Transcutaneous auricular vagal nerve stimulation alleviated the development of peripheral and central symptoms of systemic lupus erythematosus in MRL/lpr mice

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Research Article

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Abstract

Objective.

The objective of this study was to investigate the effects of transcutaneous auricular vagus nerve stimulation (taVNS) on the development of peripheral and central symptoms of systemic lupus erythematosus (SLE) in MRL/lpr mice.

Methods.

MRL/lpr mice were treated with taVNS for ten weeks, and the severity of both the central and peripheric symptoms was assessed.

Results.

taVNS activated tyrosine hydroxylase positive (TH\(^+\)) neurons in the locus coeruleus (LC), improved cognitive impairment, and alleviated depression-like behaviors. taVNS reduced the number of hippocampal microglia and protected the LC TH\(^+\) neurons in MRL/lpr mice. Treatment with taVNS also retarded the development of lymphadenectasis and splenomegaly, decreased the proportion of double-negative T (DNT) cells, as well as alleviated nephritis and renal IgG deposition in MRL/lpr mice. However, when the LC TH\(^+\) neurons were selectively lesioned, both peripheral and central therapeutic action of taVNS was eliminated. Finally, the results of liquid chromatography-tandem mass spectrometry (LC-MS) indicated taVNS mainly LC-dependently reduced the concentration of norepinephrine and adrenaline in serum.

Conclusion.

This study provides direct evidence that taVNS can retard the development of peripheral and central symptoms of SLE, which may be related to its modulating the activity of LC TH\(^+\) neurons. Our findings suggest that taVNS may be a potential non-invasive treatment approach for SLE, although further research is needed to explore its clinical applications in human patients.

1. Introduction

Systemic lupus erythematosus (SLE) is an inflammatory autoimmune disease with multiorgan involvement, which predominantly affects women of reproductive ages [1, 2]. The incidence of SLE is 43.7 (15.87 to 108.92) per 100 000 persons, and the mortality of patients with SLE is higher than that of the general population [3]. Treatment options for SLE remain inadequate. Despite well-recognized adverse
effects (such as osteoporosis, and cardiovascular disease), corticosteroids are still widely used for SLE treatment [4]. Thus, it is still necessary and urgent to development of novel therapies for SLE.

The use of non-pharmacological means to treat autoimmune diseases has recently spiked the interest of investigators, and vagus nerve stimulation (VNS) has shown one of the most promising [5]. Transcutaneous auricular vagus nerve stimulation (taVNS) is a non-invasive kind of VNS approach with the advantages of being low-cost and portable. The taVNS may modulate the immune system via the neuro-immune interaction and has been a therapy of interest for treating autoimmune diseases [6–8]. Recently, Aranow et al. reported a randomized, double-blind, sham-controlled pilot trial that taVNS reduces pain and fatigue in patients with SLE [9]. However, the effects of taVNS on SLE, including its development, remain ambiguous and vague.

MRL/lpr mice spontaneously develop an autoimmune disease that closely resembles human SLE with autoantibodies and multiple systematic manifestations (such as lupus nephritis and neuropsychiatric symptoms) [10, 11]. Here, we tested the effects of taVNS in MRL/lpr mice, and the preliminary mechanism was also investigated.

2. Methods

2.1 Animals

Female MRL/lpr mice (8 weeks old, 25 ± 3 g body weight) were purchased from and housed in the Laboratory Animal Research Center of Zhejiang Chinese Medical University at 23 ± 2 °C and 40–60% humidity with a 12-h light-dark cycle (lights on from 8:00 to 20:00). To study the effect of taVNS on lupus mice, the animals were randomly divided into six groups: taVNS-treated group (receiving electrical stimulation) (n = 9) and controls (n = 9), taVNS-treated group (n = 6) and their controls following LC lesion (n = 6), sympathetic denervation mice (n = 6) and controls (n = 6), forty-two mice in total. The sample size was chosen empirically, based on common experience in the relevant fields, to provide a sufficient level of statistical power for detecting indicated biological effects. In LC lesion + taVNS group, one animal was dead midway through the experiment. All procedures and protocols were approved by the Animal Advisory Committee of Zhejiang Chinese Medical University. All experiments were performed in accordance with the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2 Stimulation parameters of taVNS

The stimulation was delivered with an isolated pulse stimulator and the parameters were based on previous research [12]: 1 mA, 20 pulses/second, 30 s ON and 4.5 min OFF, total length of 30 min, with 330 µs pulse width. When the mice were in eight weeks, the taVNS group received transcutaneous auricular vagal nerve stimulation every other day. During the stimulation, mice were anesthetized by using 3% isoflurane in an induction chamber and maintaining under 1.5% isoflurane via a nose-cone delivery system on a heated table. As for stimulation, electrodes were placed in the concha of the left ear as the
right branch of the vagus nerve innervates the sinoatrial node and may adversely affect heart rate [13, 14]. For the control mice were anesthetized, but no electrical stimulation was delivered.

