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Vagus nerve stimulation improves locomotion and neuronal populations in a model of Parkinson's disease

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Abstract

Background—Parkinson's disease (PD) is a progressive, neurodegenerative disorder with no disease-modifying therapies, and symptomatic treatments are often limited by debilitating side effects. In PD, locus coeruleus noradrenergic (LC-NE) neurons degenerate prior to substantia nigra dopaminergic (SN-DA) neurons. Vagus nerve stimulation (VNS) activates LC neurons, and decreases pro-inflammatory markers, allowing improvement of LC targets, making it a potential PD therapeutic.

Objective—To assess therapeutic potential of VNS in a PD model.

Methods—To mimic the progression of PD degeneration, rats received a systemic injection of noradrenergic neurotoxin DSP-4, followed one week later by bilateral intrastratial injection of dopaminergic neurotoxin 6-hydroxydopamine. At this time, a subset of rats also had vagus cuffs implanted. After eleven days, rats received a precise VNS regimen twice a day for ten days, and locomotion was measured during each afternoon session. Immediately following final stimulation, rats were euthanized, and left dorsal striatum, bilateral SN and LC were sectioned for immunohistochemical detection of monoaminergic neurons (tyrosine hydroxylase, TH), α -synuclein, astrocytes (GFAP) and microglia (Iba-1).

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Results—VNS significantly increased locomotion of lesioned rats. VNS also resulted in increased expression of TH in striatum, SN, and LC; decreased SN α -synuclein expression; and decreased expression of glial markers in the SN and LC of lesioned rats. Additionally, saline-treated rats after VNS, had higher LC TH and lower SN Iba-1.

Conclusions—Our findings of increased locomotion, beneficial effects on LC-NE and SN-DA neurons, decreased α -synuclein density in SN TH-positive neurons, and neuroinflammation suggest VNS has potential as a novel PD therapeutic.

Keywords

Parkinson's disease; vagus nerve; dopamine; substantia nigra; locus coeruleus; norepinephrine

INTRODUCTION

Parkinson's disease (PD) is a progressive, neurodegenerative disorder characterized clinically by tremor, bradykinesia, rigidity, and non-motor symptoms [1]. The pathological process underlying PD begins in the peripheral nervous system, and progresses through the vagal nerve to the brainstem, before reaching the substantia nigra (SN) and cortex [2,3]. The loss of locus coeruleus noradrenergic (LC-NE) neurons is significant in PD, and occurs prior to the loss of SN dopaminergic (SN-DA) neurons [4,5]. Degeneration of these LC-NE neurons is implicated in the early cognitive dysfunction in PD and increases sensitization of SN-DA neurons [6,7], providing evidence for a link between LC-NE and SN-DA systems. One major neurodegenerative mechanism in PD is neuroinflammation, which is associated with increases in astrocytes and increased activation of microglia [8]. While it remains unknown when neuroinflammation begins in PD relative to degeneration, prolonged neuroinflammation can exacerbate neurodegeneration [9].

Existing treatment strategies for PD are purely symptomatic and incompletely address symptoms, often with intolerable side effects [10,11]. New strategies are vital to develop more effective treatments for PD. Therefore, here we study the effects of vagus nerve stimulation (VNS) on motor function, LC-NE and SN-DA systems, α -synuclein, and neuroinflammation in a rat model of PD. Rodent models of cerebral ischemia and depression show that VNS has beneficial effects on LC neurons, decreases both inflammation and oxidative stress, and increases neurotrophic factors [12,13,14]. Because dysfunction in these mechanisms is known to contribute to degeneration in PD, a multi-modal approach like VNS may be beneficial in PD patients, where pharmaceutical trials targeting one mechanism at a time have failed, including a meta-analysis examining the impact of NSAIDs on the risk of developing PD [15].

In rodents, LC-NE degeneration can be induced using the noradrenergic neurotoxin, N-Ethyl-N-(2-chloroethyl)-2-bromobenzylamine hydrochloride (DSP-4). DSP-4 is NE specific, and can readily cross the blood brain barrier [16]. While it has transient effects on peripheral NE, effects in the CNS are long-lasting [17]. NE depletion with DSP-4 potentiates memory deficits and motor symptoms in conjunction with DA lesions caused by 6-hydroxydopamine (6-OHDA) in both the striatum and the medial forebrain bundle [7,18,19]. A double lesion of LC and SN neurons can result in a greater loss of SN-DA neurons and motor function

than 6-OHDA alone, providing further evidence for a link between the two populations [7,18]. Thus, the use of DSP-4 with intrastriatal 6-OHDA in this model allows for better representation of the progression from lower brain stem to higher brain regions previously proposed by Braak et al (2003).

VNS is approved by the Food and Drug Administration for treatment-resistant forms of epilepsy (1997) and depression (2005) by modifying brain activity through the solitary nucleus (NTS) and its projections [20]. The vagus nerve is essential for parasympathetic nervous system function, and descending fibers innervate major internal organs including the GI tract, heart, lungs, and kidneys. Ascending fibers of the vagus nerve project to brainstem nuclei, including the NTS, which projects to the LC, and from LC to higher regions including the caudate/putamen and cortex. In rats, the right vagus is mostly efferent, while the left vagus is mostly afferent; thus, by targeting the left vagus as we are doing in the current study, effects are induced primarily in the brain rather than the periphery [21]. Chronic VNS in rats significantly increases firing rates of LC-NE neurons and increases NE levels in LC target regions [22,23,24]. In addition, increased NE induces neuroprotection in the hippocampus and cortical neurons [25,26,27]. While the effects of VNS on NE systems in non-PD models are well established, no published studies have looked at the effects of VNS on the SN-DA system and its relation to motor function. By using the more representative LC-NE lesion with the DAergic lesion in rats, the relationship between these neurotransmitter systems, as well as the therapeutic potential for VNS in PD, are more closely examined.

MATERIALS AND METHODS

Animals

Adult male Long Evans rats (200–225g, Charles River) were randomly divided into four treatment groups: saline non-VNS (n=8), saline VNS (n=5), lesion non-VNS (n=7), lesion VNS (n=6). These rats were housed in an AAALAC-accredited facility at the Medical University of South Carolina (MUSC), with two rats per cage until surgery, and single-housed post-surgery. The facility was kept at 20–22 °C with a 12 hour light:dark cycle, and food and water provided ad libitum. All procedures were approved by MUSC's Institutional Animal Care and Use Committee.

