Antidepressant-like Effects of Transcutaneous Auricular Vagus Nerve Stimulation: Insights from Relieves Neuroinflammation Mediated by P2X7R in Rat Model of Chronic Unpredicted Mild Stress

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Research

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Abstract

Background: As a prevalent mental health condition, depression is believed to be mediated by stress-induced neuroinflammation. Transcutaneous auricular vagus nerve stimulation (taVNS) has been used in the treatment of depression as the latest neuromodulation therapy. However, the antidepressant mechanism of the treatment at the molecular level is still unclear. Our previous study evaluated the effectiveness of the taVNS in antidepressant-like behavior. The objective of this study is to explore the role of P2X7R mediated hippocampal neuroinflammation in taVNS's antidepressant effect.

Methods: Rat depression model was established by using a chronic unpredicted mild stress (CUMS) method for five weeks. Starting from the 3rd week, taVNS intervention was applied through an electroacupuncture apparatus (HANS-100A, 2/15 Hz, 2mA) for 30 minutes every day for three weeks. Body weight test (BWT) and behavioral assessments such as open field test (OFT) and sucrose preference test (SPT) were conducted on days 0, 7, 14, 21, 28 and 35. The protein levels of P2X7R, NLRP3, caspase-1, IL-1 β and IL-18 in the hippocampus were examined using western blot. Moreover, P2X7R expressing cells were detected using immunohistochemistry.

Results: The results showed that CUMS induced body weight loss and depression-like behavior in rats. Hippocampal neuroinflammation was upregulated, which was manifested in the higher expression of P2X7R, NLRP3, caspase-1, IL-1 β and IL-18. Interestingly, 3 weeks of the taVNS significantly reduced the depression-like behaviors and strengthen the growth of the body, and the CUMS-induced expression of P2X7R, NLRP3, caspase-1, IL-1 β and IL-18 was attenuated. We also found that P2X7R was expressed in microglia of the hippocampus.

Conclusion: In summary, the taVNS has antidepressant effect. It alleviates hippocampal neuroinflammation, which may be related to the regulation of the initial signal of P2X7R.

Background

Depression is a highly prevalent mental health condition [1], it is the second-largest disability contributor affecting millions of people worldwide [1–4]. In recent years, with the advent of more and more antidepressants, people with depression have more treatment options, but about 30%-50% of patients fail to respond to those treatments [5], suggesting the complexity of the pathological mechanism of depression. Therefore, it is necessary to develop and promote more effective and tolerant treatments based on the pathogenesis of depression [6].

In the past decades, stress exposure is considered to be the leading environmental cause of depression [7–9], it is also the main experimental pathway for studying depression-like behavior in laboratory animals [10]. Furthermore, the observation of immune system alternations in depressed patients leads to a growing appreciation that stress-induced neuroinflammation characterized by overproduction of inflammatory cytokines in the brain as important pathogenesis of depression [9–14]. Inflammatory process may play an important role in the pathophysiology for at least a subgroup of patients with

depression [15]. For instance, neuroinflammation characterized by microglia activation is observed in the brain of depression patients with suicidal or moderate to severe depression episodes [6, 16]. Preclinical studies have also shown that chronic stress can lead to the activation of the innate immune system in the brain, while the increase of inflammatory cytokines, such as IL-1 β , IL-6, and IL-18 can lead to depression-like behavior in animals [12, 17–19]. Therefore, anti-inflammatory therapy may be a promising antidepressant treatment [6].

As a non-pharmaceutical treatment option for various diseases, bioelectronic medicine has progressively become the focus in recent years [20], and the treatment technology represented by transcutaneous auricular vagus nerve stimulation (taVNS) has become a promising alternative to antidepressants [21–23]. Our previous studies showed that the antidepressant effect of taVNS is mediated by the default network of nucleus tractus solitarius-limbic lobe-brain [22]. It is worth noting that the hippocampus is an important part of the limbic lobe, studies have shown that the significant reduction of hippocampal volume is related to depression [24, 25], and the abnormalities of hippocampus are related to depression causality [26].

