Activation of Alpha7 Nicotinic Acetylcholine Receptors Reduce Chronic Pain-induced Anxiety and Depression-Like Behaviors via WNT/ β -Catenin Pathway

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Research Article

Keywords: alpha7 nicotinic acetylcholine receptor, pain, depression, WNT/ β -catenin, inflammation

Posted Date: September 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-913681/v1>

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Abstract

Chronic pain frequently leads to anxiety, depression and microglia-related inflammation is the common mechanism for chronic pain and psychiatric disorders. Activation of alpha 7 nicotinic acetylcholine ($\alpha 7$ nACh) receptor suppresses microglia-related inflammation. Thus, we evaluated the activation of $\alpha 7$ nACh receptors on chronic pain-induced anxiety- and depression-like behaviors and explored the potential mechanisms. Open field, sucrose preference, and pain behavior tests (paw withdrawal threshold, and cold response to acetone) were evaluated in male Wistar rats that received spared nerve injury (SNI) and complete Freund's adjuvant (CFA) into the unilateral ankle articular cavity. The effect of intracerebroventricular (ICV) injection of $\alpha 7$ nACh receptor agonist PHA and $\alpha 7$ nACh receptor antagonist on anxiety and depression-like behaviors and cytokines expression were examined. Cytokines in medial prefrontal cortex (mPFC), basolateral amygdala (BA), and ventral hippocampus (VH) were measured using enzyme-linked immune absorbent assay. SNI surgery and CFA injection induced anxiety- and depression-like behaviors, and ICV injection of PHA reduced that anxiety- and depression-like behaviors. SNI surgery and CFA injection skewed the mPFC, BA, and VH to a pro-inflammatory response and ICV injection of PHA shift brain to an anti-inflammatory response in those sites. The anti-depressive effect and anti-inflammatory effect of PHA were reversed by WNT/ β -catenin inhibitor. Activation of alpha7 nicotinic acetylcholine receptors reduces chronic pain-induced anxiety- and depression-like behaviors, the mechanisms may be attributed to the activation of WNT/ β -catenin pathway and suppression of brain inflammation.

Background

Chronic pain is detrimental to human beings and frequently causes psychiatric disorders such as anxiety and depression [1]. Although effective treatments are available, about one third of patients with depression fail to respond to conventional antidepressant therapies [2], which leads to searching new therapeutic approaches. Recently, anxiety and depression has been regarded as an inflammatory disease, because suppression of inflammation improved the pathological behaviors. The proinflammatory process affects neuronal activity and results in anxiety- and depression-like behaviors. The inflammation mainly comes from microglia, as minocycline, a microglial inhibitor suppresses brain inflammation and alleviates the anxiety- and depression-like behaviors[3]. As a result, targeting microglia-related inflammation is an effective approach to managing psychiatric illness.

Alpha 7 nicotinic acetylcholine ($\alpha 7$ nACh) receptor, a subtype of nicotinic acetylcholine receptors, is involved in a variety of biological functions such as synaptic plasticity and neuronal survival; and is considered a prominent therapeutic target in neuropathological diseases[4]. Microglia, an immune cell in the central nervous system, express $\alpha 7$ nACh receptors, and by targeting $\alpha 7$ nACh receptor could change the microglia status and resolve microglia-related neuroinflammation[5]. In recent years, "cholinergic anti-inflammatory pathway" has been demonstrated to regulate central nervous inflammation via the $\alpha 7$ nACh receptors of microglia[6]. Activation of microglia $\alpha 7$ nACh receptor also protect the brain from various

insults by decreasing the proinflammatory effect of microglia[7]. Thus, activation of $\alpha 7$ nACh receptor may have anti-psychiatric effects.

PHA-543613 (PHA) is an $\alpha 7$ nACh receptor agonist, and we have previously shown that PHA prevent the development and maintenance of neuropathic pain by suppression of microglia-related inflammation[8]. Since chronic pain frequently results in depression and has a common biological change of inflammation in the central nervous [9], whether targeting $\alpha 7$ nACh receptors is effective for treating chronic pain-induced anxiety and depression remain to be clarified. We designed this experiment to test the effect of ICV (intracerebroventricular) injection of $\alpha 7$ nACh receptor agonist PHA on the anxiety- and depression-like behaviors induced by peripheral nerve transection surgery and articular cavity proinflammatory injection in rats and explore the potential signal pathways involved in the anxiolytic and anti-depressive effect of $\alpha 7$ nACh receptor activation.

Materials And Methods

Animals and drugs preparation

Our experiments were performed in accordance with the Animal Care and use Committee of Shanghai Jiao Tong University and animal care guidelines of the National Institutes of Health. Male Wistar rats were obtained from the Shanghai Experimental Animal Institute and were kept in a temperature and humidity-controlled environment on a 12-h light/dark cycle (light on between 8:00 AM. to 18:00 P.M.) with free access to food and water. The $\alpha 7$ nAChR agonist, PHA, and its antagonist, methyllycaconitine citrate (MLA) were purchased from Tocris Biosciences (Minneapolis, USA). PHA was dissolved in 5% dimethylsulfoxide (DMSO) at a dosage of 4 μ g in a volume of 4 μ L. MLA was dissolved in normal saline and prepared at a dosage of 4 μ g in a volume of 4 μ L. For the control group, an equal volume of 5% DMSO was used. Minocycline hydrochloride (purchased from Sigma-Aldrich Corp, St Louis, USA) was dissolved in normal saline with a concentration of 40 μ g/ μ L. SB431542 (a TGF β inhibitor; Sigma-Aldrich, St. Louis, USA) were dissolved in 4% DMSO (4 μ L, 5 nmol) and used for ICV injection[10]. AZD8055(a mTOR inhibitor; Sigma-Aldrich, St. Louis, USA) was prepared 10 mg/mL with 4% DMSO as vehicle and used 4 μ L for ICV injection[11]. IWR-1-endo[12] (IWR, a WNT/ β -catenin inhibitor), Numb[13] (a notch inhibitor) and AZD1480[14] (a JAK inhibitor) were bought from Sigma-Aldrich, St. Louis, USA and were dissolved in 4% DMSO with a concentration of 5 μ M and a volume of 4 μ L was used for ICV injection.