Surgical Procedures

A timeline of experimental design for these experiments is depicted in Figure 1. To induce NE lesions in the LC, rats were injected with DSP-4 (50mg/kg, i.p., Sigma, St Louis, MO) or with sterile saline (0.9% NaCl, i.p., Hospira, Lake Forest, IL, Figure 1). Seven days later, rats were deeply anesthetized using chloral hydrate (400mg/kg, i.p.) and administered bupivacaine at incision sites (1mg/kg, s.c.; Hospira). VNS cuff placement was conducted as previously described [28,29]. Briefly, an incision was made on top of the skull and in the left ventral cervical region for VNS animals only. Muscle groups were bluntly dissected, the left cervical vagus nerve was isolated, and a bipolar platinum-iridium cuff electrode (impedance <10 kOhms) was implanted around the isolated nerve. Leads from the cuff were tunneled subcutaneously behind the ear through the head incision, and the neck incision sutured. Both

non-VNS and VNS rats were then placed in a stereotaxic frame (Stoelting, Wood Dale, IL), and four burr holes made in the skull above the striatum (two per hemisphere) using a Dremel (Dremel, Racine, WI) at the following coordinates: Hole 1: AP:+1.6, ML:±2.4, DV: -4.2; Hole 2: AP:+0.2, ML:±3.7, DV:-5.0 [30]. For lesion rats, 6-OHDA (5µg/µl; 2µl/site, containing sterile saline and 0.02% ascorbate, Sigma) was injected into each striatal site via Hamilton syringe [19] (SGE, Melbourne, Australia), while saline-treated rats received sterile saline (2µL/site). After each injection, the needle was left in place for 5 minutes before retracting slowly. Leads from the vagus cuff in VNS rats were then coupled to a two-channel connector headcap. Four sterile bone screws were screwed into the skull and covered with acrylic (Lang Dental, Wheeling, IL) to secure the headcap.

VNS Stimulation

VNS was administered to freely-moving, unanesthetized rats in a locomotor box beginning eleven days post-surgery (to allow for 6-OHDA lesion development) after confirming successful lesion via locomotor assessment. Each rat's headcap was plugged into the stimulator (A-M System, Carlsborg, WA) and received two thirty-minute sessions of VNS per day for ten days, with daily sessions separated by 4 hours. Stimulation parameters delivered a 500ms train of 15 biphasic pulses at 30Hz every 30 seconds. Each pulse gave 0.8mA of current and lasted 100 µs. The time course and stimulation parameters were chosen based on previous studies that both demonstrated sufficient timing to produce therapeutic effects [28,31] and confirmed successful stimulation using neuronal spiking synchrony and electroencephalographic measures during stimulation [29,32]. Non-VNS rats were also placed in locomotor boxes for two thirty-minute sessions each day.

Locomotor Activity

Locomotor activity (total distance traveled) was measured each stimulation day during the afternoon session in a darkened environment using Digiscan photobeam chambers (Omnitech, Columbus, OH) [33]. Beam breaks were recorded every five minutes and reported in centimeters throughout the thirty-minute session, and the total distance traveled across the ten stimulation days was averaged for each animal to obtain an individual mean distance traveled in a blinded version of the data set.

Brain Preparation

Immediately following the afternoon locomotor session on day ten, rats were deeply anesthetized using isoflurane (Piramal Healthcare, Andhra Pradesh, India) and decapitated. The left striatum, bilateral SN, and bilateral LC were blocked and preserved in 4% paraformaldehyde for 48 hours before transferring to 30% sucrose for at least 48 hours. These blocks were frozen in OCT (Tissue-Tek, Torrance, CA) and sectioned on a cryostat (Microm, Walldorf, Germany) at 45µm for immunohistochemistry.

Immunohistochemical Staining

Immunohistochemistry was performed in the LC, striatum, and SN using rabbit polyclonal antibodies for tyrosine hydroxylase (TH, 1:1000, Pel-freez, Rogers, AR), glial fibrillary acidic protein (GFAP, 1:2000, Dako, Carpinteria, CA), or Iba-1 (1:1000, Wako, Richmond,

VA). Briefly, primary antibodies were applied to serial sections from the LC (every 3rd section, TH, GFAP, or Iba-1), striatum (every 12th section, TH), or SN (every 6th section, TH, GFAP, or Iba-1) based on previous protocols [34,35]. Endogenous peroxidase was quenched by incubating sections in 10% peroxide and 20% methanol in Tris-buffered saline (TBS). Non-specific binding was blocked with a one-hour incubation in 10% normal goat serum, followed by primary antibody at room temperature overnight. The next day, sections were incubated in biotinylated goat anti-rabbit (1:200, Vector Laboratories, Burlingame, CA) secondary antibody for one hour, followed by another hour incubation in avidin-biotin complex (Vectastain ABC kit, Vector). For development, the VIP peroxidase substrate kit (Vector) enhanced the reaction and produced a color stain. This reaction was stopped using TBS, and sections were mounted on glass slides, dehydrated, and cover-slipped using Permount (Sigma).

Immunofluorescent Staining and Imaging

Immunofluorescence was performed on every 6th section in the SN using polyclonal antibodies for sheep TH (1:1000, Abcam, Cambridge, MA) and rabbit α -synuclein (1:250, Cell Signaling, Danvers, MA) as previously described [36]. Non-specific binding was blocked for one hour using 10% normal donkey serum in phosphate-buffered saline (PBS) + 0.3% Triton-X-100, followed by primary antibody incubation overnight. The next day, sections were incubated for one hour in secondary antibody, donkey-anti-rabbit-FITC (1:200) and donkey-anti-sheep-TRITC (1:50, Jackson ImmunoResearch, West Grove, PA), followed by 10 minutes in DAPI solution, then rinsed in PBS. Sections were mounted and coverslipped using ProLong Gold (Cell Signaling). Z-stack images of TH/DAPI were taken using a Zeiss LSM 880 Quasar inverted laser scanning confocal/multiphoton microscope with 63x/1.4NA planapochromat oil immersion objective using ZEN software (Zeiss, Thornwood, NY).

Semiquantitation of Staining Density

For analysis of the each of the immunohistochemical markers, the analyzer was blinded to treatment conditions.