Vagus nerve (VN) is the 10th cranial nerve, mainly a sensory nerve, and mostly relays biofeedback to the brain [20, 27]. In the past 20 years, a large number of studies have shown that the VN can serve as a bridge between the central nervous system (CNS) and the immune system, and play an essential role in regulating inflammation [28, 29]. Anatomic research shows that the only branch of the VN on the body surface is the auricular branch of the vagus nerve (ABVN) [30], which is mainly distributed in the external auditory meatus and concha (cymba conchae and cavum conchae), and the cymba conchae is supplied exclusively by the ABVN [31]. Recently, a review infer that taVNS has anti-inflammatory properties that are exerted through activation of the Hypothalamo-Pituitary-Adrenocortical axis (HPA axis), the cholinergic anti-inflammatory pathway (CAP), and brain regions or circuits in depression [32], but the anti-inflammatory mechanism of taVNS in depression is still unknown, especially the mechanism of its anti-inflammatory in the brain.

Previously, we found in a pre-experimental study that taVNS reverses the depressive behavior of chronic unpredicted mild stress (CUMS) rats is related to the inhibition of purinergic ligand-gated ion channel 7 receptor (P2 $\times$ 7R) expression in the hippocampus. In the brain, it is reported that P2 $\times$ 7R is an ionotropic receptor mainly located in the microglia, activated by cellular danger signals such as adenosine triphosphate (ATP) [33]. In general, microglia culture stimulated with ATP has a higher expression of Nod-like receptor protein 3 (NLRP3), Asc protein, and caspase-1, and the stimulation also promotes the secretion of Interleukin-1 β (IL-1 β) and Interleukin-18 (IL-18) in an NLRP3-dependent manner [9]. More importantly, hippocampus is a critical site of P2 $\times$ 7R that may regulate behavior / emotional response to stress in the CUMS rats [8].

The goal of the study is to investigate the impact of the taVNS on behavioral deficits and neuroinflammation in rats exposed to CUMS, and to understand whether the anti-inflammatory-based antidepressant effect is mediated through regulating P2 $\times$ 7R. We examined the behaviors of the rats at

assigned time points, including anhedonia, exploration, and motor activity. P2 × 7R and related neuroinflammatory markers in the hippocampus (NLRP3 inflammatory bodies, proinflammatory cytokines) were measured at the end.

Methods

Experimental animals

Specific pathogen free (SPF) male Sprague-Dawley rats, 6-week old, weighting (180 ± 20) g, were obtained from the SPF (Beijing) biotechnology co., LTD.. All rats were housed in a controlled environment with a temperature range of 20–25 °C, $55 \pm 2\%$ humidity, and in quiet states maintained under a 12 h/12 h light/dark cycles with ad libitum access to food and water (except when indicated). After a week of adaptation, rats were randomly divided into two groups. The rats in the first group were housed in cages with five rats per cage. The rats in the second group were exposed to CUMS were housed separately in different cages for social isolation. The protocol of the animal procedures was approved by the Animal Care and Use Committee of Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (Permit No. D2019-02-11-1). All animal experiments were complied with the ARRIVE guidelines and carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Due to the effect of estrogen on stress, only male rats were used in this study.

Experimental grouping

Sucrose preference test (SPT) and open field test (OFT) were carried out on all rats to ensure baseline characteristics consistency before implementing any further interventions. Seven rats were excluded due to the inconsistent baseline characteristics. Nine rats with no significant depression-like behavior after the 2nd weeks of CUMS exposure were also excluded. Finally, a total of 32 rats were enrolled in the following experiment. These rats were randomly assigned into four groups: control group (C), model group (M), taVNS group (TA), and transcutaneous auricular non-vagus nerve stimulation (tnVNS) group (TN). There are 8 rats in each group. Rats in M, TA, and TN groups were subjected to social isolation and CUMS for 5 weeks. Starting from the 3rd week, 1 h before the CUMS procedure, rats in the TA group and TN group were administered with taVNS and tnVNS once daily, for 3 weeks. The experimental procedure is shown in Fig. 1.

Figure 1 Experimental procedures. CUMS, chronic unpredicted mild stress; taVNS, transcutaneous auricular vagus nerve stimulation; tnVNS, transcutaneous auricular non-vagus nerve stimulation.

Preparation of the CUMS model

The CUMS model has been validated as one of the most relevant rodent models of depression [34]. In this study, the CUMS model was modified according to the methods previously described [35], some appropriate adjustments were made to enhance unpredictability. Rats were subjected to 7 different stressors: restraint for 2 h (restraining in a cylinder-shaped wire net, 20 cm length and 5.5 cm diameter) [36], clip tail for 3 min, swimming in cold water for 5 min, food deprivation for 24 h, housing in a wet cage

for 24 h (200 milliliters of water mixed with 100 grams of sawdust), water deprivation for 24 h, continuous overnight illumination for 12 h. One of these stressors were performed every day in a random order for rats in the 1st to 5th week (Table 1), and the same stressor was not used for consecutive days to avoid the any prediction from the rats.