Animal models establishments

Spared nerve injury (SNI) surgery was performed according to our previous study[15] . Briefly, rats received general anesthesia with isoflurane 5% induction and 2.5% maintenance using an inhalational anesthesia system (Penlon Sigma Delta, Louisville, KY, USA). The rats were fixed on an operation table and the sciatic nerve was exposed at the lateral thigh with an animal surgery microscope (Leica M205FA, Germany); the tibia and common peroneal branches were dissected and tightly ligated with 6-0 absorbable suture and transected, leaving the sural nerve intact. The sciatic nerve of the sham group was only exposed, without ligation and transection. Then the skin was sutured with 5-0 sutures. The rat was

returned to their cages for recovery as the surgery was over. Behavior tests, drug injections, and ELISA assays were done in a blinded manner to avoid bias.

Monoarthritic model was induced by an injection of complete Freund's adjuvant (CFA) into the unilateral ankle articular cavity[16]. The experimental rats were anesthetized with isoflurane prior to inducing arthritis. The skin around the site of injection was sterilized with 75% alcohol and the right leg of the rat was held, and the fossa of the lateral malleolus of the fibula was located. A 28-gauge needle was used vertically to penetrate the skin, and turned distally to insert into the articular cavity from the gap between the tibiofibular and tarsus bone until a distinct loss of resistance. Then, a volume of 50 μ l of CFA was injected. For the sham group, the same amount of normal saline was injected into the right ankle articular cavity.

For the food intake evaluation, 50g food was provided for each rat in one cage and determined at the indicated day for 24hs. The amount of food remaining after 24hs was individually weighed and subtracted from the original quantity provided. Body weight was recorded before surgery or stimulation, before and after medical intervention. All rats were free access to water.

Pain behavior tests

For mechanical allodynia, the values were measured according to our previous study[17]. Briefly, the rats were placed in a Plexiglas box on a mesh floor 50cm above the table. After 15 min of acclimation, the mechanical withdrawal threshold was measured with an electronic apparatus (IITC Life Science, Woodland Hills, CA, USA). An increasing force was used to stimulate the lateral side of the ipsilateral hind paw. As an immediate paw withdrawal was observed, the value was recorded; the mean value was averaged from 3 repeated measures with an interval of 5 min.

Response rate to cold acetone was done according to our previous study[17]. The positive response was recorded as withdrawal or licking or shaking of the hind paw to a drop of acetone applied to the ventral surface of the paw. The acetone response was repeated for 5 times and the response rate was calculated.

Anxiety- and depression-like behavior tests

The open field test was done according to our previous study[17]. The rat was placed in a square arena (50 × 50 × 50 cm), which was divided into two areas: a peripheral area and a central (17 × 17 cm) square area. The center distance and total distance were recorded in the experiment, and center/total distance duration were calculated as measures of anxiety- and depression-like behavior, and total running distance was recorded as a measure of general motor behavior. The running track was recorded for 5 min by a camera 2 m above the box. The field was cleaned with 75% ethanol after each test.

The sucrose preference experiment followed a previous study with minor modifications[17]. Briefly, all of the rats were habituated with 1% sucrose for 48 h before the experiments. On the day of the experiment, each rat received an 8-h fluid and food deprivation and then was exposed to two bottles for 1 h, one filled

with 1% sucrose and the other tap water. The sucrose intake percentage was calculated with a formula: $(\text{sucrose}/(\text{sucrose} + \text{water})) \times 100\%$.

Intracerebroventricular catheter placement and injection

Intracerebroventricular (ICV) catheter placement and injection followed the procedures used in our previous study[17]. Briefly, rats were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg). Then, the rat was fixed on a stereotaxic apparatus. The surgery was done and a catheter was placed with stereotaxic coordinates: 0.8 mm posterior, 1.5 mm left lateral, and 4.5 mm ventral from the bregma. The guide cannula was fixed with dental cement and the rat was returned to individual cage for recovery. The ICV cannulation was verified by injection of methylene blue through the cannula after experiment. For each daily injection, rats were anesthetized with isoflurane as above; rats received bilateral microinjection of PHA and inhibitors or minocycline (160 µg) or saline (control) into the cerebral ventricle. A total volume of 4.0 µl was infused into each side over 10 min, and the injection syringe was left in place for an additional 5 min to allow for diffusion. The microinjections were performed daily for six days, followed by behavior tests.

