1 Title

2 Continuous vagus nerve stimulation exerts beneficial effects on rats with experimentally
3 induced Parkinson's disease: evidence suggesting involvement of a vagal afferent
4 pathway.

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Author contribution

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Abbreviations

CNS: central nervous system; DA: dopamine; DAPI:4,6-diamidino-2-phenylindole; DβH: dopamine β hydroxylase; GFAP: glial fibrillary acidic protein; Iba-1: ionized calcium binding adapter molecule 1; LC: Locus coeruleus; NA: noradrenaline; NTS: nucleus of the solitary tract; PBS: phosphate-buffered saline; PD: Parkinson's disease; PEA: paraformaldehyde; SD: standard deviation; SNpc: substantia nigra pars compacta; TH: tyrosine hydroxylase; TNFα: tumor necrosis factor α; VNS: vagus nerve stimulation;

6-OHDA: 6-hydroxydopamine

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1 Introduction

Parkinson's disease (PD) is a common neurodegenerative disease, characterized by the loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) [1, 2]. Neuroinflammation and the immune system play a crucial role in the pathogenesis of PD [3, 4]. Increased reactive microglia and astrocyte and elevated levels of the proinflammatory cytokines (interleukin [IL]-1β, IL-6, tumor necrosis factor α [TNFα], and interferon-γ) populate the SNpc of PD patients, implicating their key contribution to the DA neuron degeneration [3, 5-7]. Thus, proper modulation of neuroinflammation may stop or slow down the progression of PD.

Noradrenaline (NA) neuron degeneration in the locus coeruleus (LC) is also thought to be associated with development of PD [8, 9]. In postmortem brain examination of PD patients, neuronal loss was greater in the LC than in the SNpc [10]. LC is the major source of NA in the central nervous system (CNS) and regulates the midbrain DA system [11]. In addition, because the depletion of NA neurons in the LC exacerbates the nigrostriatal DA neuron degeneration in experimental PD models, enhancing NA signals

100 from the LC may exert a neuroprotective effect for DA neurons in SN [12, 13]. Thus, the
101 NA system in the LC appears to play an important role in DA neuronal loss in PD.
102 Vagus nerve stimulation (VNS) is approved for refractory epilepsy and depression.
103 Recent studies have shown that VNS exerts anti-inflammatory effects against CNS
104 disorders, such as ischemic stroke and traumatic brain injury [14-17], as well as systemic
105 inflammatory disease, including endotoxemia, rheumatoid arthritis, inflammatory bowel
106 disease, heart failure, and myocardial infarction [18-23]. VNS promotes neuroprotective
107 effects against DA loss and ameliorates the motor symptoms in rats experimentally
108 induced to display PD [24, 25]. However, a single or short-term intermittent stimulation
109 was used in the previous studies, which was far from the clinical setting of VNS. For
110 clinical application, the experiments should be conducted with long-term and continuous
111 VNS, which was possible in our stimulation system. Additionally, the vagus nerve
112 contains 80% afferent fibers that project upward from the viscera into the medulla and
113 20% efferent fibers that regulate visceral organs [26]. Although VNS activates both
114 afferent fiber and efferent fibers, it is unclear whether the vagus afferent pathway or
115 efferent pathway primarily retards the progression of PD. Thus, we would like to
116 investigate whether the left VNS in the presence of ipsilateral caudal or rostral vagotomy

exert therapeutic effects on PD model of rats with focus on anti-inflammatory effects of VNS.

In this study, we aimed to stimulated either the vagus afferent fibers or the vagus efferent fibers independently with the caudal or rostral vagotomy to reveal their direct involvement in the observed therapeutic effects of VNS in a rat model of PD.

2 Material and Methods

2.1 Animals

Adult female Sprague Dawley rats (Shimizu Laboratory Supplies, Japan) weighing 200 to 260 g at the beginning of the study were used for all experiments. A total of 51 rats were used in this study. Eight rats were used for 5 groups, respectively. Five rats were omitted because of more than 20% weight loss, 4 rats died after surgery, VNS device trouble in one rat, and one rat showed no rotation by methamphetamine administration in the behavior test at 1 week. Rats were housed in pairs per cage until surgery, and one per cage after surgery. Animal housing consisted of individual cages in a temperature- and humidity-controlled room and maintained on a semidiurnal light-dark cycle. We closely followed guidelines of the Institutional Animal Care and Use Committee of Okayama University Graduate School of Medicine (Protocols #OKU-2017449 and OKU-2020203).

135

136 *2.2* ***VNS device***

137 We used an electrical stimulation device called the SAS-200 (Unique Medical Co., Ltd.
138 Tokyo, Japan) for VNS. Details of this device have been previously described [25, 27].
139 This device allows continuous and adjustable electrical stimulation of the vagus nerve
140 while allowing free movement of rats. The mobile generator was fixed to the back of the
141 rats, along with a protective jacket covering these systems. The stimulus parameters could
142 be instantly changed by a customized software application using a standard PC via
143 Bluetooth.