Table 1
CUMS procedure during the whole experiment.

Week	1th	2th	3th	4th	5th
Monday	Restraint 2 h	Clip tail 3 min	Swimming in cold water 5 min	Housing in a wet cage 24 h	Food deprivation 24 h
Tuesday	Food deprivation 24 h	Housing in a wet cage 24 h	Restraint 2 h	Continuous overnight illumination 12 h	Water deprivation 24 h
Wednesday	Clip tail 3 min	Water deprivation 24 h	Food deprivation 24 h	Swimming in cold water 5 min	Continuous overnight illumination 12 h
Thursday	Swimming in cold water 5 min	Continuous overnight illumination 12 h	Water deprivation 24 h	Restraint 2 h	Clip tail 3 min
Friday	Housing in a wet cage 24 h	Food deprivation 24 h	Continuous overnight illumination 12 h	Water deprivation 24 h	Swimming in cold water 5 min
Saturday	Water deprivation 24 h	Swimming in cold water 5 min	Housing in a wet cage 24 h	Clip tail 3 min	Restraint 2 h
Sunday	Continuous overnight illumination 12 h	Restraint 2 h	Clip tail 3 min	Food deprivation 24 h	Housing in a wet cage 24 h

Table 1 CUMS procedure during the whole experiment.

Intervention of taVNS/tnVNS

The taVNS/tnVNS intervention was started after 2 weeks of CUMS exposure, once everyday for 3 weeks. During the intervention, the rats were anesthetized continuously with 2% isoflurane inhalation anesthesia (Matrx VIP 3000, Midmark Corporation, United States). The positive and negative electrodes were placed at the rats’ ear over the skin inside and outside of the rats’ auricular concha or margin region [37] (see Fig. 2). Electroacupuncture apparatus (HANS-100, Nanjing, China) was used for the stimulation. The

stimulation parameters are: 1) stimulation frequency: 2/15 Hz (2 and 15 Hz, switched every second); 2) stimulus intensity: 2 mA; 3) stimulation duration: 30 min [38].

Figure 2 Intervention of taVNS/tnVNS. The taVNS targets the skin receptive area are located in the auricular concha with ABVN distribution. The tnVNS targets the skin receptive area are located in the auricular margin with no ABVN distribution.

Body weight test (BWT)

Body weight is the most important and sensitive index to reflect the growth and development of the body. Clinically, the weight factor, as an important component of Hamilton Depression scale (HAMD), is also an important indicator to evaluate the status of patients with depression. In this study, we performed BWT tests on all rats on days 0, 7, 14, 21, 28, and 35, respectively.

Behavioral tests

Open field test (OFT)

OFT were performed as described before [34, 39], which is commonly used to measure the general locomotor activity and the willingness to explore in rodents [34]. The behavior is very similar to the clinical symptoms of psychomotor retardation in depression. In the OFT, the apparatus used was a square arena, which was made of an 80 × 80 × 40 cm plastic board without special smell, characterized by a black wall and a black base, and this base was divided into equal squares of 16 × 16 cm by white stripes. When the rats were gently placed in the center of the square arena, they were allowed to enjoy autonomous movement and free exploration. The crossing number (defined as at least three paws in the same square) and the rearing number (defined as the rat standing upright on its hind legs) were monitored and recorded for 3 min. After each rat finished the test, the OFT apparatus was cleaned with 75% ethanol to avoid odor interference left by the previous rat to the next one. In this study, we performed OFT tests on all rats on days 0, 7, 14, 21, 28, and 35, respectively. Crossing and rearing number to assess the ability of rats to adapt to the new environment and general sports activities were analyzed [34, 36, 40].