Density measures of TH-immunoreactivity (TH-ir) in the striatum (outline: Figure 3E) and Iba-1-ir in the LC and SN (outline: Figure 6A,E, respectively), were conducted as previously described using a QImage R3 camera system (QImaging, Surrey, BC) with Image J software (NIH, Bethesda, MD) that measured optical density on a scale from 0 to 1.0 [33,35]. Measures were obtained by subtracting background from mean staining intensity using every 12th section through the striatum, every 3rd section through the LC, and every 6th section through the SN.

Using an Olympus Fluoview confocal setup, four sections through the SN were imaged to create TH/ α -synuclein Z-stacks for density analysis in ImageJ. By scrolling through the TRITC (TH) channel of each Z-stack, fifteen TH-positive cells were outlined (average area = 0.312 mm², sixty cells per animal), and density measurements were taken from the corresponding cells in the FITC (α -synuclein) channel to obtain an individual average for each animal. Data are reported as a percentage of saline non-VNS control rats.

Stereological Cell Counting

Quantitative estimates of the total number of TH-positive neurons in the LC and SN (outline: Figure 3A,I, respectively), as well as GFAP-positive astrocytes in the LC and SN (outline: Figure 5A,E, respectively) were achieved using an unbiased, stereological cell counting method [34,37]. Cell counts in the SN included the SN pars compacta, and excluded the SN pars reticulata and ventral tegmental area. Briefly, the optical fractionator system consisted of a computer-assisted image analysis system including a Nikon Eclipse E-600 microscope (Nikon, Tokyo, Japan) and stereological software (Stereoinvestigator, MicroBrightField, Colchester, VT). The LC and SN were outlined under low magnification (10x) on every 3rd and every 6th section, respectively, through the rostro-caudal extent of each region, and the outline was measured with a systematic random design of dissector counting frames (100×100µm). Cells were counted within each counting frame using a 20x objective lens. Blinded counts were done to determine an estimated population for each animal.

Statistical Analysis

Graphical data have been displayed as mean ± standard error of the mean (SEM). Data were analyzed using a 2 (Lesion) x 2 (Stimulation) analysis of variance (ANOVA) via GraphPad Prism (GraphPad Software, La Jolla, CA) followed by Newman-Keuls post-hoc analysis corrected for multiple comparisons to determine group-wise differences. Significance was set to $p < 0.05$. Pearson correlations were conducted to determine linear association between locomotor activity and each of the immunohistochemical measures in this study, and r and p values have been reported.

RESULTS

Locomotor Activity Behavior

The effect of VNS on locomotor activity (total distance traveled) was assessed during the afternoon stimulation sessions (Figure 2). Lesion non-VNS rats demonstrated a progressive decline in locomotor activity across days, while this effect was attenuated in lesion VNS rats. Additionally, lesion non-VNS rats consistently displayed decreased locomotion compared to saline non-VNS rats across all days (Figure 2A). A 2(Lesion) x 2(Stimulation) ANOVA comparing the average distance traveled revealed a significant effect of Lesion ($F(1,22)=21.93$, $p=0.0001$), but a significant effect of Stimulation did not exist ($F(1,22)=3.611$, $p=0.0706$, Figure 2B). Post-hoc analysis corrected for multiple comparisons using Newman-Keuls revealed decreased locomotion in lesion non-VNS rats compared to saline non-VNS rats ($p < 0.001$), as well as greater locomotion in lesion VNS rats compared to lesion non-VNS rats ($p < 0.05$), with no difference between the saline non-VNS and saline VNS rats. These data suggest that VNS improves motor function in animals that have impaired NE and DA systems.

Effects of VNS on LC-NE and SN-DA neurons

To assess the effects of LC-NE and SN-DA lesions, as well as VNS treatment, TH-ir was assessed in the dorsal striatum via density to examine DA terminals, and TH-positive neurons were counted via stereology in the LC and SN (Figure 3). 2(Lesion) x

2(Stimulation) ANOVA indicated an interaction of Lesion and Stimulation on TH-ir in the dorsal striatum, with main effects of Lesion and Stimulation on TH-ir in the striatum and on TH-positive cell counts in the LC and SN (Table 1). Newman-Keuls test showed lesion non-VNS rats had lower striatal TH-ir ($p<0.0001$), SN TH-pos cells ($p<0.001$), and LC TH-pos cells ($p<0.0001$) compared to saline non-VNS rats (Figure 3A,C,E,G,I,K,M–O). Lesion VNS rats had greater TH expression in the striatum and more TH-positive cells in both the LC and SN compared to lesion non-VNS rats ($p<0.01$, Figure 3C–D,G–H,K–L), with the number of TH-positive neurons in the LC and SN being comparable to those of saline non-VNS (Figure 3A,D,I,L). These data indicate VNS has beneficial effects on both LC-NE and SN-DA populations in this model. Pearson correlations determined that a positive relationship exists between TH and locomotion (Striatum: $r=0.714$, $p<0.0001$; SN: $r=0.536$, $p=0.0048$; LC: $r=0.574$, $p=0.0022$).

Effects of VNS on α -synuclein in the SN

DAPI nuclear staining in lesion non-VNS animals at this time point in lesion development indicated a loss of TH phenotype due to fewer nuclei associated with corresponding TH-positive neurons, rather than cell death (Figure 4A,C). Since α -synuclein is implicated in both overall function of the DA transporter and inclusion bodies of PD pathology, we wanted to determine if the induced lesion altered α -synuclein in DA neurons. To examine this, α -synuclein-ir density was measured within existing TH-positive cells in the SN (Figure 4). A 2(Lesion) x 2(Stimulation) ANOVA of α -synuclein-ir density in TH-positive cells revealed a significant effect of Stimulation ($F(1,16)=5.580$, $p=0.0312$). While there was not a significant Lesion effect, there was a trend towards significance ($F(1,16)=4.135$, $p=0.0586$). Newman-Keuls analysis revealed greater α -synuclein-ir in SN TH-positive neurons of lesion non-VNS rats compared to saline non-VNS rats ($p<0.05$, Figure 4E,G,I,G inset). α -synuclein-ir was lower in SN TH-positive neurons of lesion VNS rats compared to lesion non-VNS rats ($p<0.05$, Figure 4G,H,I,H inset), with no significant difference in α -synuclein expression after VNS in SN TH-positive neurons of saline rats (Figure 4E,F,I). These data indicate that VNS has the potential to regulate α -synuclein density in TH-positive neurons in this model. α -synuclein-ir in SN TH-positive neurons did not correlate with locomotion in this study. Effects of VNS on Glial Populations in LC and SN