144

145 *2.3* ***Experimental design***

146 Rats were randomly divided into five groups: intact vagus nerve stimulation (VNS); left
147 vagus nerve stimulation in the presence of left caudal vagotomy, which we defined as
148 afferent vagus nerve fiber stimulation (aVNS); left vagus nerve stimulation with rostral
149 vagotomy, which we defined as efferent vagus nerve fiber stimulation (eVNS); sham
150 VNS (Sham); and vagotomy (Vx) groups (Fig. 1A). The time course of this study is
151 shown in Fig. 1B. First, on day 0, rats in the VNS, aVNS and eVNS groups underwent
152 VNS cuff electrode implantation at the left vagus nerve. The non-stimulation groups

(Sham and Vx) underwent the isolation of left vagus nerve but not the implantation of the cuff electrode. Second, all rats received 6-hydroxydopamine (6-OHDA) administration into the left striatum. Third, the aVNS group rats underwent vagus nerve transection at the level of the nerve distal to the electrode, while rats in the eVNS group underwent vagus nerve transection at the level of the nerve proximal to the electrode. The Vx group underwent a left vagotomy at this timepoint. Finally, the SAS-200 stimulator and a protective jacket were attached to the back of the rats.

After these surgical procedures, we switched on the stimulator. On days 7 and 14, all rats were subjected to behavioral tests, and at day 15, they were euthanized for immunohistochemical investigations.

2.4 ***Surgical procedure***

2.4.1 VNS surgery

VNS cuff placement was conducted as previously described [25], and the detailed method is described in a supplementary file.

2.4.2 6-OHDA lesion surgery

6-OHDA lesion surgery was conducted as previously described [25, 27, 28].

171 The detailed method is described in a supplementary file

172

173 *2.5* ***VNS stimulation***

174 After fixation of the SAS-200 onto the rats, the stimulation was started immediately.
175 Stimulation consisted of biphasic square pulses at 30 Hz, 500 μsec pulse width, and was
176 delivered for 30 sec every 5 min. These parameters were based on those used clinically
177 for drug-resistant epilepsy [29]. Stimulation intensity was set to 0.1 mA from day 0 to
178 day 7, and up to 0.25 mA after behavioral tests at day 7. This intensity parameter was
179 determined based on the results of our previous investigation [25].

180

181 *2.6* ***Behavioral tests***

182 The detailed methods of cylinder test and methamphetamine-induced rotation test are
183 described in a supplementary file.

184

185 *2.7* ***Brain preparation and immunohistochemical investigation***

186 The detailed methods for brain preparation, tyrosine hydroxylase and other
187 immunofluorescent staining are described in a supplementary file.

188

2.8 ***Histological evaluation of the ligated vagus nerve by transmission electron microscopy and toluidine blue***

The detailed methods for TEM and toluidine blue are described in a supplementary file.

2.9 ***Statistical analysis***

Comparisons in cylinder test or methamphetamine-induced rotation test and changes over time were analyzed using two-way repeated measures analysis of variance (ANOVA) followed by Tukey's post-hoc test with significance set at $p<0.05$. In other analyses, one-way ANOVA was used. Analyses were performed using GraphPad Prism 9.0 (GraphPad software, inc). Data are shown as mean ± standard deviation (SD).

3 **Results**

3.1 ***Mortality rate***

The mortality rate was VNS: 0%, aVNS: 0%, eVNS: 25%, Sham: 0%, and Vx: 10%. The percentage of rats omitted because of more than 20% weight loss was VNS: 25%, aVNS: 0%, eVNS: 8.3%, Sham: 11.1%, and Vx: 0%, respectively.

3.2 ***Behavioral tests***

3.2.1 Cylinder test

The contralateral bias on day 7 and 14 were as follows: day 7 ± SD, day 14 ± SD (%); VNS: 11.2 ± 8.9, 4.0 ± 4.8; aVNS: 2.2 ± 5.8, 0.7 ± 7.3; eVNS: 26.4 ± 11.5, 28.5 ± 30.1; Sham: 24.7 ± 8.6, 29.8 ± 15.0; Vx: 24.7 ± 18.1, 45.8 ± 32.9. Two-way repeated measures ANOVA revealed that there were significant treatment effects in cylinder test between day 7 and day 14 ($F_{(4, 35)} = 6.68$, $p < 0.01$) (Fig. 2A). Post hoc Tukey's test represented that the rats in the VNS group showed significant improvement on day 14 compared to the Sham and Vx group (aVNS: $p > 0.99$; eVNS: $p = 0.068$; Sham: $p = 0.048$; Vx: $p = 0.0002$ vs. VNS group, respectively). Similarly, rats in the aVNS group showed significant improvement on day 14 compared to the eVNS, Sham and Vx group (eVNS: $p = 0.027$; Sham: $p = 0.018$; Vx: $p < 0.0001$ vs. aVNS group, respectively). However, rats in the eVNS group did not exhibit any improvement in cylinder test compared to the Sham or Vx group (Sham: $p > 0.99$; Vx: $p = 0.33$ vs. eVNS group, respectively).