Sucrose preference test (SPT)

SPT was performed as described [35, 36, 41]. Anhedonia was considered to be one of the core symptoms of depressive disorder, and the condition of anhedonic-like behaviors of rats were evaluated by the SPT. Rats were trained to adapt to a 1% sucrose solution (Amresco 0335, USA) during the adaptation cycle. After the adaptation, all rats were deprived of food and water for 23 h. Then, rats were all housed in individual cages and had free access to two pre-weighed bottles containing 240 ml of sucrose solution (1% w/v) and 240 ml pure water for 1 h. At the end of the test, the bottles of 1% sucrose solution and pure water were re-weighed and recorded. In this study, we performed SPT tests on all rats on days 0, 7, 14, 21, 28, and 35, respectively. The percentage difference in sucrose preference was calculated by the following formula [42]: sucrose preference rate (%) = sucrose consumption / (sucrose consumption + water

consumption) $\times 100$. All values were presented as percentage differences. Anhedonia was expressed by sucrose preference.

Tissue collection

After completing the intervention for 5 weeks, all rats were anesthetized with an injection of pentobarbital sodium (35 mg/kg body weight, i.p.). Then 6 rats in each group were decapitated, and the hippocampus samples were collected on ice, after washing with pre-chilled sterile saline solution, hippocampus samples were stored in pre-chilled 1.5 ml cryogenic microtube and experienced a snap-frozen in liquid nitrogen. Samples were then placed at -80°C in preparation for Western blot analysis. For the other 2 rats in each group, the perfusion needle was inserted into the ascending aorta after anesthesia and clamped with forceps. After incision of the right atrial appendage, 350 ml of cold saline was infused until the liver became white. Then, 4% paraformaldehyde was cold infused with 300–500 ml until the limb was stiff. Each brain was fixed in 4% paraformaldehyde, and preparation for immunohistochemistry analysis.

Western blots

The samples were homogenized with RIPA lysis buffer with protease inhibitor cocktail for protein extraction. The samples were centrifuged at 13,000 rpm at 4°C for 20 min, and the supernatants were collected. Protein concentration was determined by the bicinchoninic acid (BCA) method. Samples were separated in 10%, 12% or 15% SDS gels and transferred to PVDF membranes (0.2 or $0.45\ \mu\text{m}$). The membranes were blocked in 5% bull serum albumin tris-buffered saline plus Tween (BSA-TBST) for 1 h at room temperature (RT) and incubated at 4°C overnight with the following primary antibodies: anti-P2 $\times$ 7R (1: 100, Santa Cruz/Sc-134224); anti-NLRP3 (1: 500, Abcam/ab214185); anti-Caspase-1 (1: 500, Abcam/ab1872); anti-IL-1 β (1: 500, Abcam/ab9787); anti-IL-18 (1: 500, Abcam/ab191860). Anti- β -actin (1: 3000, Abcam/ab6276) was used as the loading control. After the blots were washed in TBST 5 times, secondary antibodies (1: 5000, Abcam/ab6789, Abcam/ab6721) were incubated for 1 h at RT. The signal was captured on an ImageQuant LAS4000 mini image analyzer (GE Healthcare, Buckinghamshire, UK), and the band levels were quantified using Quantity One software v.4.6.2 (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry

The fixed brain was embedded in paraffin and sliced. The brain sections were blocked at room temperature with 1% goat serum and 0.3% Triton X-100 in PB for 1 h, and then incubated overnight at 4°C with primary antibodies: anti-P2 $\times$ 7R (1: 100, Santa Cruz/Sc-134224) and anti-Iba1 (1: 200, ab5076). The brain sections were incubated at room temperature with appropriate fluorescein isothiocyanate- or Cy3-conjugated secondary antibody (1: 200; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h. Nuclei were counterstained with DAPI. Images were acquired using a Panoramic MIDI slide scanner (3DHISTECH Kft., Budapest, Hungary) and analyze the images through Panoramic Viewer Software program (version 1.15.3.35149).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software package (IBM, Armonk, New York, NY, USA). The results were presented as the mean $\pm$ SD. Within each group, the data of behavioral tests at different time points were analyzed by two-way analysis of variance (ANOVA) with *Tukey's post hoc* test. Additionally, one-way ANOVA was used. Fisher's test was used to compare the different intervention groups. A *P* value of less than 0.05 was considered statistically significant.