### 2.3 In vivo electrophysiological recording

Neuronal activity recording was performed as previously described [15]. During surgery, electrodes were implanted into the LC (ML, −5.45 mm; AP, + 0.95 mm from bregma; DV, −3.60 mm from the brain surface). Recordings were made using a bundle of microelectrodes with 6 wires (25 µm, AM-Systems) with impedances of 1–2 MΩ as measured using an Omega-Tip-Z (World Precision Instruments, Inc.). Neuronal activity was sampled using a Cerebus acquisition system (Blackrock Microsystems; sampling rate 30 kHz, high-pass filtered at 250 Hz, low-pass filtered at 7500 Hz, and sorted online) grounded to screws above the cerebellum and referenced against a wire within the same brain area. To improve the signal-to-noise ratio, an online 50-Hz line noise-cancelation algorithm was also applied. The recorded neuronal data were re-sorted offline using Plexon's Offline Sorter software (Plexon Inc). The mean frequency and interspike interval histograms were generated using Neuroexplorer 5.0 software (NEX Technologies Int. Inc)

### 2.4 Lymph node score of the mice

The lymph node score was evaluated according to a previous study [16], with some modifications. The score was scaled from 0 to 4 in neck, as follows: 0, no enlargement of lymph nodes; 1, an increase of at least one lymph node that is not visible but is felt a little by palpation; 2, an increase of at least one visible lymph node which felt by palpation easily; 3, visible lymph node enlargement and the area is less than 0.5 cm²; 4, extremely enlarged lymph nodes and the area is greater than 0.5 cm². Moreover, the score was ranged on a scale from 0 to 6 according to the number of lymph nodes in bilateral axilla and bilateral groin.

### 2.5 Behavioral tests

Mice were brought to the testing room to acclimatize to the testing environment one hour in advance. All the tests were automatically monitored by ANY-maze software (Stoelting, USA). All the mice were subjected to the behavioral tests as follows:

**2.5.1 Open-field test (OFT).** The OFT was performed similarly to that reported previously [17]. Test was conducted inside a black box (40 cm × 40 cm × 40 cm). Mice were allowed to explore the field freely for 15 min, and the total distance, time and distance traveled in the center area by mice were recorded. The field was dealt with 75% ethanol solution before each mouse was introduced.

**2.5.2 Novel object recognition test (NORT).** Based on the OFT, the field of NORT introduces two kinds of objects with different shapes. In adapting phase, mice were exposed to two identical objects in the test field for 5 min and then returned to their cages for 30 min. In the following 3 min testing phase, mice were returned to the test field and one of the objects was replaced by a new object [18]. The field was cleaned with solution of 75% ethanol after each test. The discrimination index (DI) (time spent exploring the novel object / time spent exploring two objects) was calculated in this experiment.
2.5.3 Y maze test. Y maze is composed of 3 identical arms (30 cm × 10 cm × 21 cm) made of black plastic at 120° angle to one another. Three arms were randomly designated as the start arm, the novel arm, and the other arm. According to previous experiment [19], the novel arm was closed with a partition, then the mice were placed into the start arm to explore the other arm freely for 5 min (adaptation phase). After 2 min interval, the mice were returned to explore the maze freely for 2 min, with all three arms open (test phase). At the end of each adaptation or test, the baffle and the bottom of the maze were wiped with 75% ethanol solution. The percentages of the number of entries and the time spent in the novel arm were calculated from the video recording.

2.5.4 Splash test. The splash test was performed according to Hu et al [20]. The 10% sucrose solution was sprayed onto the dorsal hair of the mice in the cage with a nebulizer. Sucrose solution dirtied hair and induced grooming behavior in mice. Then, we recorded the time spent grooming in the next 5 minutes as an indicator of self-care and motivational behavior. The percentage of time spent in grooming (grooming time / 5 min) was calculated.

2.5.5 Forced swimming test (FST). The FST was performed according to xu et al [21]. In brief, each mouse was placed into a glass cylinder (25 cm in height, 17 cm in diameter) filled with 15-cm-high water (25°C). The water was replaced after each trial. All animals were forced to swim for 6 min: the first 2 min was an adaption phase for recording the latency of immobility, and the duration of immobility was recorded during the latter 4 min. The immobility time was measured when the mice are floating in the water without struggling and only making movements necessary to keep their heads above the water.