3.2.2 Methamphetamine-induced rotation test

The results of the methamphetamine-induced rotation test were as follows: day 7 ± SD, day 14 ± SD (turns / 90 min); VNS: 447 ± 270, 334 ± 177; aVNS: 919 ± 560, 618 ± 180;

eVNS: 1151 ± 725, 1248 ± 704; Sham: 1415 ± 421, 1649 ± 380; Vx: 989 ± 506, 1662 ± 602. Two-way repeated measures ANOVA revealed that there was a significant difference in methamphetamine-induced rotation test between day 7 and day 14 ($F_{(4, 35)} = 8.57$, $p < 0.01$) (Fig 2B). Rats in the VNS group showed a significant reduction in the number of rotations on day 7 compared to the Sham group (aVNS: $p = 0.38$; eVNS: $p = 0.067$; Sham: $p = 0.004$; Vx: $p = 0.25$ vs. VNS group, respectively). Rats in the VNS group showed a significant reduction in the number of rotations on day 14 compared to the eVNS, Sham, and Vx group (aVNS: $p = 0.82$; eVNS: $p = 0.0075$; Sham: $p < 0.0001$; Vx: $p < 0.0001$ vs. VNS group, respectively). Likewise, rats in the aVNS group showed a significant reduction in the number of rotations on day 14 compared to the Sham and Vx group (eVNS: $p = 0.13$; Sam: $p = 0.0018$; Vx: $p = 0.0015$ vs. aVNS group). Unlike the VNS and aVNS group, the eVNS group did not demonstrate any reduction in rotations compared to the Sham and Vx group (Sham: $p = 0.55$; Vx: $p = 0.52$ vs. eVNS group, respectively).

3.3 ***Immunohistochemical investigation***

3.3.1 TH immunostaining

In the striatum, the VNS (85.3 ± 8.8%) group had significantly preserved TH-positive fibers compared to the Sham (63.2 ± 9.7%, $p = 0.03$) and Vx (53.0 ± 17.1%, $p < 0.01$) groups, respectively (Fig. 3A). In addition, the aVNS (83.9 ± 15.6%) group had significantly preserved TH-positive fibers compared to the Vx group ($p < 0.01$), and tended to preserved more TH-positive fibers compared to the Sham group ($p = 0.05$). The eVNS group (70.4 ± 14.6%) did not show any improvement in TH-positive fiber preservation compared to the Sham ($p = 0.86$) or Vx ($p = 0.14$) groups.

In the SNpc, the VNS group (71.7 ± 17.4%) had significantly preserved TH-positive cells compared to eVNS (45.3 ± 15.7%, $p = 0.02$, Fig. 3B). Similarly, the aVNS group (74.2 ± 15.0%) displayed significant preservation of TH-positive cells in the SNpc compared to the eVNS ($p = 0.01$). There was no significant difference among the VNS, aVNS, Sham (53.6 ± 10.5%) and Vx (50.8 ± 18.1%) groups. However, aVNS tended to preserve more TH-positive cells compared to Vx group ($p = 0.06$).

3.3.2 Iba-1 staining

The number of Iba-1-positive cells in the striatum were significantly reduced in the VNS (23.1 ± 12.8 cells / field) and aVNS (25.0 ± 11.5 cells / field) group compared to the Vx (54.5 ± 22.0 cells /field, VNS vs. Vx: $p = 0.01$; aVNS vs. Vx: $p = 0.02$) group respectively

(Fig. 4A). In addition, the number of Iba-1-positive cells in the SNpc were significantly reduced in the VNS (17.3 ± 6.5 cells / field) and aVNS (14.3 ± 4.9 cells / field) group compared to the Sham (30.5 ± 7.0 cells / field, VNS vs. Sham: $p < 0.01$; aVNS vs. Sham: $p < 0.01$) and Vx (34.5 ± 9.3 cells / field, VNS vs. Vx: $p < 0.01$; aVNS vs. Vx: $p < 0.01$) groups, respectively (Fig. 4B). The eVNS group did not show significant reduction of Iba-1-positive cells in the striatum (43.6 ± 19.9 cells / field), but showed significant reduction in the SNpc (23.8 ± 5.8 cells / field) compared to Vx group ($p = 0.04$).

3.3.3 GFAP staining

The number of GFAP-positive cells in the striatum was significantly lower in the VNS (18.2 ± 6.2 cells / field) and aVNS (18.5 ± 4.4 cells /field) groups compared to the eVNS group (28.5 ± 6.0 cells /field, VNS vs. eVNS: $p = 0.04$; aVNS vs. eVNS: $p = 0.048$) respectively (Fig. 4C). In the SNpc, the number of GFAP-positive cells was significantly reduced in the VNS (12.1 ± 3.8 cells /field) and aVNS (11.1 ± 3.3 cells /field) groups compared to the eVNS (22.0 ± 5.6 cells /field, VNS vs. eVNS: $p < 0.01$; aVNS vs. eVNS: $p < 0.01$), Sham (24.5 ± 4.7 cells /field, VNS vs. Sham: $p < 0.01$; aVNS vs. Sham: $p < 0.01$) and Vx (23.3 ± 5.2 cells /field, VNS vs. Vx: $p < 0.01$; aVNS vs. Vx: $p < 0.01$) groups, respectively (Fig. 4D).