Results

Changes in body weight

Before the experimental procedures, there was no significant difference among groups. However, the body weight of each group changed significantly after the CUMS or the taVNS/tnVNS interventions. Shown in Fig. 3a, compared with the C group, the body weight of the rats in the M groups decreased significantly, especially on days 14, 21, 28 and 35 ($P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.01$, respectively). Surprisingly, after the intervention of taVNS or tnVNS, this downward trend was gradually curbed. In particular, compared with the M group, the growth of rats in the TA group was significantly promoted on days 28 and 35 ($P < 0.05$, $P < 0.01$, respectively). In addition, TA group has a significant growth advantage over TN group on days 35 ($P < 0.01$).

Behavioral Observations

Changes in crossing and rearing numbers

There was no significant difference among the groups in the crossing and rearing numbers on day 0. After the CUMS or the taVNS/tnVNS interventions, the crossing and rearing numbers of the rats in each group changed dramatically. Shown in Fig. 3b and c, compared with the C group, after continuous exposure to the CUMS, the crossing and rearing numbers of the rats in the M groups decreased significantly on days 7, 14, 21, 28 and 35 ($P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.01$, respectively). Interestingly, this trend was significantly reversed after the taVNS or tnVNS intervention. Compared with the M group, the crossing number of rats in the TA and TN group increased substantially on days 21, 28 and 35 ($P < 0.01$, $P < 0.01$, $P < 0.01$; $P < 0.05$, $P < 0.01$, $P < 0.01$). It is worth noting that the degree of reversal in TA group is more obvious than that in TN group on days 28 and 35 ($P < 0.05$, $P < 0.05$). The rearing number of the rats in the TA and TN group also increased significantly on days 28 and 35 ($P < 0.01$, $P < 0.01$; $P < 0.01$, $P < 0.05$).

Changes in sucrose preference

Similar to what was observed in the OFT tests, there was no difference in the sucrose preference of rats in the groups before the interventions. When the CUMS procedure or the taVNS/tnVNS was involved at different time points, the rats' sucrose preference in each group changed. Shown in Fig. 3d, compared with the C group, the sucrose preference of the rats in the M groups decreased significantly on days 14, 21, 28, and 35 ($P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.01$, respectively). Similarly, after the taVNS or

tnVNS intervention, especially the former, this phenomenon was significantly reversed. Compared with the M group, the sucrose preference of the rats in the TA group increased dramatically on days 21, 28, and 35 ($P < 0.01$, $P < 0.01$, $P < 0.01$), however this significant increase occurred only on day 35 ($P < 0.01$) in the TN group, and this kind of increment in TA group is more obvious than that in TN group on days 28 and 35 ($P < 0.05$, $P < 0.01$).

Figure 3 Differences showing the effects of stress/antidepressant treatments on depressive like behaviors in depression rats induced by CUMS. taVNS, transcutaneous auricular vagus nerve stimulation; tnVNS, transcutaneous auricular non-vagus nerve stimulation. **(a)** Results of two-way ANOVA with time points and groups of changes in body weight were as follows: time points, $F = 1625.631$, $P = 0.000$; groups, $F = 198.626$, $P = 0.000$; interaction, $F = 19.606$, $P = 0.000$; $n = 8$ per group. **(b)** Results of two-way ANOVA with time points and groups of changes in number of crossings were as follows: time points, $F = 145.834$, $P = 0.000$; groups, $F = 254.304$, $P = 0.000$; interaction, $F = 23.402$, $P = 0.000$; $n = 8$ per group. **(c)** Results of two-way ANOVA with time points and groups of changes in number of rearing were as follows: time points, $F = 91.951$, $P = 0.000$; groups, $F = 158.558$, $P = 0.000$; interaction, $F = 11.024$, $P = 0.000$; $n = 8$ per group. **(d)** Results of two-way ANOVA with time points and groups of changes in sucrose preference were as follows: time points, $F = 34.547$, $P = 0.000$; groups, $F = 75.690$, $P = 0.000$; interaction, $F = 5.764$, $P = 0.000$; $n = 8$ per group. Results are expressed as the mean $\pm$ SD. * $P < 0.05$ compared with the C group; ** $P < 0.01$ compared with the C group; ▲▲ $P < 0.01$ compared with the M group; # $P < 0.05$ compared with the TN group; □□ $P < 0.01$ compared with the TN group.

P2 × 7R expression in microglia

In agreement with a large number of reports in the literature, double immunofluorescence labeling of brain sections showed that P2 × 7R was indeed expressed in the hippocampus of rats and dominantly colocalized with microglial marker Iba1 (see Fig. 4).