Inflammatory cytokines measurements with enzyme-linked immune-absorbent assay

After the last behavior tests, rats were deeply anesthetized with pentobarbital sodium (100 mg/kg) followed by decapitation according to our previous study[17]. Tissues were collected for ELISA followed our previously study. Briefly, the brains were placed in a chilled matrix and micro-dissected on a chilled glass plate. The medial prefrontal cortex (mPFC) was obtained from a 2-mm-thick slice ranging from approximately 5 to 3 mm anterior of Bregma. The ventral hippocampus (VH) was isolated from a 4-mm-thick section ranging from approximately 3.2 to 7.2 mm posterior of Bregma and was separated from cortex and underlying brain structures. The basolateral amygdala (BA) was dissected out ranging from approximately 1.2 to 3.2 mm posterior of Bregma just lateral to the optic tracks. After the samples were collected, these tissues were homogenized with normal saline and centrifuged at 4000 rpm, 4 °C for 10 min. The supernatants were used for IL-10, IL-1β, TNF-α and IL-18 assay using ELISA kits (Nanjingjiancheng Biocompany, Nanjing, China) and the procedures followed the manufacturer's instructions.

Statistical analysis

The data are shown as mean ± SD and were analyzed with one-way or two-way analysis of variance (ANOVA) with repeated measures. Bonferroni post hoc analysis was conducted if a statistically significant difference was found. Nonparametric Wilcoxon or unpaired “*t*” test was performed for comparison of non-repeated measures. *P* < 0.05 was considered statistically significant. The statistical analysis was performed using GraphPad Prism software (GraphPad Prism 5.0, GraphPad Software Inc., San Diego, CA, USA).

Results

SNI surgery induced anxiety- and depression-like behaviors after surgery

The anxiety- and depression-like behaviors were measured from POD14 onward, and the results showed that SNI surgery decreased center distance (group $F_{1,14} = 25.7$, $P = 0.001$; time $F_{1,14} = 0.367$, $P = 0.329$; interaction $F_{1,14} = 0.001$, $P = 0.971$; Fig 1A) and center/total distance (group $F_{1,14} = 23.3$, $P = 0.001$; time $F_{1,14} = 0.492$, $P = 0.329$; interaction $F_{1,14} = 0.037$, $P = 0.851$; Fig 1C) compared to sham group in the open field test from POD14 to POD 21 after surgery. SNI surgery did not affect total running distance in the open field test after surgery (group $F_{1,14} = 0.274$, $P = 0.609$; time $F_{1,14} = 0.430$, $P = 0.523$; interaction $F_{1,14} = 0.059$, $P = 0.811$; Fig 1B). In the sucrose preference test, SNI decreased sucrose intake percentage from POD14 to POD21 (group $F_{1,14} = 64.9$, $P = 0.001$; time $F_{1,14} = 0.076$, $P = 0.787$; interaction $F_{1,14} = 0.116$, $P = 0.739$; Fig 1D). In addition, SNI decreased food intake (group $F_{1,14} = 35.6$, $P = 0.001$; time $F_{3,14} = 1.86$, $P = 0.152$; interaction $F_{1,14} = 11.5$, $P = 0.001$; Fig 1E) and weight gain (group $F_{1,14} = 58.3$, $P = 0.001$; time $F_{1,14} = 76.8$, $P = 0.329$; interaction $F_{1,14} = 134$, $P = 0.001$; and Fig 1F respectively) compared to sham after surgery from POD7 to POD21. The paw withdrawal threshold and response to acetone were recorded before and after SNI surgery. The results showed that SNI surgery decreased paw withdrawal threshold (group $F_{1,14} = 335$, $P = 0.001$; time $F_{1,14} = 75.6$, $P = 0.001$; interaction $F_{1,14} = 61.7$, $P = 0.001$; and Fig 1G) and increased response to acetone (group $F_{1,14} = 162$, $P = 0.001$; time $F_{1,14} = 16.5$, $P = 0.001$; interaction $F_{1,14} = 18.8$, $P = 0.001$; and Fig 1H) after surgery from POD7 to POD21.

ICV injection of $\alpha 7$ nACh receptor agonist PHA and microglia inhibitor MIN decreased anxiety- and depression-like behaviors after SNI surgery

The behavior results showed that SNI surgery decreased center distance and center/total distance from POD10 to POD18, and ICV PHA or MIN improved that decrement of center distance (group $F_{4,45} = 29.7$, $P = 0.001$; time $F_{1,45} = 12.0$, $P = 0.001$; interaction $F_{4,45} = 2.95$, $P = 0.030$; and Fig 2A) and center/total distance (group $F_{4,45} = 23.7$, $P = 0.001$; time $F_{1,45} = 14.4$, $P = 0.001$; interaction $F_{4,45} = 4.38$, $P = 0.005$; Fig 2C) on POD18. SNI decreased sucrose intake percentage from POD10 to POD18 and ICV PHA or MIN decreased that effect on POD18 (group $F_{4,45} = 35.6$, $P = 0.001$; time $F_{1,45} = 11.7$, $P = 0.001$; interaction $F_{4,45} = 5.60$, $P = 0.001$; Fig 2D). The total running distance was not different among four groups in the open field test (group $F_{4,45} = 0.642$, $P = 0.635$; time $F_{1,45} = 0.027$, $P = 0.870$; interaction $F_{4,45} = 0.058$, $P = 0.994$; Fig 2B). SNI surgery decreased food intake and weight gain from POD10 to POD18 compared to sham group, and ICV PHA or MIN decreased that effect on food intake (group $F_{4,45} = 7.82$, $P = 0.001$; time $F_{2,45} = 9.94$, $P = 0.001$; interaction $F_{8,45} = 2.09$, $P = 0.045$; Fig 2E) and weight gain (group $F_{4,45} = 70.4$, $P = 0.001$; time $F_{2,45} = 12.9$, $P = 0.001$; interaction $F_{8,45} = 23.2$, $P = 0.001$; Fig 2F) on POD18. Although SNI decreased paw withdrawal threshold and increased response to acetone, ICV PHA or MIN did not affect paw withdrawal threshold (group $F_{4,45} = 407$, $P = 0.001$; time $F_{3,45} = 206$, $P = 0.001$; interaction $F_{12,45} = 25.8$, $P = 0.001$; Fig 2G) and response to acetone (group $F_{4,45} = 125$, $P = 0.001$; time $F_{3,45} = 102$, $P = 0.001$; interaction $F_{12,45} = 16.4$, $P = 0.001$; Fig 2H). The time course for surgery and experiment was shown in Fig 2I.