3.3.4 DβH staining

The density of DβH-positive neurons in the LC was significantly higher in the VNS group (91.8 ± 6.7%) compared to the eVNS (54.9 ± 4.6%, $p < 0.01$), Sham (52.5 ± 19.2%, $p < 0.01$) and Vx (46.5 ± 14.5%, $p < 0.01$) groups, respectively (Fig. 5). Similarly, the density of DβH-positive neurons in the aVNS group (86.8 ± 11.3%) was significantly higher compared to the eVNS ($p = 0.01$), Sham ($p < 0.01$) and Vx ($p < 0.01$) groups, respectively.

3.4 ***Relationship between the behavioral data and TH staining***

The relationship between the behavioral data (cylinder test and methamphetamine-induced rotation) and TH-positive fibers in striatum and neurons in SNpc (lesion/control side ratio) were evaluated. There is a significant negative correlation between contralateral bias of cylinder test and TH-positive fibers (A: n = 40, r square = 0.2773, $p < 0.01$) and neurons (B: n = 40, r square = 0.1846, $p < 0.01$). There is also a significant negative correlation between methamphetamine-induced rotation and TH-positive fibers (C: n = 40, r square = 0.4396, $p < 0.01$) and neurons (D: n = 40, r square = 0.1702, $p < 0.01$).

3.5 *Histological evaluation of the ligated vagus nerve*

Some degree of maintenance of morphology of myelinated nerve on 14 days after vagus nerve ligation both at proximal and distal sides was confirmed. The detailed results are described in a supplementary file (Suppl. figure 1, 2).

4 **Discussion**

In this study, we demonstrate that VNS and aVNS exerted therapeutic effects in a rat model of PD. Fourteen days of continuous VNS and aVNS suppressed the progression of PD-like behavior, preserved TH-positive cells in the SNpc, inhibited the activation of glial cells in the SNpc, and increased the density of rate limiting enzyme for NA in the LC, compared to the Sham or Vx groups. In contrast, eVNS (left VNS in the presence of rostral vagotomy) did not promote any beneficial effects on PD pathogenesis and motor function, except for suppression of iba-1 positive cells in SNpc. Our results suggest that the vagal afferent stimulation plays an important role in VNS therapy for PD.

4.1 ***Vagus afferent pathway***

309 The vagus afferent pathway is thought to innervate nuclei of the solitary tract (NTS).
310 Additionally, the VNS is believed to exert beneficial effects on LC-NA neurons via the
311 NTS [30]. To reveal whether VNS affects LC-NA neurons via the vagus afferent pathway,
312 we performed the anti-DβH immunofluorescent staining and investigated the rate limiting
313 enzyme of LC-NA pathway. We found that both VNS and aVNS increased the density of
314 DβH in LC-NA pathway, however, eVNS, which does not involve afferent vagus nerve
315 activation, failed to increase DβH. Previous studies have shown that neuronal activity of
316 the LC is modulated by VNS [31, 32] and that VNS prevents LC-NA neuron loss in a rat
317 model of PD [24]. These published reports applied bidirectional (both afferent and
318 efferent), as well as single or intermittent VNS. We simulated continuous, independent
319 afferent or efferent stimulation by means of ipsilateral caudal or rostral vagotomy to
320 investigate the effects of VNS on LC-NA pathway, and demonstrated increased density
321 of DβH in the LC maybe related to the observed therapeutic outcomes in experimental
322 PD.

323 The locus coeruleus projects throughout the central nervous system, including the
324 midbrain dopaminergic groups [33], and LC-NA neurons are shown to afford anti-
325 inflammatory and neuroprotective effects on SN-DA neurons [34, 35]. These protective
326 effects may be mediated by various mechanisms, including suppression of microglial

activation, reduction of pro-inflammatory cytokines, enhancement of neurotrophic factors, and reduction of oxidative stress [36-39]. Although glial cells constitute the frontline immune defense in the CNS, chronically overactive glial cells can start to produce pro-inflammatory mediators, leading to deleterious neuroinflammation and SN-DA neuron degeneration [3, 4]. Our present study indicates that VNS and aVNS, which favorably affect LC-NA pathway, suppressed the activation of microglia and astrocytes in the SNpc and maintained SN-DA neuron viability. These results suggest that afferent vagus nerve activation plays a crucial role in the protective effects of VNS on PD pathology.

4.2 **Vagus efferent pathway**

In our study, efferent VNS did not exert any beneficial effects in a rat model of PD. The prevailing concept stipulates that vagus efferent stimulation reduces systemic inflammation via the so-called 'cholinergic anti-inflammatory pathway', in which acetylcholine and alpha-7 nicotinic acetylcholine receptors (α7nAChR) are involved [40, 41]. The cholinergic output from the vagus nerve regulates immune reactions and suppresses pro-inflammatory cytokine release from macrophages through α7nAChR activation [42]. This pathway is also reported to induce anti-inflammatory and neuroprotective effects on neuroinflammation [43]. In the CNS, α7nAChR is expressed

345 on microglia and astrocytes, and activation of this receptor regulates pro-inflammatory
346 cytokine release from microglia and protects the astrocytes from oxidative stress-induced
347 apoptosis [44-46]. Furthermore, vagal efferent fibers may synapse with the splenic nerve
348 to inhibit the release of TNF-α by splenic macrophages against systemic inflammation
349 [47].