To examine neuroinflammation, astrocytes in the LC and SN were labeled via GFAP (Figure 5). The majority of astrocytes in the saline groups are in a resting state, as indicated by their smaller cell bodies and long processes (inset Figure 5A,B,E,F). However, in lesion non-VNS rats, astrocytes are predominantly activated, as shown by their enlarged cell bodies and shortened processes (inset Figure 5C,G), indicating increased inflammation in this group. After VNS, astrocytes appear to be predominantly in a resting state in lesion rats (inset Figure 5D,H), indicating a decrease in inflammation. Analysis of total GFAP-positive cells was conducted using unbiased stereology. A 2-way ANOVA revealed an interaction of these effects in the SN, with main effects of Lesion and Stimulation in both the LC and SN (Table 1). Newman-Keuls post-hoc test showed total GFAP-positive cells were higher in both regions for lesion non-VNS rats compared to saline non-VNS rats (Figure 5A,C,E,G,I,J, LC: $p<0.05$, SN: $p<0.0001$). Total GFAP-positive cells were fewer in lesion VNS rats compared to lesion non-VNS rats (Figure 5C,D,G,H,I,J, LC: $p<0.05$, SN: $p<0.001$), and counts in

lesion VNS rats were similar to saline non-VNS rats (Figure 5A,D,E,H). Together, these results indicate that VNS reduced inflammation in lesion rats, and provide evidence for an anti-inflammatory effect of VNS. Pearson correlations indicate a negative relationship between GFAP-positive cells and locomotion (SN: $r=-0.668$, $p=0.0002$; LC: $r=-0.410$, $p=0.0378$).

In order to determine the effects of VNS on microglia, the pan microglial marker Iba-1 was used [38] in the LC and SN (Figure 6). Iba-1-ir density was conducted to assess microglia using a 2-way ANOVA, which revealed an interaction between these factors in the LC, with main effects of both Lesion and Stimulation in the LC and SN (Table 1). Both regions showed greater Iba-1-ir in lesion non-VNS rats compared to saline non-VNS rats, as indicated by Newman-Keuls post-hoc (LC: $p<0.01$, SN: $p<0.0001$, Figure 6A,C,E,G,I,J). Iba-1-ir was lower after VNS in lesion rats for both regions (LC: $p<0.01$, SN: $p<0.001$, Figure 6C,D,G,H,I,J), providing further evidence that VNS reduced inflammation in this lesion model. Pearson correlations establish a negative relationship between Iba-1-ir and locomotion (SN: $r=-0.728$, $p<0.0001$; LC: $r=-0.501$, $p=0.0092$). Taken together, these data indicate that VNS therapy exerted anti-inflammatory effects that may contribute to neuronal survival and maintenance, and related behavioral improvements.

DISCUSSION

This is the first study that has examined the therapeutic potential of VNS in PD. Both LC-NE and SN-DA degeneration were incorporated using a DSP-4/intrastriatal 6-OHDA rat model. Striatal injections were chosen over SN or medial forebrain bundle to better recapitulate the progressive clinical nature of PD [2,19]. The PD-like deficits observed in this model include decreased locomotion, lower TH-ir in the striatum, fewer TH-positive neurons in LC and SN, increased α -synuclein in remaining TH-positive neurons in SN, and increased inflammation in LC and SN as measured by increased astrocytes (GFAP) and increased activation of microglia (Iba-1-ir). Following VNS in lesioned rats, each of these measures improved.

PD patients have decreased output of the basal ganglia, resulting in a decreased ability to initiate movement. Similar behavioral outcomes were demonstrated in the current study by reduced locomotor activity of lesion non-VNS rats compared to saline non-VNS rats. However, our results showed that VNS increased locomotion in lesioned rats, providing the first look at the effects of VNS on the SN-DA system and locomotion. Previous studies using similar stimulation paradigms have focused on a wide variety of behavioral components related to other diseases [13,14,39,40]. Longitudinal locomotor data shows that in lesion non-VNS rats, locomotion continues to decline over the course of the study. However, in lesion VNS rats, this decline is attenuated, and locomotor activity at the conclusion of the study is comparable to saline-treated rats. These findings complement previous studies showing that by stimulating the white matter tracts of the dorsal column, locomotion and striatal DA were increased in 6-OHDA-treated rats [41], demonstrating that stimulation of various regions within the central nervous system can affect brain regions involved with motor function.

VNS is thought to have beneficial effects on LC-NE neurons via the NTS [22,24], and in this model, may slow LC-NE degeneration and ultimately the progression of PD-like symptoms. To determine whether VNS has effects on LC-NE neurons in our model, we looked at TH-positive cells in this region. Our study showed that while lesion non-VNS animals had fewer TH-positive neurons in the LC, VNS treatment resulted in a greater number of LC TH-positive neurons this population, making them comparable to saline non-VNS rats. While other groups have shown that ablation of the LC blocks VNS effects [13,42], here, we demonstrate approximately a 50% reduction in TH-positive LC neurons. It is possible that increased activation of the remaining cells was sufficient to reduce inflammation and increase locomotion, and it has been shown that remaining LC-NE cells after DSP-4 lesion can act in a hyperactive manner to re-innervate targets [43]. Additionally, neuronal loss after DSP-4 occurs later than NE terminal loss in LC target regions [44,45], suggesting that VNS in our model may act in a neuroprotective manner to prevent LC-NE loss in lesion VNS rats. Future studies are necessary to determine whether the improvements observed in our model are due to protective effects of VNS or increased activation of remaining LC-NE neurons.