PHA decreased anxiety- and depression-like behaviors after SNI surgery by the activation of $\alpha 7$ nACh receptors and suppression of brain inflammation

The results showed that ICV co-administration of $\alpha 7$ nACh receptor antagonist MLA decreased anti-anxiety- and anti-depressive effect of PHA in center distance ($F = 19.8$, $P = 0.001$; Fig 3A) and center/total distance ($F = 14$, $P = 0.001$; Fig 3C) in the open field test, and the total distance running ($F = 0.427$, $P = 0.789$; Fig 3B) was not different between groups after SNI surgery on POD18. PHA increased sucrose intake percentage and food intake and weight gain after SNI, while co-administration MLA decreased that effect of PHA on sucrose intake percentage ($F = 15.5$, $P = 0.001$; Fig 3D), food intake ($F = 8.63$, $P = 0.001$; Fig 3E) and weight gain ($F = 35.5$, $P = 0.001$; Fig 3F). MLA did not affect the effect of PHA on paw withdrawal threshold (group $F_{3,36} = 0.548$, $P = 0.653$; time $F_{2,36} = 680$, $P = 0.001$; interaction $F_{8,45} = 1.05$, $P = 0.401$; Fig 3G) and response to acetone (group $F_{3,36} = 1.22$, $P = 0.316$; time $F_{2,36} = 196$, $P = 0.001$; interaction $F_{8,45} = 0.625$, $P = 0.710$; Fig 3 H).

In addition, ICV PHA increased IL-10 in the mPFC ($F = 4.79$, $P = 0.003$; Fig 4A), BA ($F = 13.4$, $P = 0.001$; Fig 4E) and VH ($F = 11.9$, $P = 0.001$; Fig 4I) compared to the Vehicle group, and MLA decreased those effect. In contrast, SNI increased IL-1 β in the mPFC, BA and VH, compared to Naive group, and ICV PHA decreased IL-1 β in the mPFC ($F = 26.7$, $P = 0.001$; Fig 4B), BA ($F = 39.2$, $P = 0.001$; Fig 4F) and VH ($F = 27.4$, $P = 0.001$; Fig 4J) compared to the Vehicle group, whereas the effect was compromised by MLA. Similarly, SNI increased TNF- α in the mPFC, BA and VH compared to Naive group, and ICV PHA decreased their content in the mPFC ($F = 23.0$, $P = 0.001$; Fig 4C), BA ($F = 23.4$, $P = 0.001$; Fig 4G) and VH ($F = 22.1$, $P = 0.001$; Fig 4K) compared to the Vehicle group, while MLA decreased the effects of PHA. Further, SNI increased IL-18 in the mPFC, BA and VH compared to Naive group, and ICV PHA decreased their content in the mPFC ($F = 28.9$, $P = 0.001$; Fig 4 D), BA ($F = 15.7$, $P = 0.001$; Fig 4 H) and VH ($F = 21.0$, $P = 0.001$; Fig 4 L) compared to the Vehicle group, whereas those effect was compromised by MLA.

PHA decreased anxiety- and depressive-like behaviors after CFA injection by the activation of $\alpha 7$ nACh receptors and suppression of brain inflammation

The time course for surgery and experiment was similar as Fig 2I, except for CFA injection replaced the SNI surgery. The results showed that CFA induced anxiety-like behaviors in the open field test and PHA improved those behaviors, whereas those effect of PHA on center distance ($F = 16.9$, $P = 0.001$; Fig 5A) and center/total distance ($F = 14.3$, $P = 0.001$; Fig 5C) was compromised by MLA. The total running distance moved were not different among those groups ($F = 0.145$, $P = 0.964$; Fig 5B). CFA decreased sucrose intake percentage, food intake and weight gain, and ICV PHA improved the behavior results, whereas, the effect of PHA on sucrose intake percentage ($F = 21.6$, $P = 0.001$; Fig 5D), food intake ($F = 7.8$, $P = 0.001$; Fig 5E) and weight gain (group $F_{3,36} = 7.68$, $P = 0.001$; time $F_{2,36} = 14.5$, $P = 0.316$; interaction $F_{6,36} = 9.74$, $P = 0.001$; Fig 5F) was compromised by MLA. CFA decreased mechanical allodynia and increased response to cold, whereas ICV PHA and MLA did not affect those response to mechanical stimulation (group $F_{3,36} = 0.051$, $P = 0.985$; time $F_{2,36} = 86.4$, $P = 0.001$; interaction $F_{6,36} = 0.187$, $P = 0.980$; Fig 5G) and acetone (group $F_{3,36} = 0.423$, $P = 0.738$; time $F_{2,36} = 52.4$, $P = 0.001$;