350 However, there is still debate about the true mechanism and function of the vagal
351 efferent pathway. A plausible view advances the mechanism that the cholinergic anti-
352 inflammatory pathway is mediated by the greater splanchnic nerve from the thoracic
353 sympathetic ganglion, rather than by the vagal efferent parasympathetic nerve [48-50].
354 Indeed, VNS with ligation of the vagus efferent side (selective afferent VNS) exerted
355 anti-inflammatory effects through the splanchnic nerve [48]. Another explanation for the
356 lack of neuroprotective effects by the efferent VNS in present study may be due to specific
357 stimulation level in that the efferent vagus nerve is more activated by low-frequency
358 stimuli (1-5 Hz) rather than the higher frequency we used here (30 Hz). Such discrepant
359 outcomes arising from varying the stimulation frequency has been reported elsewhere
360 [40]. Low-frequency but not high-frequency stimuli may reinforce the cholinergic anti-
361 inflammatory pathway. Further investigation to optimize the VNS-parameters should be
362 done in the future.

In addition, vagotomy, in other words axotomy, is associated with degeneration of axonal structures at distal to a site of injury [51]. So, VNS in the presence of rostral vagotomy, which we defined “eVNS” in our study may be insufficient to activate the vagal efferent fiber.

4.3 **Continuous stimulation**

Here, we applied continuous long-term VNS as opposed to past studies utilizing restricted to intermittent and short-term stimulation [24, 52]. We developed a compact and mobile electrical stimulation device that could manage both continuous VNS and free movement of experimentally-induced parkinsonian rats. While we previously reported the therapeutic effects of continuous VNS in PD rats [25], here we demonstrate the crucial role of afferent vagus pathway in mediating the beneficial outcomes of VNS. Our preclinical approach mimicked actual clinical practice parameters of VNS. The observation that afferent vagus pathway closely participates in the continuous VNS therapeutic effects warrants a careful consideration of targeting the stimulation to the vagus afferent fibers to optimize the clinical recovery of PD patients.

4.4 *Histological changes after vagus nerve ligation and efficacy of electrical stimulation*

In this study, some degree of maintenance of morphology of myelinated nerve on 14 days after vagus nerve ligation both at proximal and distal sides was confirmed. Axonal degeneration of severed peripheral nerves usually leads to Wallerian degeneration within hours to days, with dramatic changes such as mitochondrial dysfunction, increase of axonal autophagy, and axon disintegration [53], it is highly likely that the surviving cells are part of the total neuronal cell population. However, some axons of the peripheral nerves are reported to maintain some transport and action potentials even after damage [54], suggesting that some of their functions are preserved. Furthermore, electrical stimulation of the proximal side of severed peripheral nerve cells has been reported to preserve cerebral anatomy and function [55, 56]. Distal end stimulation after peripheral nerve transection has also been reported to promote regeneration of motor and sensory nerves [57, 58]. Although the present study did not directly evaluate the effects of electrical stimulation, we believe that both afferent and efferent electrical stimulation after vagotomy are likely to be effective.

4.5 Study Limitations

In the present study, we based our VNS-parameters on our previous study [25]. In that experiment, although the effects of stimulus intensity were evaluated from 0.1 - 1.0 mA, stimulus frequency, pulse width and on-off interval was not evaluated. The ideal stimulus parameters should be explored for treatment of PD, including the length of treatment. Moreover, we simultaneously conducted the 6-OHDA lesion surgery and the VNS therapeutic intervention to achieve highest therapeutic effects of VNS stimulation. This protocol may not be able to represent the clinical stage of PD in which about 70% of nigrostriatal dopamine neurons are already depleted when motor symptoms appear. The 6-OHDA infusion to the striatum is a well-established method for experimental induction of PD [59]. This central toxin model yields a progressive degeneration of nigrostriatal dopamine neurons, which is a typical feature of PD [60]. However, the current research on PD suggests that systemic inflammation, particularly in the gut, contributes to PD pathogenesis [61]. The 6-OHDA-induced parkinsonian model reproduces only limited aspects of PD pathogenesis, thus, to investigate the therapeutic potential of VNS for PD, further experiments using systemic toxin-induced models, such as the rotenone [62, 63], or a genetic model [64], should be considered to closely reveal any VNS interaction with the vagal nerve and the systemic or gut inflammation. Additionally, the lack of

electrophysiological data and the small number of DβH samples (n=5 each) due to the difficulty in sectioning and staining by the small structure of LC might limit the value of this study. About the way of vagotomy, vagus nerve was cut in this study. Wallerian degeneration of the proximal cell body of vagus nerve affected the effects of VNS itself, especially for the chronic experiment. This might be a structural problem, although we compared the 5 groups including only vagotomy group. Further experiments with chemical or electrical vagotomy might be useful to evaluate therapeutic effects of efferent VNS. We did not evaluate the side-effect of VNS on brain or body temperature in our study. It is reported that 30s on-5min off cycle VNS induce the decrease of brain temperature by 1℃ [65]. This hypothermic effect could be the confounder in our study because the hypothermia could suppress the inflammation caused by intrastriatal administration of 6-OHDA. Also, we did not evaluate whether VNS with caudal or rostral vagotomy affects brain or body temperature. Effects of VNS, selective VNS and vagotomy on brain or body temperature should be investigated. The 0.5s on-30s off cycle VNS, which did not affect the brain temperature [66], might be useful to eliminate the impacts of hypothermic effect by VNS. Additionally, the difference of VNS laterality and the control group with non-stimulated cuff electrode should be explored in the future.