Previous studies have demonstrated that the LC-NE neurons have direct projections to the SN-DA neurons, thus having protective effects [46,47,48]. Therefore, we also examined effects of VNS on the nigrostriatal pathway in this model since degeneration of this pathway in PD results in development of motor symptoms. Our results showed decreased TH-ir in the dorsal striatum of lesion non-VNS rats compared to saline non-VNS rats, as well as fewer TH-positive neurons in the SN. After VNS, however, greater TH-ir was present in the dorsal striatum with increased TH-positive cells in the SN of lesion rats compared to lesion non-VNS rats, possibly suggesting that increased TH after VNS in these areas contributes in part to beneficial effects on locomotor activity. While the addition of LC-NE degeneration to nigrostriatal lesions in some studies exacerbates SN-DA neuronal degeneration [7,18], others have shown potentiation of motor symptoms only, with similar SN-DA loss [19]. Increased number of SN TH-positive neurons in this model may be due to increases in the LC [2,25], since a direct connection from the LC to the SN has been previously demonstrated via tract tracing and electrophysiological studies [46,47]. Additionally, in previous studies utilizing the MPTP mouse model of PD, inhibition of the NE transporter within the SN prior to MPTP administration resulted in an attenuation of PD-like behavioral and neuronal decline [48], demonstrating that the LC-NE neurons provide protection to the SN-DA neurons and improve motor function. The mechanisms underlying the potential neuroprotection of NE on DA neurons in our model is unknown, yet future studies will be conducted to better understand this relationship. The use of NET inhibitors alone after MPTP administration have failed to show improvements in primate studies on L-dopa induced dyskinesia [49] and in cognitive function in clinical trials [50,51], suggesting that modification of the NE system alone is insufficient to improve all PD symptoms. Thus, the advantage of VNS over single target drug therapy is a multi-mechanistic approach affecting neuronal health in the LC and SN, but also reducing α -synuclein expression in TH-positive neurons and neuroinflammation.

While not currently well-understood, it has been suggested that non-pathological α -synuclein is typically localized to terminals and plays a role in DA neurotransmission, while pathological α -synuclein aggregates in cell bodies contributes to PD progression [52]. In the

current study, widely distributed, punctate α -synuclein expression can be observed in all treatment groups, indicating localization to neuronal processes, and thus resembling a non-pathological expression pattern [53]. However, lesion non-VNS rats displayed higher α -synuclein in remaining TH-positive neurons, and lesion VNS rats had significantly decreased α -synuclein in TH-positive neurons compared to lesion non-VNS rats, making them comparable to saline rats. The greater expression of α -synuclein in remaining TH-positive cells of lesion non-VNS rats indicates that either protein transport to the terminal is dysfunctional, or the cells are unable to degrade the additional α -synuclein in cell bodies as they normally would [36], demonstrating an expression pattern similar to that of PD patients [52,54]. Previous studies have also shown that increases in α -synuclein expression can increase microglial activation and may contribute to inflammation [55]. Thus, VNS treatment results in reduced α -synuclein in TH-positive neurons that may be in part due to the anti-inflammatory properties of VNS (further discussed below), although future studies must be conducted to determine the relationship between inflammation and α -synuclein in this model.

Neuroinflammation is a mechanism implicated in neurodegeneration, particularly in PD, as the auto-oxidative properties of SN-DA neurons make them more vulnerable to inflammatory insults [56]. Inflammation was higher in lesioned rats in this study as measured by GFAP-positive counts (astrocytes) and Iba-1-ir density (microglia), and VNS decreased both measures of inflammation in the SN and LC of this model. Although astrocytes are a major source of glutamate uptake to limit excitotoxicity, in inflammatory states, astrocytes alter transcription of neuroprotective genes, and glutamate uptake is decreased despite an increase in astrocyte number [9,57,58]. Additionally, microglia are the resident macrophages of the brain and become activated during inflammatory states, leading to swelling of microglial bodies and shortening of processes, as observed by increased Iba-1-ir in lesion non-VNS rats [59,60]. While acute microglial activation may result in neuroprotective secretions from microglia, prolonged activation results in pro-inflammatory secretions that are thought to contribute to neurodegeneration in chronic diseases such as PD [9,61,62]. VNS in a rat model of cerebral ischemia reduced inflammation via reduced glial activation and increased release of anti-inflammatory molecules, having a protective effect on neuronal populations and improving behavior [14,63,64]. Thus, the reduced inflammation after VNS in lesioned rats may contribute to the neuronal and behavioral improvements observed in this study.

CONCLUSIONS

Overall, this study demonstrates that VNS has multi-modal treatment potential in PD as demonstrated by improving behavior and neuronal populations, and decreased α -synuclein expression and inflammation in a progressive lesion model of PD. VNS may complement existing treatment options, as current surgical options are more invasive than VNS, and pharmacological treatments are limited and rely on proper absorption that is often disrupted in PD patients with a high incidence of gastrointestinal symptoms. Further studies are required to validate these results and determine the precise mechanism of action for VNS, and to better understand the treatment potential for PD.

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Conflicts of Interest: none

References

1. Steece-Collier K, Maries E, Kordower J. Etiology of Parkinson's disease: Genetics and environment revisited. *Proc Natl Acad Sci USA*. 2002; 99:13972–4. [PubMed: 12391311]
2. Braak H, Rub U, Gai WP, Del Tredici K. Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *J Neural Transm (Vienna)*. 2003; 110:517–36. [PubMed: 12721813]
3. Holmqvist S, Chutna O, Bousset L, Aldrin-Kirk P, Li W, Bjorklund T, et al. Direct evidence of Parkinson pathology spread from the gastrointestinal tract to the brain in rats. *Acta Neuropathol*. 2014; 128:805–20. [PubMed: 25296989]
4. Chan-Palay V. Alterations in the locus coeruleus in dementias of Alzheimer's and Parkinson's disease. *Prog Brain Res*. 1991; 88:625–30. [PubMed: 1726030]
5. Del Tradici K, Braak H. Dysfunction of the locus coeruleus-norepinephrine system and related circuitry in Parkinson's disease-related dementia. *J Neurol Neurosurg Psychiatry*. 2013; 84:774–83. [PubMed: 23064099]
6. Freed DM. On the involvement of the locus coeruleus in Parkinson's disease. *J Neuropsychiatry Clin Neurosci*. 1990; 2:114–5. [PubMed: 2136056]
7. Marin C, Aguilar E, Bonastre M. Effect of locus coeruleus denervation in levodopa-induced motor fluctuations in hemiparkinsonian rats. *J Neural Transm*. 2008; 115:1133–9. [PubMed: 18528630]
8. Abbott NJ. Inflammatory mediators and modulation of blood-brain barrier permeability. *Cell Mol Neurobiol*. 2000; 20:131–47. [PubMed: 10696506]
9. Glass CK, Saijo K, Winner B, Marchetto MC, Gage FJ. Mechanisms underlying inflammation in neurodegeneration. *Cell*. 2010; 140:918–34. [PubMed: 20303880]
10. Worth PF. When the going gets tough: how to select patients with Parkinson's disease for advanced therapies. *Pract Neurol*. 2013; 13:140–52. [PubMed: 23487815]
11. Halim A, Baumgartner L, Binder DK. Effect of deep brain stimulation on autonomic dysfunction in patients with Parkinson's disease. *J Clin Neurosci*. 2011; 18:804–6. [PubMed: 21489800]
12. Follasa P, Biggio F, Gorini G, Caria S, Talani G, Dazzi L, et al. Vagus nerve stimulation increases norepinephrine concentration and the gene expression of BDNF and bFGF in the rat brain. *Brain Res*. 2007; 1179:28–34. [PubMed: 17920573]
13. Furmaga H, Shah A, Frazer A. Serotonergic and noradrenergic pathways are required for the anxiolytic-like and antidepressant-like behavioral effects of repeated vagal nerve stimulation in rats. *Biol Psychiatry*. 2011; 70:937–55. [PubMed: 21907323]
14. Jiang Y, Li L, Liu B, Zhang Y, Chen Q, Li C. PPAR γ upregulation induced by vagus nerve stimulation exerts anti-inflammatory effect in cerebral ischemia/reperfusion rats. *Med Sci Monit*. 2015; 21:268–75. [PubMed: 25619160]
15. Samii A, Etminan M, Wiens M, Jafari S. NSAID use and the risk of Parkinson's disease. *Drugs & Aging*. 2009; 26:769–79. [PubMed: 19728750]
16. Kostrzewa RM. Evolution of neurotoxins: from research modalities to clinical realities. *Curr Protoc Neurosci*. 2009; 46:1.18.1–10.