Figure 4 Representative images of immunofluorescence for P2 × 7R (green) and Iba1 (red) in the hippocampus of rats. Nuclei were counterstained with DAPI (blue). Selected white boxes were zoomed in right panels. Arrowhead indicates representative colocalization of P2 × 7R and Iba1. Scale bar, 50 μ m and 20 μ m (zoomed images).

Expression of P2 × 7R, NLRP3, Caspase-1, IL-1 β and IL-18 in the hippocampus by Western blots analysis

There was a significant difference in P2 × 7R expression among the groups (Fig. 5a). The expression of P2 × 7R was significantly higher in the M group ($P < 0.01$) compared to that in the C group. The difference was also found between the C and TN groups ($P < 0.01$). In comparison with the M group, the higher expression of P2 × 7R was significantly attenuated in the TA and TN groups ($P < 0.01$, $P < 0.01$). Importantly, the expression level of P2 × 7R in the hippocampus of the TA group was lower than that in the TN group.

Compared to the control group, the expression of NLRP3 in the M group was significantly up-regulated ($P < 0.01$). Of note, both taVNS and tnVNS reversed the up-regulation of hippocampal NLRP3 when compared with that in the M group with statistical significance ($P < 0.01$, $P < 0.01$) (Fig. 5b).

There was a significant difference in caspase-1 expression among the groups (Fig. 5c). The expression of caspase-1 was significantly higher in the M group ($P < 0.01$) compared to that in the C group. In comparison with the M group, the higher expression of caspase-1 was significantly reversed in the TA and TN group ($P < 0.01$, $P < 0.01$).

The result showed that, compared to the control group, the expression of IL-1 β in the M group was significantly up-regulated ($P < 0.01$). Neither the taVNS nor the tnVNS significantly reversed the up-regulation of hippocampal IL-1 β when compared with that in the M group, nonetheless the reducing trend was the same (Fig. 5d).

There was a significant difference in IL-18 expression among the groups (Fig. 5e). The expression of IL-18 was significantly higher in the M group ($P < 0.01$) compared to that in the C group. Significance was also found between the C and TN groups ($P < 0.01$). In comparison with the M group, the expression of IL-18 was significantly reversed in the TA group ($P < 0.01$, $P < 0.01$).

Figure 3 Differences showing the effects of stress/antidepressant treatments on the expression of P2 $\times$ 7R, NLRP3, caspase-1, IL-1 β and IL-18 in hippocampus of depression rats induced by CUMS. **(a)** P2 $\times$ 7R ($n = 6$ per group) using western blot analysis; **(b)** NLRP3 ($n = 6$ per group) using western blot analysis; **(c)** Caspase-1 ($n = 6$ per group) using western blot analysis; **(d)** IL-1 β ($n = 6$ per group) using western blot analysis; **(e)** IL-18 ($n = 6$ per group) using western blot analysis. Results are expressed as the mean $\pm$ SD. * $P < 0.05$ compared with the C group; ** $P < 0.01$ compared with the C group; $\blacktriangle P < 0.05$ compared with the M group; $\blacktriangle\blacktriangle P < 0.01$ compared with the M group.

Discussion

Although the pathogenesis of depression is not clear, many studies have shown that stress-induced neuroinflammation is closely related to depression [13, 14, 43]. The anti-inflammatory treatment has become a new option for antidepressation, and recent meta-analyses have supported this concept [44, 45]. Previous studies have shown that taVNS can effectively treat depression [21, 22, 23]. Based on the relationship between VN and the immune system [20, 27], we speculate that the regulation of neuroinflammation may play an essential role in the antidepressant effect of taVNS. However, due to the lack of evidence and the heterogeneity of methods, this conjecture is still controversial. In this study, we established a rat depression model by CUMS method, which was proved to be parallel to the symptoms of depression and accurately summarized the human condition [40, 46]. We provided convincing findings on the antidepressant-like effect of taVNS at the molecular level and suggested a new conceptual framework for exploring new treatments for depression.