interaction $F_{6,36} = 0.531$, $P = 0.783$; Fig 5H). the ELISA results showed that CFA did not affect IL-10 content in the mPFC BA and VH, and ICV PHA increased IL-10 content in the mPFC ($F = 7.29$, $P = 0.001$) and BA ($F = 8.68$, $P = 0.001$), and those effect was decreased by MLA (Fig 6A). CFA increased IL-1 β content in the mPFC BA and VH, and ICV PHA decreased IL-1 β increment in mPFC ($F = 29.0$, $P = 0.001$), BA ($F = 7.29$, $P = 0.001$), and VH ($F = 24.7$, $P = 0.001$), and the effect was compromised by MLA (Fig 6B). CFA increased TNF- α content in the mPFC BA and VH, and ICV PHA decreased TNF- α content in the mPFC ($F = 19.5$, $P = 0.001$), and BA ($F = 18.4$, $P = 0.001$), the effect was abolished by MLA (Fig 6C). CFA increased IL-18 content in the mPFC BA and VH, and ICV PHA decreased IL-18 content in the mPFC ($F = 14.1$, $P = 0.001$) and VH ($F = 16.7$, $P = 0.001$), while the effect of PHA was compromised by MLA (Fig 6D).

PHA decreased anxiety- and depressive-like behaviors via WNT/ β -catenin signal pathway

PHA increased center distance, center/total distance ratio, sucrose intake percentage, food intake and weight gain after SNI surgery and signal pathway inhibitors of TGF β , mTOR, WNT/ β , notch, JAK were co-administered to explore the potential the anti-anxiety anti-depressive mechanism of PHA. The results found WNT/ β -catenin inhibitor IWR reversed the anti-anxiety and anti-depressive effect of PHA on center distance ($F = 4.05$, $P = 0.002$; $t = 3.35$, $P = 0.004$; Fig 7A), center/total distance ratio ($F = 4.11$, $P = 0.002$; $t = 3.44$, $P = 0.003$; Fig 7C), sucrose intake percentage ($F = 4.55$, $P = 0.001$; $t = 3.22$, $P = 0.006$; Fig 7D), food intake percentage ($F = 6.17$, $P = 0.001$; $t = 3.24$, $P = 0.004$; Fig 7E) and weight gain ($F = 6.10$, $P = 0.001$; $t = 3.16$, $P = 0.006$; Fig 7F). The total running distance were not different among six groups ($F = 0.458$, $P = 0.837$; Fig 7B) Surprisingly, mTOR inhibitor AZD8055 also reversed the effect of PHA on food intake percentage ($t = 3.53$, $P = 0.002$; Fig 7E respectively) and weight gain ($t = 3.21$, $P = 0.005$; Fig 7 F). And TGF β , notch and JAK pathway inhibitors did not affect the anti-anxiety anti-depressive behaviors of PHA.

PHA decreased brain inflammation via WNT/ β -catenin and mTOR pathway

The cytokines were measured after behavior tests. The results showed that PHA increased IL-10 and decreased IL-18 after SNI surgery, and those effect on IL-10 (Fig 8A) in mPFC ($t = 4.78$, $P = 0.001$), BA ($t = 4.34$, $P = 0.001$) and VH ($t = 3.53$, $P = 0.003$) and on IL-18 (Fig 8 D) in mPFC ($t = 3.49$, $P = 0.003$), BA ($t = 4.53$, $P = 0.001$) and VH ($t = 3.62$, $P = 0.002$) were abolished by WNT/ β -catenin inhibitor IWR. In addition, PHA decreased IL-1 β content in mPFC and VH after SNI surgery, and the change was compromised by mTOR inhibitor AZD8055 in the mPFC ($t = 3.72$, $P = 0.002$) and VH ($t = 4.04$, $P = 0.001$) and WNT/ β -catenin inhibitor IWR in the mPFC ($t = 3.32$, $P = 0.004$) and VH ($t = 4.64$, $P = 0.001$) (Fig 8B). Similarly, PHA decreased the content of TNF in mPFC, BA and VH after SNI surgery, and the content change of TNF α in mPFC ($t = 3.21$, $P = 0.005$) and BA ($t = 4.83$, $P = 0.001$) was reversed by mTOR inhibitor AZD8055 and in mPFC ($t = 3.55$, $P = 0.002$), BA ($t = 4.24$, $P = 0.001$) and VH ($t = 4.44$, $P = 0.002$) was reversed by WNT/ β -catenin inhibitor IWR (Fig 8C).

Discussion

Our results showed that ICV injection of $\alpha 7$ nACh receptor agonist PHA decreased anxiety- and depression-like behaviors induced by SNI and CFA, the mechanisms could be due to the activation of

WNT/ β -catenin signal pathway and suppression of brain inflammation.

Pain and anxiety, depression comorbidity have been observed in clinics and investigated in animal models[18]. In the study, we found that SNI-induced anxiety- and depression-like behaviors after surgery. The results were confirmed by decreased center and center/total distance in the open field tests, and decreased sucrose and food intake, and weight gain compared to naïve rats. SNI surgery did not affect the total distance moved in the open field test, which excluded the possibility that the effect was attributed to motor impairment. Pain behaviors of paw withdrawal threshold and response to acetone suggest the successful establishment of pain animal models. In addition, animals received CFA injection, which is proinflammatory and induces pain, also present anxiety- and depression-like behaviors, which confirmed that chronic pain induces affective disorders. Clinical study has demonstrated that chronic pain frequently results in anxiety and depression[1], and chronic pain contributes to the recurrence, longer duration and increased sadness of depressive episodes[19]. In addition, pain and psychiatric symptoms are intertwined and co-exacerbate physical and psychological symptoms. The mechanism may be explained by some overlap biological process[20]. For example, injuries to sensory pathways have been demonstrated to affect the same brain regions that are involved in mood disorders[9].