5 Conclusion

Continuous left VNS in the presence of left caudal vagotomy exerts beneficial effects in a rat model of PD, including preservation of DA neurons in nigrostriatal systems and increased density of DβH in the LC. In contrast, left VNS with rostral vagotomy did not show any therapeutic effects. These results suggest that the afferent vagal pathway plays an important role in VNS treatment for PD.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Figure Legends

Figure 1: Experimental protocol

(A) Rats were divided into 5 groups: intact vagus nerve stimulation (VNS), afferent VNS (aVNS), efferent VNS (eVNS), sham operated (Sham) and vagotomy (Vx). (B) On day 0, a cuff electrode was implanted into the left vagus nerve in the VNS group, aVNS group and eVNS group. After the VNS surgery, 6-hydroxydopamine (6-OHDA) was administered into the left striatum. Then, the aVNS and eVNS groups underwent vagal transection on the side opposite side to which the stimulation would be performed. The Vx group underwent a left vagotomy. Electrical stimulation was started immediately after surgery. Stimulation intensity was 0.1 mA until behavioral tests on day 7, and 0.25 mA from day 7 to day14. All rats were then euthanized on day 15.

Figure 2: Results of behavioral tests

Comparisons between cylinder test or methamphetamine-induced rotation test and changes over time were analyzed. (A) In the intact vagus nerve stimulation (VNS) group, the improvement of contralateral bias was observed on day 14 compared to the sham operated (Sham) group and Vagotomy (Vx) group ($p < 0.05$). In the afferent VNS (aVNS) group, the improvement of contralateral bias was observed in day 14 compared to the

efferent VNS (eVNS) group, Sham and Vx group ($p < 0.05$). (B) The number of rotations on day 7 was significantly lower in the VNS group compared to the Sham group ($p < 0.05$). The number of rotations on day 14 was significantly lower in the VNS group compared to the eVNS, Sham and Vx group ($p < 0.05$). The number of rotations on day 14 was significantly lower in the aVNS group compared to the Sham and Vx group ($p < 0.05$). The data are presented as mean ± standard deviation and analyzed by two-way repeated measure ANOVA and Tukey's post hoc tests. n = 8 rats in each group.

Figure 3: Results of tyrosine hydroxylase (TH) staining of the striatum (STR) and substantia nigra pars compacta (SNpc)

(A) TH-positive fibers in the lesioned STR were significantly preserved in the intact vagus nerve stimulation (VNS) group compared to the sham operated (Sham) group and vagotomy (Vx) group ($p < 0.05$). TH-positive fibers in the lesioned STR were significantly preserved in the afferent VNS (aVNS) group compared to the Vx group ($p < 0.05$). (B) TH-positive neurons in the lesioned SN were significantly preserved in the VNS and aVNS group compared to the efferent VNS (eVNS) group ($p < 0.05$). The data are presented as mean ± standard deviation and analyzed by one-way ANOVA and Turkey's post hoc tests. n = 8 in each group.

Figure 4: Results of ionized calcium binding adaptor molecule 1 (Iba-1: green), glial fibrillary acidic protein (GFAP: red) and 4,6-diamidino-2-phenylindole (DAPI: blue) staining of the lesion-side striatum (STR) and substantia nigra pars compacta (SNpc). (A) The intact vagus nerve stimulation (VNS) and afferent VNS (aVNS) group inhibited the microglial proliferation in the STR compared to vagotomy (Vx) group ($p < 0.05$). (B) The VNS and aVNS group inhibited the microglial proliferation in the SNpc compared to the sham operated (Sham) group and Vx group ($p < 0.05$). The microglial proliferation was also inhibited in the efferent VNS (eVNS) group compared to the Vx group ($p < 0.05$). (C) The VNS and aVNS group showed inhibited astrocytic proliferation in the STR compared to the eVNS group. (D) VNS and aVNS reduced astrocytic activation in the SNpc compared to the eVNS, Sham and Vx group. The data are presented as mean ± standard deviation and analyzed by one-way ANOVA and Turkey's post hoc tests. n = 8 in each group.

Figure 5: Result of dopamine β hydroxylase (DβH) staining

The intact vagus nerve stimulation (VNS) and afferent VNS (aVNS) groups showed significantly increased density of DβH-positive regions in the locus coeruleus (LC)

compared to efferent VNS (eVNS) group, sham operated (Sham) group and vagotomy (Vx) group. The data are presented as mean ± standard deviation and analyzed by one-way ANOVA and Turkey's post hoc tests. n = 5 in each group.