Depression-like behaviors induced by CUMS and taVNS exhibits the antidepressant-like effect

CUMS is considered to be one of the most effective and reliable methods to establish a rodent model of depression [34, 35, 47]. This method simulates a variety of stressors of depression. Animals exposed to the CUMS will show a series of depression-like behaviors such as behavioral despair, anhedonia, less exploration, and less locomotion [48]. OFT and SPT are standard methods to assess such behaviors. In this study, we selected SPT and OFT to evaluate depression-like behaviors in rats. It was found that the CUMS significantly decreased the rat's exploration and locomotion abilities in OFT and the percentage of sucrose preference in SPT. It is worth mentioning that both the taVNS and tnVNS can alleviate the CUMS-induced depression-like behaviors in the rat. The antidepressant-like effect of taVNS is earlier and stronger. In addition, taVNS has a significant promoting effect on improving the growth of CUMS exposed rats, which is unreachable by tnVNS. The results are, to some extent, consistent with our previous clinical studies [22, 23].

The antidepressant-like effect of taVNS may be related to the regulation of the initiation signal of neuroinflammation

P2 × 7R is an ion channel that is activated by ATP after stress exposure [49]. It is widely distributed in the central nervous system, not only in neurons but also in glial cells [50, 51]. P2 × 7R is known for promoting the assembly of the NLRP3 inflammasome, releasing the proinflammatory IL-1 β and IL-18-dependent caspase-1 from innate immune cells after exposure to LPS and ATP [52]. Previous studies have shown that the P2 × 7R-mediated effect occurs mainly through the activation of the neuroinflammatory response, which is involved in the pathological process of depression [53–55]. Furthermore, P2 × 7R gene knockout may have an antidepressant-like effect in mice [56, 57]. In this study, we detected the related indexes of P2 × 7R-mediated inflammation. Interestingly, we found that P2 × 7R, NLRP3, caspase-1, IL-1 β , and IL-18 were up-regulated in rats' hippocampus after exposure to the CUMS. It is inferred that the rats' depression-like behaviors were related to the increase of P2 × 7R-mediated neuroinflammation in the hippocampus. The preliminary data suggested that P2 × 7R could be a potential target for depression treatment. After the taVNS intervention, inflammatory cascade, including P2 × 7R and its mediators, were down-regulated at different levels. This observation is consistent with our previous hypothesis that the taVNS is anti-inflammatory, and it plays an antidepressant-like role by modulating the down-regulation of hippocampal P2 × 7R expression (see Fig. 6).

Furthermore, our results suggested that similar to taVNS, tnVNS is also anti-inflammatory and plays an antidepressant-like role in the modulation. Although this results is not consistent with the original intention of this study, similar results have been reported in our previous clinical study [22, 23].

Anatomically, the auricle area of rats is much smaller than that of humans, and due to the limitation of stimulation electrode, a relatively large surface electrode generates a diffuse electric stimulation field. Therefore, the stimulation of rat auricular margin is not always so accurate during the experiment, this limitation may lead to more or less stimulation of ABVN by tnVNS.

Figure 6 The antidepressant-like effect of taVNS may be related to the regulation of the initiation signal $P2 \times 7R$ of neuroinflammation. The high levels of ATP released from neurons or astrocytes reach $P2 \times 7R$ located in microglia. $P2 \times 7R$ stimulation elicits K^+ efflux and triggers the assembly and activation of NLRP3 inflammatory. Activated NLRP3 inflammatory mediates the activation of caspase-1, while caspase-1 in turn stimulates the production of IL-1 β and IL-18. In this pathway, taVNS/tnVNS may regulate its upstream signal $P2 \times 7R$ and play an antidepressant role.

The present study showed that the CUMS-induced depression-like behavior induced in the rats was related to the $P2 \times 7R$ -mediated neuroinflammation in the hippocampus. Importantly, our results indicated that the taVNS intervention effectively relieved the rats' depression-like behavior promptly. The antidepressant effect is anti-inflammatory, and it is achieved by down-regulating the $P2 \times 7R$. The study may provide a new idea for exploring neuromodulation techniques in the treatment of depression from the perspective of anti-inflammation.

Abbreviations

ABVN

Auricular branch of the vagus nerve; ANOVA: Analysis of variance; ATP: Adenosine triphosphate; BWT: Body weight test; CAP: Cholinergic anti-inflammatory pathway; CNS: Central nervous system; CUMS: Chronic unpredictable mild stress; HAMD: Hamilton Depression scale; HPA axis: Hypothalamo-Pituitary-Adrenocortical Axis; IL-18: Interleukin-18; IL-1 β : Interleukin-1 β ; NLRP3: Nod-like receptor protein 3; OFT: Open field test; $P2 \times 7R$: Purinergic ligand-gated ion channel 7 receptor; SPF: Specific pathogen free; SPT: Sucrose preference test; taVNS: Transcutaneous auricular vagus nerve stimulation; tnVNS: Transcutaneous auricular non-vagus nerve stimulation; VN: Vagus nerve;

Declarations

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Availability of data and materials

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

Fundraising: PJR and YW; study design: PJR and YW; study performance: YW, SYL, XH, YZ, XG, BZ, JLZ, LWH and JYW; data analysis and paper writing: YW; manuscript modification: PJR, HM and RHW. All authors read and approved the final manuscript.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this manuscript.