2.6. Renal histopathology and IgG deposition

For histology, mouse kidneys were fixed in 4% Paraformaldehyde for 48h at 4°C, and 5 mm paraffin sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). Using VS120-S6-W (OLYMPUS) and xvViewer software to obtain the images of stained kidney sections. Glomerular lesions were graded on a scale of 0–3 in ten glomeruli from the H&E-stained cross-sections per mouse and additionally count the number of cells in ten glomeruli per mouse [22, 23]. To quantify the glomerular mesangial matrix area from the PAS-stained, Image-Pro Plus 6.0 software was used to measure the area of the glomerular tuft and quantify the PAS-positive area within the tuft [24], calculating the percentage of PAS-positive tuft area (PTA) to the total glomerular tuft area (GTA). IgG deposition in glomeruli was assessed by immunofluorescent staining, the mean fluorescence intensity (MFI) of each glomerulus was calculated using the Image-Pro Plus 6.0 software and the MFI of five glomeruli was averaged [23].

2.7. Flow cytometry analysis

Fresh splenic tissue was taken from a subset of animals, mashing the spleen through 70 µM cell strainer in RPMI buffer to obtain the single-cell suspension of the spleen. After centrifugation, discarding the supernatant slowly, Red Blood Cell Lysis Buffer was added and mixed to lyse the red blood cells. 5 minutes later, an equal volume of 1640 is added to the centrifugal tube for terminating lysis. Centrifugating and discarding the supernatant again, cells were then resuspended with RPMI buffer and counted. According to the number of cells, the concentration of cells was diluted about 10^7 cells/ml.
Splenocytes were stained with different cocktails of antibodies for 30 min at 4°C to study immune populations of T cells. The fluorochrome-conjugated antibodies used for surface staining were anti-mouse CD3 FITC (MULTISCIENCES), anti-mouse CD4 PE (MULTISCIENCES), anti-mouse CD8 APC (MULTISCIENCES). Data were acquired through FACS analysis using the FC 500 MC system (Beckman Coulter, Fullerton, CA, USA) and analyzed by FlowJo version 10 software (Tree Star, Inc, Ashland, OR).

2.8. Immunofluorescence staining

Mice were euthanized with an overdose of 1% pentobarbital sodium and perfused with icy normal saline and 4% paraformaldehyde. The brains were taken and soaked in 4% paraformaldehyde for 48 h, cryoprotected in 20% sucrose solution for another 24 h and 30% sucrose solution for another 48 h. The brains were sliced into 18 µm sections and were mounted on adhesion microscope slides (CITOTEST). The sections were incubated with the following antibodies at 4°C overnight: anti-cFos (1:400, Abcam; #ab190289), anti-cFos (1:400, Abcam; #ab208942), anti-Tyrosine Hydroxylase and (1:500, Abcam; ab6211) anti Iba1 (1:500, Wako; #019-19741). Then, the sections were thoroughly washed and incubated at 37°C for 30 min with an Alexa Fluor 488-conjugated antibody (1:200, Abcam; #ab150077) or Alexa Fluor 647-conjugated antibody (1:200, Abcam; #ab150115). After washing, the cell nuclei were counterstained with DAPI (Meilunbio; #MA0222-L). The sections were observed using an Olympus VS120-S6-W microscope.

2.9. Stereotaxic surgery

The administration of 6-hydroxydopamine (6-OHDA (Sigma-Aldrich), a neurotoxin that specifically denervates catecholaminergic neurons) was performed according to previously published work [25]. Mice were anaesthetized in an induction chamber (3% isoflurane) and placed in a stereotaxic frame (RWD). After skull was exposed 6-OHDA (5 µg/µl) injected bilaterally into the locus coeruleus (LC) at the following stereotaxic coordinates: LC, −5.45 mm from bregma, 0.95 mm lateral from midline, and −3.60 mm vertical from cortical surface, at a rate of 0.13 µl/min over 8 min. The needle was left in place for an additional 4 min after the injection and then withdrawn the needle slowly. Animals were sutured, removed from the stereotaxic apparatus to incubator and allowed to recover.

2.10. Quantification of neurotransmitters in serum by liquid chromatography-tandem mass spectrometry (LC-MS)

Mice serum was prepared for neurotransmitter detection. For LC-MS, 100 µL of acetonitrile was added to 50 µL serum and 50 µL of internal standard, followed by vortexing for a minute. The mixture was centrifuged at 4 °C, at 12000 rpm/min for 10 min for protein precipitation. The supernatant was collected and dried using a Vacuum centrifugal concentrator until dryness was achieved. The samples were resuspended in 100 µL double distilled water and centrifuged at 4 °C, at 12000 rpm/min for 10 min. 80 µL supernatant was transferred to LC-MS vials and ready for analysis.

2.11. Sympathetic denervation
6-OHDA (Sigma-Aldrich) was dissolved in sterile saline containing 0.01% ascorbate (Meilunbio). In 8 weeks old MRL/lpr mice, 100 mg/kg 6-OHDA was administered by intraperitoneal injection in day 1, day 3 and day 5; 150 mg/kg 6-OHDA was administered in day 7.