17. Jonsson G, Hallman H, Ponzio F, Ross S. DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine) —A useful denervation tool for central and peripheral noradrenaline neurons. *Eur J Pharmacol.* 1981; 72:173–88. [PubMed: 6265244]
18. Shin E, Rogers JT, Devoto P, Bjorklund A, Carta M. Noradrenaline neuron degeneration contributes to motor impairments and development of L-DOPA-induced dyskinesia in a rat model of Parkinson's disease. *Exp Neurol.* 2014; 257:25–38. [PubMed: 24747357]
19. Ledreux A, Boger HA, Hinson VK, Cantwell K, Granholm AC. BDNF levels are increased by aminoindan and rasagiline in a double lesion model of Parkinson's disease. *Brain Res.* 2016; 1631:34–45. [PubMed: 26607251]
20. Schachter SC, Saper CB. Vagus nerve stimulation. *Epilepsia.* 1998; 39:677–86. [PubMed: 9670894]
21. Yuan H, Silberstein S. Vagus nerve and vagus nerve stimulation, a comprehensive review: part I. Headache. 2016; 56:71–8. [PubMed: 26364692]
22. Fornai F, Ruffoli R, Giorgi FS, Paparelli A. The role of locus coeruleus in the antiepileptic activity induced by vagus nerve stimulation. *Eur J Neurosci.* 2011; 33:2169–78. [PubMed: 21535457]
23. Verlinden T, Rijkers K, Hoogland G, Herrler J. Morphology of the human cervical vagus nerve: implications for vagus nerve stimulation treatment. *Acta Neurol Scand.* 2016; 133:173–82. [PubMed: 26190515]
24. Dorr A, Debonnel G. Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. *J Pharmacol Exp Ther.* 2006; 318:890–8. [PubMed: 16690723]
25. Tononi G, Cirelli C. Sleep function and synaptic homeostasis. *Sleep Med Rev.* 2006; 10:49–62. [PubMed: 16376591]
26. Biggio F, Gorini G, Utzeri C, Olla P, Marrosu F, Mocchetti I, et al. Chronic vagus nerve stimulation induces neuronal plasticity in the rat hippocampus. *Int J Neuropsychopharmacol.* 2009; 12:1209–21. [PubMed: 19309534]
27. Madrigal J, Feinstein D, Dello Russo C. Norepinephrine protects cortical neurons against microglial-induced cell death. *J Neurosci Res.* 2005; 81:390–6. [PubMed: 15948176]
28. Peña DF, Childs JE, Willett S, Vital A, McIntyre CA, Kroener S. Vagus nerve stimulation enhances excitation of conditioned fear and modulates plasticity in the pathway from the ventromedial prefrontal cortex to the amygdala. *Front Behav Neurosci.* 2014; 8:327. [PubMed: 25278857]
29. Engineer ND, Riley JR, Seale JD, Vrana WA, Shetake JA, Sudanagunta SP, et al. Reversing pathological neural activity using targeted plasticity. *Nature.* 2011; 470:101–4. [PubMed: 21228773]
30. Paxinos, G., Watson, C. *The Rat Brain in Stereotaxic Coordinates.* 2. San Diego: Academic Press; 1986.
31. Nichols JA, Nichols AR, Smirnakis SM, Engineer ND, Kilgard MP, Atzori M. Vagus nerve stimulation modulates cortical synchrony and excitability through the activation of muscarinic receptors. *Neuroscience.* 2011; 189:207–14. [PubMed: 21627982]
32. Porter BA, Khodaparast N, Fayyaz T, Cheung RJ, Ahmed SS, Vrana WA, et al. Repeatedly pairing vagus nerve stimulation with a movement reorganizes primary motor cortex. *Cerebral Cortex.* 2012; 22:2365–74. [PubMed: 22079923]
33. Boger HA, Middaugh LD, Patrick KS, Ramamoorthy S, Denehy ED, Zhu H, et al. Long-term consequences of methamphetamine exposure in young adults are exacerbated in glial cell line-derived neurotrophic factor heterozygous mice. *J Neurosci.* 2007; 27:8816–25. [PubMed: 17699663]
34. Boger HA, Middaugh LD, Huang P, Zaman Z, Smith AC, Hoffer BJ, et al. A partial GDNF depletion leads to earlier age-related deterioration of motor function and tyrosine hydroxylase expression in the substantia nigra. *Exp Neurol.* 2006; 202:336–47. [PubMed: 16889771]
35. Farrand AQ, Gregory RA, Scofield MD, Helke KL, Boger HA. Effects of aging on glutamate neurotransmission in the substantia nigra of Gdnf heterozygous mice. *Neurobiol Aging.* 2015; 36:1569–76. [PubMed: 25577412]
36. Granholm AC, Zaman V, Godbee J, Smith M, Ramadan R, Umphlet C, et al. Prenatal LPS increases inflammation in the substantia nigra of Gdnf heterozygous mice. *Brain Pathol.* 2011; 21:330–48. [PubMed: 20969653]