Consent for publication

Not applicable.

Ethical approval

All experimental procedures were approved by the animal Care and Use Committee of Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (Permit No. D2019-02-11-1).

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Figures

Figure 1

Experimental procedures. CUMS, chronic unpredicted mild stress; taVNS, transcutaneous auricular vagus nerve stimulation; tnVNS, transcutaneous auricular non-vagus nerve stimulation.

Figure 2

Intervention of taVNS/tnVNS. The taVNS targets the skin receptive area are located in the auricular concha with ABVN distribution. The tnVNS targets the skin receptive area are located in the auricular margin with no ABVN distribution.

Figure 3

Differences showing the effects of stress/antidepressant treatments on depressive like behaviors in depression rats induced by CUMS. taVNS, transcutaneous auricular vagus nerve stimulation; tnVNS, transcutaneous auricular non-vagus nerve stimulation. (a) Results of two-way ANOVA with time points and groups of changes in body weight were as follows: time points, $F=1625.631$, $P=0.000$; groups, $F=198.626$, $P=0.000$; interaction, $F=19.606$, $P=0.000$; $n=8$ per group. (b) Results of two-way ANOVA with time points and groups of changes in number of crossings were as follows: time points, $F=145.834$, $P=0.000$; groups, $F=254.304$, $P=0.000$; interaction, $F=23.402$, $P=0.000$; $n=8$ per group. (c) Results of two-way ANOVA with time points and groups of changes in number of rearing were as follows: time points, $F=91.951$, $P=0.000$; groups, $F=158.558$, $P=0.000$; interaction, $F=11.024$, $P=0.000$; $n=8$ per group. (d) Results of two-way ANOVA with time points and groups of changes in sucrose preference were as follows: time points, $F=34.547$, $P=0.000$; groups, $F=75.690$, $P=0.000$; interaction, $F=5.764$, $P=0.000$; $n=8$ per group. Results are expressed as the mean $\pm$ SD. * $P<0.05$ compared with the C group; ** $P<0.01$

compared with the C group; ▲▲P<0.01 compared with the M group; ☐P<0.05 compared with the TN group; ☐☐P<0.01 compared with the TN group.

Figure 4

Representative images of immunofluorescence for P2X7R (green) and Iba1 (red) in the hippocampus of rats. Nuclei were counterstained with DAPI (blue). Selected white boxes were zoomed in right panels. Arrowhead indicates representative colocalization of P2X7R and Iba1. Scale bar, 50 μm and 20 μm (zoomed images).

Figure 5

Differences showing the effects of stress/antidepressant treatments on the expression of P2X7R, NLRP3, caspase-1, IL-1β and IL-18 in hippocampus of depression rats induced by CUMS. (a) P2X7R (n = 6 per group) using western blot analysis; (b) NLRP3 (n = 6 per group) using western blot analysis; (c) Caspase-1 (n = 6 per group) using western blot analysis; (d) IL-1β (n = 6 per group) using western blot analysis; (e) IL-18 (n = 6 per group) using western blot analysis. Results are expressed as the mean ± SD. *P<0.05

compared with the C group; ** $P < 0.01$ compared with the C group; ▲ $P < 0.05$ compared with the M group; ▲▲ $P < 0.01$ compared with the M group.

Figure 6

The antidepressant-like effect of taVNS may be related to the regulation of the initiation signal P2X7R of neuroinflammation. The high levels of ATP released from neurons or astrocytes reach P2X7R located in microglia. P2X7R stimulation elicits K⁺ efflux and triggers the assembly and activation of NLRP3 inflammatory. Activated NLRP3 inflammatory mediates the activation of caspase-1, while caspase-1 in

turn stimulates the production of IL-1 β and IL-18. In this pathway, taVNS/tnVNS may regulate its upstream signal P2X7R and play an antidepressant role.