2.12. Statistics

Mice in which injection was not successful or dead midway through the experiment were excluded from analysis and each criterion was pre-established. Prism 8 (GraphPad Software) was used for statistical analyses. Two-way analysis of variance (ANOVA) was used to analyze the development of lymph node scores. Unpaired Student's t-test was used for other data with homogeneity of variance, while the Mann-Whitney test was used for ranked data or data with a heterogeneity of variance. All data were expressed as mean ± SEM; significance was defined as p < 0.05. All behavioral tests and renal histopathology were conducted in a blinded manner.

3. Results

3.1. Brain nuclei activated by acute taVNS in 8-week-old MRL/lpr mice

To investigate the brain areas that were activated by the taVNS, the expression of cFos, a classic neurons activity maker, was stained (Fig. 1A) and the stimulation sites of our taVNS were shown in Fig. 1B. We found that the number of cFos positive cells in paracochlear glial substance (PCGS, Figures S1B and S1G), lateral cerebellar nucleus (LCN, Figures S1C and S1G), LC (Figures S1D and S1G), paraventricular hypothalamic nucleus (PVN, Figures S1E and S1G) and dorsomedial hypothalamic nucleus (DMH, Figures S1F and S1G) were increased in the taVNS group when compared with control. According to the previous study, LC was one of potential key targets of taVNS, considered proof of vagal activity [26]. Since the LC is the major site of norepinephrine production in the brain, thus, we evaluated the efficiency of our stimulus pattern via the number of activations of tyrosine hydroxylase-positive (TH⁺) neurons in LC. We found 30–70% TH⁺ neurons were activated significantly after taVNS (Figs. 1C and 1D). To further confirm the activation of LC in response to the stimulus pattern, a bundle of microelectrodes consisting of 6 wires was implanted into the LC of MRL/lpr mice for recording neuronal activity (Fig. 1E). Notably, compared to baseline, taVNS increased the neural activities in the LC (Figs. 1F – 1H). Thus, these results support that our stimulus pattern is sufficient to modulate the brain network, especially in the LC.

3.2. taVNS alleviated the neuropsychiatric SLE (NPSLE)-like symptoms in MRL/lpr mice

Apart from peripheral symptoms of SLE, MRL/lpr mice also develop NPSLE-like symptoms [27]. To evaluate the effects of taVNS on the NPSLE-like symptoms of MRL/lpr mice, we performed the behavioral tests at the age of 16 weeks in MRL/lpr mice (Fig. 2A). In OFT (Fig. 2D), the total distance (Fig. 2B) and the percentage of central distance (Fig. 2C) between the controls and taVNS groups were comparable,
while taVNS benefited the cognitive performance in Y-maze (Fig. 2E) and NORT (Fig. 2F). Moreover, taVNS also relieved the depression-like behaviors evidenced by the increase of time of grooming in the slash test (Fig. 2G) and the decrease of immobility time in FST (Fig. 2H). Microglia are involved in many central nervous system (CNS) disorders by increasing phagocytic activity and inflammatory cytokine production [28]. It has been found that the activation of microglia was related to the NPSLE in MRL/lpr mice [29]. Here we further found taVNS reduced the number of microglia in the hippocampus of MRL/lpr mice (Fig. 2I). Previous studies demonstrated that the expression of TH in LC was significantly lower in MRL/lpr mice [30]. To determine whether the taVNS relieved this impairment, we observed the expression of TH in the two groups of mice, found the number of TH⁺ cells were significantly higher in the mice that received taVNS (Fig. 2J). Thus, these results indicate that taVNS alleviated the cognitive disorder and depression-like behaviors in MRL/lpr mice via the reduction of microglia in the hippocampus and the protection to TH⁺ cells in LC.

3.3. taVNS alleviated the peripheral symptoms in MRL/lpr mice

Lymphadenectasis and splenomegaly are the main peripheral SLE symptoms in MRL/lpr mice [31, 32]. We found the taVNS delayed the development of lymphadenectasis (Fig. 3A) and reduced splenomegaly (Fig. 3B). Besides, glomerulonephritis is another important clinical manifestation of systemic inflammation in MRL / lpr mice and human SLE patients [33]. To clarify the impact of taVNS in lupus mice, we performed the histopathological analysis of kidneys from MRL/lpr mice (Figs. 3C – 3E). We found mice that received taVNS showed mild glomerulonephritis (Figure. 3F). Next, we calculated the mean number of nuclei in ten glomeruli to assess cell proliferation, we found that the taVNS-treated mice had fewer nuclei than the controls (Figure. 3G). Another index of glomerulonephritis is mesangial proliferation which shown by PAS staining. Compared with the controls, the percentage of PAS-positive tuft area in glomeruli had a decreasing trend in taVNS-treated mice (Figure. 3H). Besides, taVNS also alleviated renal IgG deposition (Figure; 3I). Taken together, these findings suggest that taVNS performs a protective effect against lupus nephritis in MRL/lpr mice.