Figure 6: Correlation analyses between behavioral data and TH-immunohistochemical evaluations

Correlation analyses between cylinder test (contralateral bias, %) and the ratio (lesion side/control side) of TH-positive fibers in the striatum (A) and TH-positive neurons in the substantia nigra pars compacta (SNpc) (B) are shown. Correlation analyses between methamphetamine-induced rotation and TH-positive fibers in the striatum (C) and TH-positive neurons in the substantia nigra pars compacta (D) are shown. All the data show the significant positive correlation between the behavioral data and TH-immunohistochemical data.

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1.1 Surgical procedure

1.1.1 VNS surgery

Rats received anesthesia with 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam and 5.0 mg/kg of butorphanol by intraperitoneal injection and placed in supine position on a heating pad maintaining the rats' body temperature at 37 ℃. Rats received subcutaneous injection of 1 % xylocaine, a skin incision in the midline section of the neck, and blunt dissection of the muscles that exposed the left cervical vagus nerve. After isolating the vagus nerve from the carotid artery, a cuff electrode was fitted to the vagus nerve with 5-0 silk threads. A cuff lead was tunneled subcutaneously to the back of rats to connect to a stimulator later.

1.1.2 6-OHDA lesion surgery

After a cervical VNS surgery, all anesthetized rats were moved to a stereotaxic instrument (Narishige, Japan). The rats underwent a midline head skin incision and a small hole was drilled in their skull. Twenty μg of 6-OHDA (4 μl of 5 mg/ml dissolved in saline containing 0.2 mg/ml of ascorbic acid; Sigma, USA) was injected into the left striatum (1.0 mm anterior and 3.0 mm lateral to the bregma and 5.0 mm ventral to the surface of the brain with the tooth-bar set at -1.3 mm) with a 28 G Hamilton syringe that delivered

742 an injection rate of the drug at 1 μl/min. Syringe withdrawal commenced after a 5-minute
743 absorption time following the injection.
744
745 ***2.1 Behavioral test***
746 *2.1.1 Cylinder test*
747 We performed a cylinder test on days 7 and 14 to assess the degree of forepaw asymmetry.
748 Rats were placed in a transparent cylinder (diameter: 20cm, height: 30cm) for 3 minutes
749 and the number of forepaw contacts on the cylinder wall was recorded. The score of the
750 cylinder test was calculated as a contralateral bias: ([number of contacts with contralateral
751 limb]-[number of contacts with ipsilateral limb])/(number of total contacts) × 100 [1].
752
753 *2.1.2 Methamphetamine-induced rotation test*
754 Subsequent to the cylinder test, we performed the methamphetamine-induced rotation
755 test. Rats received an intraperitoneal injection of methamphetamine (3.0mg/kg;
756 Dainippon Sumitomo Pharma, Japan). We assessed the number of full 360° turns in the
757 direction ipsilateral to the lesion for 90 minutes with a video camera. This test indicates
758 the degree of 6-OHDA-induced unilateral nigrostriatal dopamine depletion [2].
759

3.1 Brain preparation

On day 15, animals underwent euthanasia with an overdose of pentobarbital (100mg/kg). Rats then received transcardiac perfusion with 150 ml of cold phosphate-buffered saline (PBS) and 150 ml of 4% paraformaldehyde (PFA) in PBS. Brains were carefully removed and incubated in 4% PFA in PBS overnight at 4 °C, and subsequently stored in 30% sucrose at 4 °C. Thereafter, we sectioned the brains coronally at a thickness of 40 µm for immunohistochemistry.

4.1 Immunohistochemical investigations

4.1.1 Tyrosine hydroxylase immunostaining

To assess nigrostriatal dopaminergic neuron loss, we performed tyrosine hydroxylase (TH) staining of the bilateral striatum and SNpc. Free-floating sections were blocked with 3% hydrogen peroxide in 70% methanol for 10 minutes. After three washes in PBS for 5 minutes, sections were incubated overnight at 4 °C with rabbit anti-TH antibody (1:500; Chemicon, Temecula, CA, USA) with 10% normal horse serum in phosphate-buffered saline with Triton X-100 (PBS-Triton). After several rinses in PBS, sections were incubated for 1 hour in PBS-Triton with biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch Lab, West Grove, PA, USA), followed by 30 minutes in avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA).

778 Subsequently, the sections were treated with 3,4-diaminobenzidine (DAB; Vector) and
779 hydrogen peroxide, then mounted on albumin-coated slides, and covered with glass slips.
780 We assessed the density of TH-positive fibers in the striatum with a computerized analysis
781 system as described previously [3]. Three sections randomly selected at 0.5 ± 1.0 mm
782 anterior to the bregma were used. The two areas adjacent to the needle tract of the lesion
783 side and the symmetrical areas in the contralateral side were taken at 8× magnitude. The
784 images were converted into ImageJ (National Institutes of Health, Bethesda, MD, USA).
785 We defined the threshold of the TH-positive fibers on the lesion side, and then applied
786 the same threshold to the intact side. Each TH-positive density was calculated by (mean
787 value) × (area fraction), and then the ratio of the lesion to the intact portion of each section
788 was calculated. The averages were used for analysis. For analysis of SNpc, we manually
789 counted the number of all the TH-positive neurons in three sections at 4.8, 5.3, 5.8 mm
790 posterior to the bregma in the SNpc. We calculated the ratio of the lesion to the intact
791 portion, then used the averages for analysis.
792
793 *4.1.2 Immunofluorescent staining*
794 To evaluate inflammation in the nigrostriatal pathway, we performed ionized calcium-
795 binding adapter molecule 1 (Iba-1) and glial fibrillary acidic protein (GFAP) staining of