37. Granholm AC, Ford KA, Hyde LA, Bimonte HA, Hunter CL, Nelson M, et al. Estrogen restores cognition and cholinergic phenotype in an animal model of Down syndrome. *Physiol Behav.* 2002; 77:371–85. [PubMed: 12419414]
38. Reinert KRS, Umphlet CD, Quattlebaum AF, Boger HA. Short-term effects of an endotoxin on substantia nigra dopamine neurons. *Brain Res.* 2014; 1557:164–70. [PubMed: 24513404]
39. Hays SA, Khodaparast N, Hulse DR, Ruiz A, Sloan AM, Rennaker RL, et al. Vagus nerve stimulation during rehabilitative training improves functional recovery after intracerebral hemorrhage. *Stroke.* 2014; 45:3097–100. [PubMed: 25147331]
40. Grimonprez A, Raedt R, Baeken C, Boon P, Vonck K. The antidepressant-like effect of vagus nerve stimulation is mediated through the locus coeruleus. *J Psychiatr Res.* 2015; 68:1–7. [PubMed: 26228393]
41. Fuentes R, Petersson P, Siesser W, Caron M, Nicolelis M. Spinal cord stimulation restores locomotion in animal models of Parkinson's disease. *Science.* 2009; 323:1578–82. [PubMed: 19299613]
42. Krahl S, Clark K, Smith D, Browning R. Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. *Epilepsia.* 1998; 39:709–14. [PubMed: 9670898]
43. Fritschy J, Grzanna R. Restoration of ascending noradrenergic projections by residual locus coeruleus neurons: compensatory response to neurotoxin-induced cell death in the adult rat brain. *J Comp Neurol.* 1992; 321:421–41. [PubMed: 1506478]
44. Cassano T, Gaetani S, Moregesi M, Macheda T, Laconca L, Dispaquale P, et al. Monoaminergic changes in locus coeruleus and dorsal raphe nucleus following noradrenaline depletion. *Neurochem Res.* 2010; 34:1417–26.
45. Ross S, Stenfors S. DSP4, a selective neurotoxin for the locus coeruleus noradrenergic system. A review of its mode of action. *Neurotox Res.* 2015; 27:15–30. [PubMed: 24964753]
46. Lategan AJ, Marien MR, Colpaert FC. Suppression of nigrostriatal and mesolimbic dopamine release in vivo following noradrenaline depletion by DSP-4: a microdialysis study. *Life Sci.* 1992; 50:995–9. [PubMed: 1372673]
47. Grenhoff J, Nisell M, Ferre S, Aston-Jones G, Svensson TH. Noradrenergic modulation of midbrain dopamine cell firing elicited by stimulation of the locus coeruleus in the rat. *J Neural Transm Gen Sect.* 1993; 93:11–25. [PubMed: 8373553]
48. Rommelfanger K, Weinshenker D, Miller G. Reduced MPTP toxicity in noradrenaline transporter knockout mice. *J Neurochem.* 2004; 91:1116–24. [PubMed: 15569255]
49. Hansard M, Smith L, Jackson M, Cheetham S, Jenner P. Dopamine, but not norepinephrine or serotonin, reuptake inhibition reverses motor deficits in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated primates. *J Pharmacol Exp Ther.* 2002; 303:952–8. [PubMed: 12438514]
50. Hinson V, Delambo A, Elm J, Turner T. A randomized clinical trial of atomoxetine for mild cognitive impairment in Parkinson's disease. *Mov Disord Clin Practice.* 2017; 4:416–23.
51. Weintraub D, Mavandadi S, Mamikonyan E, Siderowf A, Duda J, Hurtig H, et al. Atomoxetine for depression and other neuropsychiatric symptoms in Parkinson disease. *Neurology.* 2010; 75:448–55. [PubMed: 20679638]
52. Bendor J, Logan T, Edwards RH. The function of α -synuclein. *Neuron.* 2013; 79:1044–66. [PubMed: 24050397]
53. Iwai A, Masliah E, Yoshimoto M, Ge N, Flanagan L, de Silva H, et al. The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron.* 1995; 14:467–75. [PubMed: 7857654]
54. Huang L, Deng M, He Y, Lu S, Liu S, Fang Y. β -Asarone increases MEF2D and TH levels and reduces α -synuclein level in 6-OHDA-induced rats via regulating the HSP70/MAPK/MEF2D/Beclin-1 pathway: chaperone-mediated autophagy activation, macroautophagy inhibition and HSP70 up-expression. *Behav Brain Res.* 2016; 313:370–9. [PubMed: 27444243]
55. Zhang QS, Heng Y, Yuan YH, Chen NH. Pathological α -synuclein exacerbates the progression of Parkinson's disease through microglial activation. *Toxicol Lett.* 2017; 265:30–7. [PubMed: 27865851]

56. Hwang O. Role of oxidative stress in Parkinson's disease. *Exp Neurobiol.* 2013; 22:11–7. [PubMed: 23585717]
57. Scofield MD, Kalivas PW. Astrocytic dysfunction and addiction: consequences of impaired glutamate homeostasis. *Neuroscientist.* 2014; 20:610–622. [PubMed: 24496610]
58. Sofroniew MV. Molecular dissection of reactive astrogliosis and glial scar formation. *Trends Neurosci.* 2009; 32:638–647. [PubMed: 19782411]
59. Streit WJ, Mrak RE, Griffin WST. Microglia and neuroinflammation: a pathological perspective. *J Neuroinflammation.* 2004; 1:14. [PubMed: 15285801]
60. Graeber MB, Streit WJ. Microglia: biology and pathology. *Acta Neuropathol.* 2010; 119:89–105. [PubMed: 20012873]
61. Hald A, Lotharius J. Oxidative stress and inflammation in Parkinson's disease: is there a causal link? *Exp Neurol.* 2005; 193:279–90. [PubMed: 15869932]
62. Herrera AJ, Tomas-Camardiel M, Venero JL, Cano J, Machado A. Inflammatory process as a determinate factor for the degeneration of substantia nigra dopaminergic neurons. *J Neural Transm.* 2005; 112:111–9. [PubMed: 15599609]
63. Liu B, Hong JS. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *J Pharmacol Exp Ther.* 2003; 304:1–7. [PubMed: 12490568]
64. Breidert T, Callebert J, Heneka MT, Landreth G, Launay JM, Hirsch EC. Protective action of the peroxisome proliferator-activated receptor-gamma agonist pioglitazone in a mouse model of Parkinson's disease. *J Neurochem.* 2002; 82:615–24. [PubMed: 12153485]

Highlights

VNS increases locomotor activity in a Parkinson's disease lesion model. TH-positive neurons in the LC and SN are increased after vagus nerve stimulation. VNS reduces α -synuclein density within TH-positive neurons in the SN of this model. Stimulation results in reduced astrocytes and microglial activation in lesioned rats.