3.4. taVNS reduced the population of DNT cells in MRL/lpr mice

It has been reported that the increased population of DNT cells was closely related to the disease severity in both MRL/lpr mice and SLE patients [34–36]. Here we analyzed different populations of CD3⁺ T cells by flow cytometry. As shown by Fig. 4A, after live gating, CD3⁺ T cells were then gated from total splenocytes, and then the population of different types of T cells was further analyzed. Compared to the controls, the taVNS group showed a decreasing tendency in splenic CD3⁺ T cells (Figure. 4B). The population of DNT cells was decreased in the taVNS group (Figure. 4C), while CD8⁺ T cells were increased in the taVNS group (Figure. 4D). The population of CD4⁺ T cells were comparable between the two groups (Figure. 3E).
3.5. The loss of LC neurons eliminates the protective effects of taVNS in MRL/lpr mice

To investigate whether the activation of LC contributed to ameliorating peripheral symptoms and neuropsychiatric symptoms in MRL/lpr mice, we used 6-OHDA to specifically deplete TH+ neurons of LC in young MRL/lpr mice (6 weeks of age) (Fig. 5A). Three weeks after administration 6-OHDA, the number of TH+ neurons in the LC were reduced significantly (Fig. 5B). In behavioral tests, the improvement of cognitive function and depression-like behaviors were also eliminated after administration of 6-OHDA (Figs. 5C – 5H). In addition, with a ten-week treatment, we found taVNS did not delayed the development of lymphadenectasis (Fig. 5I) and spleen index revealed no significant differences in splenomegaly between the groups (Fig. 5J). Moreover, renal pathology showed the lesion of LC prevented kidney protection from taVNS (Figs. 5K – 5Q). Besides, compared with Fig. 2, lesion of LC TH+ neurons seem did not significantly affect the disease development of MRL/lpr mice. Thus, we conclude that the LC TH+ neurons is necessary for the beneficial effects of taVNS on SLE.

3.6. taVNS may reduce the activity of the sympathetic nerves in MRL/lpr mice

In SLE, the increase of sympathetic activity and the decrease of parasympathetic activity have been extensively documented [37, 38], which may cause autonomic nerve system (ANS) dysfunction. Based on previous reports, the LC may increase descending inhibitory drive to sympathetic preganglionic neurons and then reduce the level of sympathetic activity [39, 40]. Besides, the activation of PVN may drive the excitement of the hypothalamic-pituitary-adrenal axis (HPA axis), which modulates peripheral inflammation by regulating glucocorticoids [41–43]. As LC and PVN were activated after taVNS, we hypothesize that taVNS may act by modulating the HPA axis or ANS. Thus, we tested the level of several neurotransmitters and corticosterone (CORT) in serum by LC-MS (Figs. 6A and B). Compared with the controls, we found taVNS reduced the level of norepinephrine (NE) (Fig. 6C) and adrenaline (AD) (Fig. 6D) but had no effect on acetylcholine (Ach) (Fig. 6E), dopamine (DA) (Fig. 6F) and CORT (Fig. 6G). Besides, these modulating effects of taVNS was eliminated while the LC neurons were lesioned (Figs. 6C - G). Interestingly, we observed that after locus coeruleus lesioned, administration of taVNS led to a decrease in the levels of Ach and DA in the serum. Thus, these results indicated that taVNS alleviated lupus-like syndrome in MRL/lpr mice via regulates the balance of the ANS. To further confirm whether the overactivity of sympathetic nerves aggravates disease progression, we injected MRL/lpr mice with 6-OHDA to denervate sympathetic nerves (Fig. 6H). We found sympathetic denervation reduced the lymph node score (Fig. 6I) and slightly reduced the spleen index (Fig. 6J). Moreover, sympathetic denervation also reduced the pathology score of lupus nephritis and its related glomerular cell proliferation (Figs. 6K - M).

4. Discussion
SLE is an inflammatory autoimmune disease affecting both peripheral and central organs. taVNS is a non-invasive kind of VNS approach for autoimmune diseases. This study mainly found that: (1) taVNS with our stimulus parameters activated several brain areas, especially the TH⁺ neurons in LC; (2) Treatment of taVNS retarded the development of lymphadenectasis and splenomegaly, decreased the proportion of double-negative T cells, as well as alleviated nephritis and renal IgG deposition in MRL/lpr mice; (3) Treatment of taVNS also improved cognitive impairment, alleviated depression-like behaviors, and reduced the number of hippocampal microglia, protected the TH⁺ neurons of locus LC in MRL/lpr mice; (4) The selective lesion of LC TH⁺ neurons eliminates the protective effects of taVNS in MRL/lpr mice. (5) Treatment of taVNS alleviated lupus-like syndrome in MRL/lpr mice via regulates the balance of the ANS. Thus, our study provides direct evidence that taVNS retarded the development of peripheral and central symptoms of SLE, which may be related to modulating the activity of TH⁺ neurons in LC.