Recently, anxiety and depression have been demonstrated to be an inflammatory disease, and inhibition of inflammation reduces anxiety- and depression-like behaviors[3]. In clinical study, neuroinflammation of cytokines has been analyzed in CSF of post-mortem brain tissue, and results found immune cells are involved in the immune response using both postmortem tissue and positron emission tomography (PET) imaging[21]. The specialized immune cells in the brain are called microglia that comprise 5–10% of total brain cells and their main functions are to maintain CNS homeostasis and to cope with damage or infection[22]. In many neurodegenerative and neuropsychiatric diseases, microglia are activated and contribute to pathology by promoting neuroinflammation[23]. In anxious and depressive conditions, microglia are activated and induce cascade proinflammatory response in the brain and contribute to the anxiety- and depression-like behaviors[24]. Evidence in rodents found that after insult, microglial indoleamine 2, 3-dioxygenase and kynurenine pathway are activated and generate excessive neurotoxic quinolinic acid, microglia shift to proinflammatory profile, and generates a host of cytokines, including interleukin IL-1 β , TNF- α and so on[25]. The proinflammatory quinolinic acid and cytokines from microglia inhibit hippocampal neurogenesis and induce anxiety- and depression-like behaviors, whereas those effects were compromised by minocycline, a microglia inhibitor. Thus, suppression of the brain microglia-related inflammation is an effective approach to treating anxiety and depression.

PHA is a $\alpha 7$ nACh receptor agonist, and our results showed that ICV injection of PHA decreased anxiety- and depression-like behaviors, including open field test, sucrose preference test and food intake induced by SNI, the reason could be attributed to the activation of $\alpha 7$ nACh receptors, as co-administration of $\alpha 7$ nACh receptor antagonist MLA abolished those effects. Consistently, our previous study has demonstrated that IT injection of PHA prevents the development and maintenance of chronic pain. The reason is attributed to suppression of proinflammatory response in the spinal cord. In addition, PHA decreased inflammatory cytokines release from microglia in vitro by activation of $\alpha 7$ nACh receptors in

primary microglia cultures[8]. The inflammatory cytokines in the brain majorly come from microglia, as microglia suppression prevents the development of anxiety- and depression-like behaviors in animals[3]. In our study, we included a group of rats received MIN, a microglia inhibitor, and results showed that ICV MIN decreased SNI-induced anxiety- and depression-like behaviors and brain proinflammatory response. This confirmed that anxiety and depression are a microglial inflammatory disease and suppression of microglia-related inflammation is effective for treating anxiety and depression, which is consistent with our previous study.

The cholinergic anti-inflammatory pathway controls cytokine production and inflammation in the body[26]. Experimental evidence suggests that cholinergic anti-inflammatory signaling requires $\alpha 7$ nACh receptors expressed on cytokine-producing microglia in the central nervous system. $\alpha 7$ nACh receptor agonists inhibit cytokine release and shows protective effect in a variety of experimental lethal inflammatory models and has been exploited to counteract abnormal chronic and hyper-activated pro-inflammatory conditions[6]. Our study confirmed that suppression of inflammation in the brain is a promising therapeutic target for treating anxiety and depression by activation of $\alpha 7$ nACh receptors. In order to confirm that the effect of $\alpha 7$ nACh receptor activation is not limited to SNI-induced depression, we included CFA model to prove the general effect of $\alpha 7$ nACh receptor agonist in chronic pain-induced anxiety and depression. Our results showed that CFA-induced anxiety- and depression-like behaviors, including decreased center distance, center/total distance, sucrose intake percentage and food intake, and ICV PHA provided anti-anxiety and anti-depressive effect induced by CFA. In addition, the effect of PHA was reversed by MLA, which confirmed that PHA's anti-anxiety and anti-depressive effect by activation of $\alpha 7$ nACh receptors. Further, PHA decreased CFA-induced brain inflammation, including increased IL-10, and decreased IL-1 β , TNF- α and IL-18 in the brain.

To further explore the mechanisms of the anxiolytic and anti-depressive effect of PHA, signal pathway inhibitors were administered together. The results showed that WNT/ β -catenin inhibitor IWR reversed the anti-depressive effect of PHA in the open field test and sucrose preference test, and both WNT/ β -catenin inhibitor IWR and mTOR inhibitor suppressed food intake and weight gain, whereas TGF- β , Notch, and JAK pathway inhibitors did not affect that effect. This suggests the anxiolytic and anti-depressive effect of PHA through WNT/ β -catenin, while feeding behavior of food and weight gain are affected by both WNT/ β -catenin and mTOR signal pathway. WNT/ β -catenin are secreted glycoproteins that signals through the frizzled receptors and are essential for normal embryonic development[27, 28]. Study have demonstrated WNT/ β -catenin signaling is associated with anxiety and depression response. The WNT/ β -catenin pathway in nucleus accumbens is down-regulated in the mouse model of social defeat model of depression, and knock down of the signaling renders mice more susceptible to social defeat stress and promotes anxiety and depression-like behaviors[29]. In addition, different classes of anti-anxiety and anti-depressant treatments, including SSRIs, SNRI, and chronic electroconvulsive shock, present anti-anxiety and anti-depressive effect by activation of WNT/ β -catenin pathway[30]. However, our study suggests that activation of $\alpha 7$ nACh receptors showed anxiolytic and anti-depressive effect via the WNT/ β -catenin pathway.