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Figure 1. Experimental design timeline

Rats were randomly given either DSP-4 (50 mg/kg, i.p.) or saline. Seven days post-injection, the rats underwent vagus nerve cuff implantation, followed by intracranial 6-OHDA administration (5 μ g/ μ l, 2 μ l/site) or saline. Starting 11 days after lesioning, the rats with a vagus cuff received stimulation 2x a day for 30min each session. All animals, regardless of treatment group, underwent locomotor assessment (LA) during the pm session. After 10 days of stimulation, the rats were euthanized and brains processed for immunohistochemistry (IHC).

Figure 2. Locomotion was increased in lesioned rats with VNS

Total distance was measured in cm, and locomotion across all ten days shows decreased locomotor activity in lesion non-VNS animals compared to saline non-VNS animals (A). Average total distance traveled across the ten days was analyzed and displayed as a scatter plot with the group mean +SEM denoted by lines and error bars (B). Lesion non-VNS rats displayed less locomotion than saline non-VNS ($***p<0.001$), whereas VNS resulted in increased locomotion after lesion ($*p<0.05$).

Figure 3. VNS effects on TH in the LC, striatum, and SN of lesioned rats

Photomicrographs of LC (A–D, scale=100μm), striatum (E–H, scale=1mm), and SN (I–L, scale=0.5mm). Saline VNS rats had greater TH-positive cell counts than saline non-VNS rats in the LC (A–B, $**p<0.01$), but TH-ir was not increased in the striatum (E–F), nor increased TH-positive neurons in the SN of saline VNS rats (I–J). TH was significantly lower in lesion non-VNS rats in all three brain regions (LC counts: C, $****p<0.0001$; striatal TH-ir: G, $****p<0.0001$; SN counts: K, $***p<0.001$). Lesion VNS rats had greater TH in all three regions (LC counts: D, $**p<0.01$; striatal TH-ir: H, $**p<0.01$; SN counts: L, $**p<0.01$). TH results are quantified in M (LC), N (Striatum), and O (SN).

Figure 4. α-synuclein immunoreactivity is reduced by VNS in SN TH-positive cells of lesioned rats

Lesion non-VNS rats had a phenotypic loss of TH compared to saline non-VNS rats (A, C), although lesion VNS rats had comparable DAPI staining to saline non-VNS rats (A, D). Lesion non-VNS rats also display greater overall α-synuclein-ir in remaining TH-positive neurons compared to saline non-VNS rats (E, G, $*p < 0.05$). VNS treatment did not significantly alter α-synuclein-ir in saline rats (F), and lowered α-synuclein-ir in lesioned rats compared to lesion non-VNS rats (H, $*p < 0.05$). The inset images in E–H show the localization of α-synuclein in the TH-positive cell denoted with the box. As can be seen in G, α-synuclein is localized to the TH-positive cell body in lesion non-VNS rats, with no visible localization of α-synuclein to TH-positive cells in the other treatment groups. Scale for A–D (shown in A) = 10μm. Scale for E–H (shown in E) = 25μm. Quantification of results in I.

Figure 5. GFAP-positive astrocytes were reduced by VNS in the LC and SN of lesioned rats
 The measurement area for stereology is outlined for the LC (A) and the SN (E).
 Photomicrographs of LC (A–D, scale=50µm) and SN (E–H, scale=100µm). VNS did not alter GFAP-positive astrocytes in saline animals (LC: A, B; SN: E, F). Lesioned rats had significantly more GFAP-positive astrocytes than saline rats (LC: C, * $p<0.05$; SN: G, **** $p<0.0001$). Total astrocyte counts in lesioned rats were lower after VNS (LC: D, * $p<0.05$; SN: H, *** $p<0.001$). Quantification of total GFAP-positive cells in I (LC) and J (SN). Insets demonstrate primarily resting state astrocytes in saline and lesion VNS rats (A, B, D, E, F, H); however, in lesion non-VNS rats more astrocytes are activated than in the other groups (C, G).

Figure 6. Iba-1-ir was reduced by VNS in the LC and SN of lesioned rats

The measurement area for microglia density analysis is outlined for the LC (A) and the SN (E). Photomicrographs of LC (A–D, scale=50µm) and SN (E–H, scale=100µm). VNS had no effect on Iba-1-ir in the LC of saline rats (A, B), but resulted in reduced Iba-1-ir in the SN (E, F, * $p<0.05$). Lesioned rats had significantly higher Iba-1-ir (LC: C, ** $p<0.01$; SN: G, **** $p<0.0001$). VNS reduced Iba-1-ir in lesioned rats (LC: D, ** $p<0.01$; SN: H, *** $p<0.001$). Quantification of results in I (LC) and J (SN).

Table 1

2-way ANOVA statistical results for immunohistochemical data.

Lesion		VNS Treatment		Interaction	
Region	F(1,22)	p-value	F(1,22)	p-value	F(1,22) p-value
TH					
Striatum	113.3	<0.0001 ****	6.049	0.0222 *	6.849 0.0157 *
SN	25.56	<0.0001 ****	6.857	0.0157 *	1.812 0.1920
LC	48.44	<0.0001 ****	20.80	0.0002 ****	0.089 0.7682
GEAP					
SN	32.33	<0.0001 ****	8.933	0.0068 **	5.916 0.0236 *
LC	7.870	0.0103 *	10.71	0.0035 **	0.135 0.7172
Iba-1					
SN	79.65	<0.0001 ****	22.12	0.0001 ****	2.404 0.1353
LC	7.530	0.0118 *	4.432	0.0469 *	4.304 0.0499 *

Significant p-values:
* p<0.05,
** p<0.01,
*** p<0.001,
**** p<0.0001