SLE is a chronic autoimmune disease that affects many parts of the body. Lymphadenectasis and splenomegaly are ubiquitous in SLE patients [31, 32]. Here we found taVNS retarded the development of lymphadenectasis and splenomegaly. In spleen, DNT cells are substantially increased in SLE patients and in murine models of the disease [34–36], and we found taVNS decreased the proportion of DNT cells. In previous study, via splenic nerve denervation, spleen has been proved to be the key segment mediate kidney protection by whole nerve electrical VNS or afferent VNS [44], our results further explained this mechanism. Furthermore, nephritis and renal IgG deposition are frequent and severe manifestations in SLE, we found taVNS alleviated nephritis and renal IgG deposition in MRL/lpr mice as well. Of note, a recent clinical study showed that taVNS relieves pain and fatigue, reduced the plasma levels of substance P (a proinflammatory neuropeptide) [9]. This study partly supported our results, indicated taVNS have therapeutic potential to SLE symptoms.

NPSLE, including depression and cognitive function, affects 40–90% of SLE patients [29]. The previous studies showed the pathomechanism of NPSLE was different from systemic disease in SLE. For example, despite transferring bone marrow from healthy control mice to MRL/lpr mice, Stock et al. [45] found the recipient mice still had a behavioral phenotype consisting of depressive-like behavior and visuospatial memory deficits. Wen et al. [46] found central features of NPSLE including increased blood-brain barrier permeability, brain cell apoptosis, and upregulated cytokine expression persisted in B cell-deficient and B cell-depleted MRL/lpr mice. Here we found the treatment of taVNS also improved cognitive impairment and alleviated depression-like behaviors, suggesting taVNS induces a different pathway that targets NPSLE while improving inflammation and autoimmune reactions. It has been reported that microglia activation may play an important role in the development of neuropsychiatric symptoms [47, 48]. Here we found the treatment of taVNS reduced the number of hippocampal microglia in MRL/lpr mice. Previous studies have shown that taVNS can significantly decrease the number of activated microglia in hippocampal sections in the chronic unpredicted mild stress rat model [49], which is consistent with our studies. Thus, these results suggest taVNS may protect against neuropsychiatric symptoms by reducing the number of hippocampal microglia.
The mechanism of taVNS is complex and it remains unclear how these therapeutic effects are mediated. Previous study has found that as the potential to alleviate depressive-like behavior in rats. This effect could be achieved by reducing the expression of NF-κB p65 and IL-1β in the hippocampus through the α7nAChR/NF-κB signaling pathway [49]. Additionally, taVNS has been shown to suppress peripheral inflammation through the activation of the cholinergic anti-inflammatory pathway [50–52], primarily mediated by the activation of the α7 nicotinic acetylcholine receptor (α7nAChR) [53]. By cFos staining, we found taVNS activated several brain areas, especially the LC TH⁺ neurons. LC mainly contains noradrenergic neurons and is related to vagal activity [54]. Noradrenaline has an important role in many psychiatric and neurodegenerative disorders and most of the noradrenaline released in the brain is supplied by LC [55, 56]. Besides, LC neurons loss is an early feature of depression and neurodegenerative diseases [57, 58], which may also exist in MRL/lpr mice [30]. In current study, we found taVNS protected TH⁺ neurons in LC, indicating that the therapeutic effects of taVNS may be related to its activation of LC noradrenergic cells. To confirm the necessity of LC activation for the therapeutic effects of taVNS, we injected 6-OHDA to selectively damage LC TH⁺ neurons in the young MRL/lpr mice. As expected, we found that the therapeutic effects, both central and peripheric, of taVNS were eliminate in these LC lesion mice. As the HPA axis and ANS are the two main pathways for brain modulating the immune system, we preliminarily further investigate whether taVNS affected these ANS-related neurotransmitters and HPA axis-related CORT in serum. We found that taVNS reduced the level of NE and AD but had no effect on Ach, DA, and CORT in serum. As NE and AD are two neurotransmitters which were correlated with sympathetic activity [59, 60], our data indicate taVNS may suppress the hyperactivity of sympathetic activity in SLE. These preliminary results suggest the therapeutic effects of taVNS on SLE may be mediated by the improvement of ANS dysfunction rather than the activation of the HPA axis. However, the underlying neuronal circuit mechanism of taVNS remains waiting to be studied, such as the neural projection between PVN and LC during taVNS [61] as well as the role of PCGS, LCN and DMH.