796 the striatum and SNpc. In addition, to explore the rate limiting enzyme of NA in the LC,
797 dopamine β hydroxylase (DβH) fluorescent staining was also performed. Sections of 40
798 μm thickness of the striatum, SNpc and LC were used. For LC analysis, three sections at
799 10 ± 1.0 mm posterior to the bregma were randomly selected. The slices were washed 3
800 times in PBS, followed by incubation with 10% normal horse serum and primary
801 antibodies; rabbit anti-Iba-1 antibody (1:250; Wako Pure Chemical Industries, Osaka,
802 Japan), rabbit anti-GFAP antibody (1:1000; Novus Biologicals, Littleton, CO, USA) and
803 rabbit anti- DβH antibody (1:500; Chemicon) in phosphate-buffered saline with Tween
804 20 (PBST) for 24 hours at 4 °C, respectively. After several rinses in PBS, sections were
805 incubated for 1 hour in fluorescein isothiocyanate (FITC; 1:200; Jackson
806 ImmunoResearch)-conjugated affinity-purified donkey anti-rabbit IgG (H+L) and 4,6-
807 diamidino-2-phenylindole (DAPI; 2 drops/mL, R37606; Thermo Fisher, Waltham, MA,
808 USA) in a dark chamber. The sections were then extensively washed with PBS and
809 coverslipped. The immunofluorescent staining was visualized using an inverted
810 fluorescence phase-contrast microscope BZ-X710 (Keyence, Japan). The numbers of Iba-
811 1-positive cells and GFAP-positive cells of the striatum and SNpc in the lesion side were
812 manually counted (each 500×500 μm square) to evaluate glial reaction. Three different
813 sections were randomly selected, which were at a level corresponding to the TH staining.

The average number of cells was used for analyses. In the LC, images were semiquantitatively analyzed by ImageJ as previously described [4]. Reference background levels were obtained from non-immunoreactive area portions adjacent to the LC region by determining the optical density on a 0 - 255 gray scale (0 being white and 255 black). The area fraction of the immunoreactive portion in the LC region was calculated at 2 - 3 slices for each rat, then the ratio of the area fraction of the lesion to the intact in each slice were calculated. The average of the percentages was used for analyses. All the immunohistochemical data were obtained and evaluated by a blinded examiner.

5.1 *Histological evaluation of the ligated vagus nerve by transmission electron microscopy and toluidine blue*

To confirm the condition of the vagus nerve after ligation, 2 mm portions of the proximal and distal ends of the ligated vagus nerve were evaluated on days 0, 7, and 14, respectively. Histological evaluation using transmission electron microscopy (TEM) and toluidine blue staining was performed to confirm preservation of vagus nerve morphology. TEM was performed as described previously [5]

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4.4 *Histological evaluation of the ligated vagus nerve*

At day 0 after vagus nerve ligation, most myelin sheaths are clearly stained on both the proximal and distal sides in toluidine blue staining. At 7 and 14 days, the staining of the myelin sheaths was blurred, but there were still scattered myelin sheaths that retained their morphology. There were slightly more normal myelin sheaths on day 7 than on day 14. Transmission electron microscopy showed more clearly the changes in axons and myelin sheaths after vagus nerve ligation. On the proximal side, axons with retained myelin sheaths were relatively abundant in all time points, that is, on day 0, 7, and 14. On the other hand, Wallerian degeneration began in some myelin sheaths from day 0 in the distal side. While degenerated axons and myelin sheaths clearly increased over time, a small number of axons with retained myelin sheaths were still seen on day 14 after vagus nerve ligation (suppl. figure 1,2).

Supplementary figure legend

Supplementary figure 1

Toluidine blue staining after vagus nerve ligation (upper row; central end, lower row: peripheral end, left, middle and right column: 0, 7 and 14 days after vagal nerve ligation, respectively)

Axons with retained myelin morphology were observed in all time points (day 0, 7, and 14) both the proximal and distal ends (yellow arrows), whereas the retention of myelin sheath morphology deteriorated over time. Yellow arrows indicate nerves with preserved myelin sheath and axon morphology.

Supplementary figure 2

Transmission electron micrographs after vagus nerve ligation (upper row; central end, lower row: peripheral end, left, middle and right column: 0, 7 and 14 days after vagus nerve ligation, respectively)

At the proximal side, axons with retained myelin sheaths were relatively abundant in all time points. At the distal side, Wallerian degeneration began in some myelin sheaths even on day 0, but a small number of axons with retained myelin sheaths were still seen on day 14. Red arrows indicate myelin sheaths where Wallerian degeneration began. Yellow arrows indicate nerves with preserved myelin sheath and axon morphology.