Our result showed that the mTOR inhibitor decreased the effect of PHA on food intake and weight gain. the reason may be attributed to that mTOR controls the body metabolism and increases food intake and results in weight gain[31]. Thus, food intake and weight gain were also affected by mTOR inhibitors in our experiments. The inflammatory cytokine concentration was measured and the results showed WNT/ β -catenin inhibitor IWR reversed the IL-10, IL-1 β , TNF- α and IL-18 content change induced by PHA, which confirmed that PHA present its anti-inflammation and anxiolytic and anti-depressive effect via WNT/ β -catenin signal pathway. Surprisingly, IL-1 β , TNF- α content change were reversed also by mTOR pathway inhibitors. This indicates that mTOR pathway is also anti-inflammatory in anxiety- and depression-like animals induced by chronic pain. Although previous study has demonstrated that the activation of mTOR reversed anxiety- and depression-like behaviors, the mechanisms may be explained by suppression of inflammation[32, 33]. However, mTOR pathway inhibitor did not affect anxiolytic and anti-depressive effect of PHA, and this suggested mTOR maybe a downstream signal of WNT/ β -catenin, or at least not a major contributor of the anxiolytic and anti-depressive effect of PHA[33]. PHA activates WNT/ β -catenin and results in the increment of IL-10 and the decrement of IL-1 β , TNF- α , IL18 release, and at the same time, activates mTOR which result in enhanced decrement of IL-1 β and TNF- α release. As previous study has demonstrated IL-10 can resolve the proinflammatory response, the anti-anxiety and anti-depressive effect of PHA could be attributed to the activation of WNT/ β -catenin and increased IL-10 release[34].

In the study, several cytokines were chosen to evaluate the brain inflammatory response. Previous studies have demonstrated that IL-10, IL-1 β , TNF- α and IL-18 were related to anxiety, depression, and modulation of inflammation can induce or decrease anxiety- and depression-like behaviors. In our study, both SNI and CFA increased IL-1 β , TNF- α and IL-18 in the brain, and ICV PHA increased IL-10, and decreased IL-1 β , TNF- α and IL-18 content. This confirmed increased anti-inflammatory IL-10 suppressed pro-inflammatory response after chronic pain insult[35]. In addition, followed our previous study, tissue of mPFC BA and VH were acquired to testify the inflammatory profile after SNI or CFA, because those sites have been proven to be involved in anxiety and depression and showed increased cytokines expression after chronic pain insult[36]. Our research confirmed that those sites are proper and effective for the study of anxiety and depression.

Conclusions

Our study suggests that the activation of $\alpha 7$ nACh receptor presents anxiolytic and anti-depressive effects, and the reason could be attributed to the activation of WNT/ β -catenin pathway and suppression of inflammation in the brain.

Abbreviations

$\alpha 7$ nACh: alpha 7 nicotinic acetylcholine; ANOVA: analysis of variance; BA: basolateral amygdala; CFA: complete Freund's adjuvant; DMSO: dimethylsulfoxide; ELISA: enzyme-linked immune-absorbent assay; ICV: intracerebroventricular; IWR: IWR-1-endo; MIN: minocycline; MLA: methyllycaconitine citrate; mPFC:

medial prefrontal cortex; POD: postoperative day; PHA: PHA-543613; SNI: spared nerve injury; VH: ventral hippocampus;

Declarations

Acknowledgement

The authors would like to thank all the person who participated in this study.

Funding

This research is funded by a grant from the National Natural Science Foundation of China (NSFC, Grant No. 81270414), Natural Science Foundation of Shanghai. China (20ZR1434600) and Natural Science Foundation of Hubei. China (2019CFB411).

Authors' contributions

Xingrui Gong and Yongmei Chen did the surgery and behavior tests and ELISA experiment. Meihau Cai did the statistical analysis. Mazhong Zhang designed the experiment. Rongmei Fan revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Experimental Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine and Hubei University of Arts and Science, and were conducted under the guidelines of National Institute of Health.

Availability of data and materials

Available upon request.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

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Figures

Figure 1

SNI surgery induced anxiety and depression-like behaviors. SNI surgery decreased center distance (A) and center/total distance (C) compared to sham group in the open field test from POD14 to POD 21 after surgery. (B) SNI surgery did not affect total running distance in the open field test after surgery. (D) SNI decreased sucrose intake percentage from POD14 to POD21. In addition, SNI decreased food intake (E) and weight gain (F) compared to sham after surgery from POD7 to POD21. SNI surgery decreased paw withdrawal threshold (G) and increased response to acetone (H) after surgery from POD7 to POD21. n = 8. “a” denotes the statistically significant difference compared to the sham group. SNI spared nerve injury, POD postoperative days