In summary, this study found the treatment of taVNS activated the neurons in the LC, and it relieved peripheral as well as central symptoms of SLE in MRL/lpr mice. LC-MS analysis further showed that taVNS reduced the levels of NE and AD in the serum. Selective lesion of LC TH⁺ neurons reversed these above-mentioned effects of taVNS. Together, our study indicates taVNS as a promising new method to treat SLE, which is related to activating LC TH⁺ neurons and improving the ANS. Further studies are still needed to define the relationship among taVNS, ANS and immune system in SLE.

Abbreviations
Declarations

Author Contributions

Z.X., C.W. and H.Lv. designed the study. H.Lv., Y.L., K.W., A.X. and M.L. performed the experiments and collected samples. Z.X., P.W. and H.Lv. analyzed the data. H.Lv. and X.Y. performed electrophysiological recording. H.Lv. and Y.L. performed behavior tests. H.Lv. and M.L. performed immunofluorescence staining. H.Lv. and P.W. performed renal pathological analysis. Z.X. and H.Lv. wrote the manuscript draft and did the manuscript revision in consultation with H.Lu.

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Availability of data and materials

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

Ethics approval and consent to participate

All experimental procedures were conducted in accordance with the Ethics Committee of Chongqing Medical University.

Consent for publication

Not applicable.

Competing interests

The author(s) declare that there is no conflict of interest.

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**Figures**
Figure 1

Brain nuclei activated by acute taVNS in 8-week-old MRL/lpr mice. (A) Timeline of cFos staining experiment. (B) Sites of electrostimulation in the auricle. (C) Percentage of cFos positive cells in TH+ neurons in LC of taVNS-treated mice and their controls (control, n = 5; taVNS, n = 5). (D) Representative immunofluorescence images of TH (green), cFos (pink), and DAPI (blue) in the LC obtained from taVNS-treated mice and their controls. (E) Schematic of in vivo recording in LC before and after taVNS. (F)
Example traces and averaged spike waveform of a recorded LC neuron before and after taVNS. (G) Mean frequency of LC neurons before and after taVNS. (H) Inter-spike intervals (IRI) of LC neurons before and after taVNS. *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student’s t-test was used for C; paired t-test was used for G and H.

Figure 2
taVNS alleviated the neuropsychiatric SLE-like symptoms in MRL/1pr mice.

(A) Timeline and sequence of behavior test. (B) Result of total distance in open field test of taVNS-treated mice and their controls (control, n = 9; taVNS, n = 9). (C) Result of the percentage of central distance in open field test of taVNS-treated mice and their controls (control, n = 9; taVNS, n = 9). (D) Example trajectories of taVNS-treated mice and their controls. (E) Diagram of the Y-maze (left) and result of the percentage of time in the novel arm in Y maze of taVNS-treated mice and their controls (right) (control, n = 9; taVNS, n = 9). (F) Diagram of the novel object recognition test (left) and result of the percentage of time in the novel object in novel object recognition test of taVNS-treated mice and their controls (right) (control, n = 9; taVNS, n = 9). (G) Diagram of the splash test (left) and result of the time of grooming behavior following a 10% sucrose solution spray in the splash test of taVNS-treated mice and their controls (right) (control, n = 6; taVNS, n = 7). (H) Diagram of the forced swim test (left) and result of the immobility time in the forced swim test of taVNS-treated mice and their controls (right) (control, n = 7; taVNS, n = 9). (I) Representative immunofluorescence images of microglia (left) and the number of microglia per 0.1mm$^2$ (right) in hippocampus obtained from taVNS-treated mice and their controls (control, n = 4; taVNS, n = 4). (J) Representative immunofluorescence images of TH (left) and the number of TH cells per 10000μm$^2$ (right) in LC obtained from taVNS-treated mice and their controls (control, n = 3; taVNS, n = 3). *p < 0.05, **p < 0.01; Mann-Whitney U test was used for C, unpaired Student’s t-test was used for B, and E– J.
Figure 3

**taVNS alleviated the peripheral symptoms in MRL/lpr mice**

(A) Representative images of 17-week-old MRL/lpr mice from two groups and change in lymph node score obtained from taVNS-treated mice and their controls from week 8 to week 17 (control, n = 10; taVNS, n = 9). (B) Representative images of spleen (left) and spleen index (right) obtained from taVNS-treated mice and their controls (control, n = 9; taVNS, n = 9). (C – E) Representative hematoxylin–eosin (HE) staining (C), periodic acid–Schiff (PAS) staining (D), and IgG immunofluorescence staining (E) of kidney sections. (F – I) Pathology score (F), nuclear/glomerulus count (G), normalized PTA/GTA (H), and normalized fluorescence intensity (I) of taVNS-treated mice compared to controls.
staining (D) and IgG immunofluorescence (E) images of kidney glomeruli obtained from taVNS-treated mice and their controls. (F) Pathology severity score of taVNS-treated mice and their controls (control, n = 4; taVNS, n = 4). (G) Glomerular cell proliferation (represented by the number of nuclei per glomerulus) of taVNS-treated mice and their controls (control, n = 4; taVNS, n = 4). (H) The level of mesangium expansion (the ratio of PAS-positive tuft area to the total glomerular tuft area) of taVNS-treated mice and their controls (control, n = 4; taVNS, n = 4). (I) Relative MFI of the IgG deposited in glomeruli of taVNS-treated mice and their controls (control, n = 4; taVNS, n = 4). *p < 0.05, **p < 0.01; two-way analysis of variance (ANOVA) was used for A; unpaired Student’s t-test was used for B and F–I.

