolene against memory loss in various AD animal models[49,53,54,63], intranasal DNRF also significantly inhibited depression, 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 DNRF 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 DNRF could be developed as an effective drug to treat depression and/or 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[100-102]. Our recent research work indicated that intranasal DNRF achieved higher therapeutic efficacy in the brain compared to oral and subcutaneous forms administration, with minimal or no side effects after chronic use [49,64,65]. Intranasal DNRF significantly ameliorated memory loss in adult 5xFAD mice as a disease-modifying drug[49]. Increasing studies suggest ryanodine receptor overactivation and associated Ca^{2+} dysregulation is an upstream critical pathology leading to multiple downstream pathologies including mitochondria dysfunction[103], oxidative stresses[104], pathological inflammation[105], and neuron damage by pyroptosis106. Eventually, these pathologies result in depression and/or anxiety psychiatric disorders[30,83,107]. This study demonstrated that inhibition of RyRs overactivation by dantrolene inhibits inflammation and ameliorates depression and anxiety behavior robustly. This study further strengthens the indication that upstream RyRs overactivation and Ca^{2+} dysregulation and associated downstream inflammation play important roles in depression and anxiety psychiatric disorders. Like esketamine, the intranasal DNRF 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 DNRF, in comparison to commonly used oral or intravenous approaches, is that it significantly increases the brain-blood concentration ratio of dantrolene[49,64], especially in aged mice[65]. This promotes its CNS therapeutic effects, while minimizing its side effects or organ toxicity[49]. Our previous study demonstrated no side effects on nose structure and smell function[49,108], liver structure[49] and function, or muscle function[49,53,67] after up to 10 months of chronic treatment in adult 5XFAD mice[49], 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). 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[109,110.] 2). We did not measure the contents of reactive oxygen species (ROS) concentrations in brains which is usually the upstream pathology to activate NLRP3 inflammasome[111]. 3). We did not measure other biomarkers demonstrating effects of intranasal DNRF on programed cell death by pyroptosis in brains. 4). We did not study the dose-response of dantrolene to treat depression and anxiety behaviors. Physiological calcium release from ER is important for many physiological functions in cells, which means over-inhibition of calcium release from the ER may be harmful to cells, as we described previously. The adequate dose of intranasal DNRF to treat psychiatric disorders must be investigated thoroughly before its clinical use in patients.

Conclusion

This study demonstrated that intranasal DNRF 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. This study may inspire future studies to repurpose intranasal dantrolene DNRF in treating depression and anxiety psychiatric disorders, especially in AD patients.

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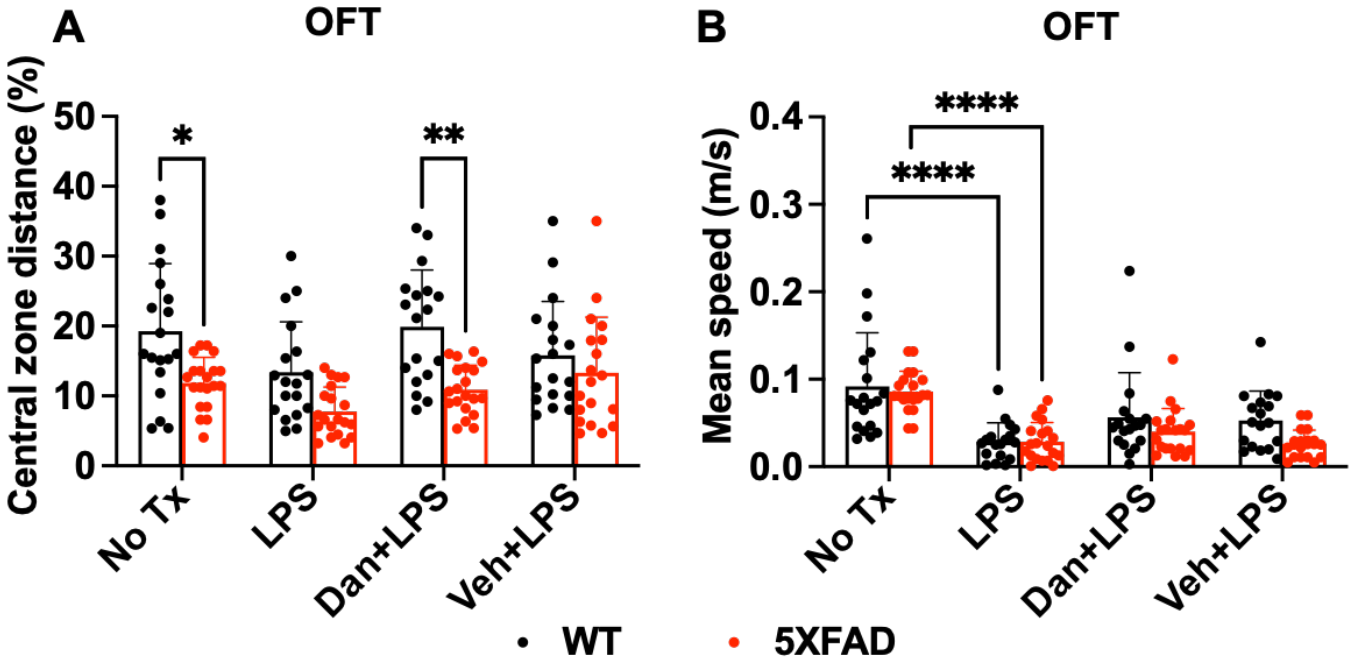
Author contributions

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. write 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.



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 in Ryanodex formulation (DNRF, 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<0.001, ****P<0.0001.