Figure 2

ICV injection of $\alpha 7$ nACh receptor agonist PHA and MIN decreased anxiety- and depression-like behaviors after SNI surgery. ICV PHA or MIN improved that decrement of center distance (A) and center/total distance (C) after SNI surgery on POD18. (B) SNI surgery did not affect total running distance in the open field test after surgery. (D) SNI decreased sucrose intake percentage from POD10 to POD18 and ICV PHA or MIN decreased that effect on POD18. SNI surgery decreased food intake (E) and weight gain (F) from POD10 to POD18 compared to sham group, and ICV PHA or MIN decreased that effect on POD18. ICV PHA or MIN did not affect paw withdrawal threshold (G) and response to acetone (H) after SNI surgery. (I) Experimental procedures and timeline. "a" denotes the statistically significant difference compared to the sham + vehicle group. "b" denotes the statistically significant difference compared to the SNI + vehicle group. $\alpha 7$ nACh alpha 7 nicotinic acetylcholine, PHA PHA-543613, ICV intracerebroventricular, MIN minocycline

Figure 3

MLA decreased anti-anxiety and anti-depressive effect of PHA. ICV injection of $\alpha 7$ nACh receptor antagonist MLA decreased anti-depressive effect of PHA in center distance (A) and center/total distance (C) in the open field test and the total distance (B) running was not different between groups after SNI surgery on POD18. PHA increased sucrose intake percentage (D) and food intake (E) and weight gain (F) after SNI, while ICV MLA decreased that effect. MLA and PHA did not affect the paw withdrawal threshold (G) and response to acetone (H). “a” denotes the statistically significant difference compared to the SNI + vehicle group. “b” denotes the statistically significant difference compared to the SNI + PHA group. MLA methyllycaconitine citrate

Figure 4

The effect of PHA on brain cytokines production. ICV PHA increased IL-10 in the mPFC (A), BA (E) and VH (I) compared to SNI+Vehicle group, and MLA decreased that effect. SNI increased IL-1β in the mPFC (B), BA (F) and VH (J) compared to Naive group, and ICV PHA decreased IL-1β in the mPFC, BA and VH compared to SNI+Vehicle group, while MLA decreased the effect of PHA. SNI increased TNF-α (C, G and K) and IL-18 (D, H and L) in the mPFC, BA and VH compared to Naive group, and ICV PHA decreased their content in the mPFC, BA and VH compared to SNI+Vehicle group, while MLA decreased the effects of PHA. “a” denotes the statistically significant difference compared to the SNI + vehicle group. “b” denotes the statistically significant difference compared to the SNI + PHA group. ELISA enzymelinked immune-absorbent assay.

Figure 5

PHA decreased anxiety and depressive-like behaviors induced by CFA. CFA decreased center distance (A) and center/total distance (C) in the open field test, and did affect total running distance moved (C), compared to naïve group, and PHA improved the behaviors in the open filed tests, whereas MLA decreased the effect of PHA. CFA decreased sucrose intake percentage (D), food intake (E) and weight gain (F), and PHA improved that effect, and the effects of PHA was abrogated by MLA. CFA decreased mechanical anodynia (G) and increased response (H) to cold, whereas ICV PHA and MLA did not affect that response. “a” denotes the statistically significant difference compared to the CFA + vehicle group. “b” denotes the statistically significant difference compared to the CFA + PHA group. “c” denotes the statistically significant difference compared to the Naive group.

Figure 6

PHA decreased CFA-induced brain inflammation by activation of $\alpha 7$ nACh receptors. (A) ICV PHA increased IL-10 content in the mPFC and BA after CFA injection, and the effect of PHA was decreased by ICV MLA. (B) CFA increased IL-1 β content in the mPFC BA and VH, and ICV PHA decreased IL-1 β increment in those sites, and the effect of PHA was decreased by MLA after CFA injection. (C) CFA increased TNF- α content in the mPFC BA and VH, and ICV PHA decreased TNF- α content in the mPFC, BA and VH, the effect of PHA was abolished by MLA. (D) CFA increased IL-18 content in the mPFC BA and VH, and ICV PHA decreased IL-18 content in the mPFC and VH, while the effect of PHA was decreased by MLA. "a" denotes the statistically significant difference compared to the CFA + vehicle group. "b" denotes the statistically significant difference compared to the CFA + PHA group. "c" denotes the statistically significant difference compared to the Naive group.

Figure 7

PHA decreased anxiety- and depressive-like behaviors via WNT/ β -catenin signal pathway. IWR (WNT/ β -catenin inhibitor) reversed the anti-depressive effect of PHA on center distance (A), center/total distance ratio (C), sucrose intake percentage D, food intake percentage (E) and weight gain (F), while did not affect total running distance(B) in the open field test after SNI surgery. However, AZD8055 (mTOR inhibitor) also reversed the effect of PHA on food intake percentage (E) and weight gain (F), Whereas SB431542(TGF β inhibitor), Numb (notch inhibitor) and AZD1480 (JAK inhibitor) did not affect anti-depressive behaviors of PHA. “a” denotes the statistically significant difference compared to the SNI group. “b” denotes the statistically significant difference compared to the PHA group.

Figure 8

PHA decreased brain inflammation via WNT/ β -catenin and mTOR pathway. PHA increased IL-10 (A) and decreased IL-18 (D) in mPFC, BA and VH after SNI surgery, and IWR decreased those effect. (B) PHA decreased IL-1 β content in mPFC and VH after SNI surgery, the effect was reversed by AZD8055 and IWR. (C) PHA decreased the content of TNF in mPFC, BA and VH after SNI surgery, and the content change of TNF α in mPFC and BA was reversed by AZD8055 and IWR. "a" denotes the statistically significant difference compared to the SNI group. "b" denotes the statistically significant difference compared to the PHA group.