Figure 4

taVNS reduced the population of DNT cells in MRL/lpr mice. (A) Representative flow cytometry plot demonstrating the percentage of CD3+ , CD4+ and CD8+ population in the spleen obtained from taVNS-treated mice and their controls. (B) Percentage change of CD3+ T cells in spleen of taVNS-treated mice and their controls (control, n = 6; taVNS, n = 6). (C) Percentage change of DNT cells in spleen of taVNS-treated mice and their controls (control, n = 6; taVNS, n = 6). (D) Percentage change of CD4+CD8+CD3+ T cells in spleen of taVNS-treated mice and their controls (control, n = 6; taVNS, n = 6). (E) Percentage change of CD4+CD8+CD3+ T cells in spleen of taVNS-treated mice and their controls (control, n = 6; taVNS,
The loss of LC neurons eliminates the protective effects of taVNS in MRL/lpr mice (A) Diagram and timeline of LC injection of 6-OHDA. (B) Representative immunofluorescence images of TH (left) and the
number of TH+ cells per 0.1mm² (right) in LC obtained from 6-OHDA treated mice and their controls (control, n = 4; 6-OHDA, n = 4). (C – D) Results of total distance (C) and the percentage of central distance (D) in open field test of taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). (E) Result of the percentage of time in the novel arm in Y maze of taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). (F) Result of the percentage of time in the novel object in novel object recognition test of taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). (G) Result of the time of grooming behavior following a 10% sucrose solution spray in the splash test of taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). (H) Result of the immobility time in the forced swim test of taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). (I) Representative lymphadenectasis images in neck and change in lymph node score obtained from taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). (J) Representative image of spleen (left) and spleen weight (right) obtained from taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). (K) Representative HE staining images of kidney obtained from taVNS-treated mice and their controls following LC lesion. (L) Pathology severity score of taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). (M) Glomerular cell proliferation (represented by the number of nuclei per glomerulus) of taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). (N) Representative PAS staining images of kidney obtained from taVNS-treated mice and their controls following LC lesion. (O) The level of mesangium expansion (the ratio of PAS-positive tuft area to the total glomerular tuft area) of taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). (P) Representative IgG immunofluorescence images of kidney obtained from taVNS-treated mice and their controls following LC lesion. (Q) Relative MFI of the IgG deposited in glomeruli of taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). *p < 0.05, **p < 0.01, ***p < 0.001; two-way analysis of variance (ANOVA) was used for I; unpaired Student's t-test was used for B – H, J, L, M, O and Q.
**Figure 6**

**taVNS may reduce the activity of the sympathetic nervous in MRL/lpr mice.** (A) Schedule of experiments for investigating which pathway between HPA axis and ANS mediates the protective effect of taVNS against SLE. (C-G) The concentrations of Serum NE (C), AD (D), Ach (E), DA (F) and CORT (G) in taVNS-treated mice and their controls (control, n = 6; taVNS, n = 6), as well as taVNS-treated mice and their controls following LC lesion (LC lesion, n = 6; LC lesion + taVNS, n = 5). (H) Schedule of experiments for investigating

(I) Representative lymphadenectomy images (left) and lymph node score (right) obtained from denervated mice and their controls in 16-week-old (controls, n=5; denervated mice, n=5). (J)
Representative images of spleen (left) and spleen index (right) obtained from denervated mice and their controls (controls, n=5; denervated mice, n=5). (K) Representative HE staining images of kidney obtained from denervated mice and their controls (controls, n=5; denervated mice, n=5). (L) Pathology severity score of denervated mice and their controls (controls, n=5; denervated mice, n=5). (M) Glomerular cell proliferation (represented by the number of nuclei per glomerulus) of denervated mice and their controls (controls, n=5; denervated mice, n=5). *p < 0.05, **p < 0.01; unpaired Student’s t-test was used for C (Control vs. taVNS) – G (Control vs. taVNS), J and M; Welch’s t test was used for C (LC lesion vs. LC lesion + taVNS), G (LC lesion vs. LC lesion + taVNS), I and L.

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