

Research Report

No Direct Projection is Observed from the Substantia Nigra to the Dorsal Vagus Complex in the Rat

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Abstract.

Background: Parkinson's disease (PD) is a neurodegenerative disorder that is characterized by degeneration of dopaminergic neurons in the substantia nigra (SN). Destruction of the SN can lead to gastric dyskinesia accompanied by decreased expression of choline acetyltransferase (ChAT) and increased expression of tyrosine hydroxylase (TH) in the dorsal vagus complex (DVC), which includes the dorsal motor nucleus of the vagus (DMV) and nucleus tractus solitarius (NTS). However, it is unclear if the SN and DVC are directly connected.

Objective: To investigate the neural projection from the SN to the DVC in rats.

Methods: Retrograde and anterograde tracing techniques combined with double-labeling immunofluorescence technique were used.

Results: Destruction of the SN significantly decreases ChAT immunoreactivity (IR) and increases TH-IR in the DVC. After injection of the retrograde tracer fluoro-gold (FG) into the DVC, FG-labeled neurons were observed in the hypothalamic paraventricular nucleus (PVN), lateral hypothalamus (LH), inferior olive (IO), and locus coeruleus (LC). No FG-positive cells were observed in the SN or striatum. Furthermore, after injection of anterograde tracer biotinylated dextran amine (BDA) into the SN, BDA-positive fibers were observed in the caudate putamen (Cpu), globus pallidus (GP), LC, and LH but not in the DVC.

Conclusion: This study is the first to demonstrate that neurons in the SN do not directly innervate the DVC in rats. The DVC might be indirectly innervated by the SN through the hypothalamus and/or the LC. These data provide important morphological insights into the potential mechanism underlying the gastroparesis observed in PD patients.

Keywords: Retrograde tracing, anterograde tracing, substantia nigra, dorsal vagus complex, gastroparesis

INTRODUCTION

Parkinson's disease (PD) is a chronic, progressive neurodegenerative disorder that is characterized by the

loss of dopaminergic neurons in the substantia nigra (SN) [1]. Most PD patients experience gastrointestinal (GI) dysfunction such as dysphagia, delayed gastric emptying, bloating, and constipation [2–4]. The dorsal motor nucleus of the vagus (DMV) is located in the dorsal motor complex (DVC) including the nucleus tractus solitarius (NTS), DMV and area postrema (AP) of medulla oblongata, it plays an important role in the regulation of upper GI function, including gastric motility. The main neurotransmitter in DMV motor neurons is acetylcholine (ACh), but norepinephrine

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(NE), dopamine (DA) and others are also present in the DMV [5, 6]. In addition to the SN, neural degeneration is observed in the DMV, locus coeruleus (LC), and hypothalamus in PD patients [7–10]. Rats subjected to bilateral destruction of the SN manifest reduced motor coordination accompanied by typical disorders of gastric emptying [11] and alteration of Ach and DA in the DMV [12]. The DMV receives dopaminergic innervation from the NTS [13]. The dopaminergic neurons in the hypothalamus project to the DVC [14]. The DMV is also innervated by the LC and the raphe nucleus [15, 16]. However, the neural connection between the DVC and SN has not been addressed. The aim of the present study is to investigate the neural projection from the SN to the DVC using retrograde and anterograde tracing techniques in rats. These results may provide morphological insights into the mechanism underlying delayed gastric emptying in PD patients.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (250–300 g) were purchased from the Laboratory Animal Services Center of Capital Medical University. All experiments were performed in accordance with the guidelines established by the National Institutes of Health (NIH, USA) and were approved by the Animal Care and Use Committee of Capital Medical University, Beijing, China. All efforts were made to minimize animal suffering, and the minimal number of animals necessary to produce reliable scientific data was used.

6-OHDA models

The methods used have been described previously [12, 17]. Briefly, rats were anesthetized by intraperitoneal (i.p.) injection with chloral hydrate (0.4 g/kg) and placed on a Kopf stereotaxic instrument. Two small areas of the skull were exposed (coordinates: AP, –5.6 mm; ML, \pm 2.0 mm; DV, –7.5 mm), and 6-OHDA (4 μ g in 2 μ l of 0.9% saline containing 0.05% ascorbic acid) was injected with a 10 μ l Hamilton syringe. Control groups were injected with 0.05% ascorbic acid/saline. The rats injected with 6-OHDA in the SN were referred to as 6-OHDA rats. After 6 weeks, 6-OHDA administration produced relatively uniform nigrostriatal lesions.

Retrograde and anterograde tracing

The rats were anesthetized and placed in a Kopf stereotaxic frame [15].

Retrograde tracing

The dorsal surface of the medulla was exposed by removing the atlanto-occipital membrane. A final volume of 0.1 μ l of 4% fluoro-gold (FG) (Biotium/80014) in saline was injected into the left DVC at the level of the obex (coordinates: AP, –0.84 mm; ML, –0.5 mm; DV, –0.28 mm) by applying slight pressure on a 5 μ l Hamilton microsyringe attached to a glass micropipette (internal tip diameter = 15–25 μ m) [16]. Three days later, the animals were sacrificed. The medulla (injection site) and the SN were processed for FG and directly visualized under a UV microscope.

Anterograde tracing

Approximately 4 μ l of 20% BDA (Eugene/n7167) (3 mg dissolved in 15 μ l 0.01 M PBS, pH 7.4) was injected into the left SN (coordinates: AP, –5.6 mm; ML, –2.0 mm; DV, –7.5 mm) with the same Hamilton microsyringe. After 7 days, the animals were killed by decapitation, and the brains were removed for detection of the anterograde tracing fibers.

Tissue preparation and immunofluorescence staining

After deep anesthetization with chloral hydrate, rats received a thoracotomy and were perfused through the left ventricle with 250 ml of saline followed by 300 ml of 4% paraformaldehyde in 0.01 M PBS (pH 7.4). The brains were immediately removed and immersed in 4% paraformaldehyde for a 12-h post-fixation period and then placed in 30% sucrose in 0.01 M PBS (pH 7.4) for a 48-h dehydration period. Serial coronal sections were cut to a thickness of 20 μ m with a cryostat (Leica CM1850, St. Gallen, Switzerland). The tissue sections were air-dried overnight at room temperature.

Cryosections including the SN and DVC of 6-OHDA rats were permeabilized with 0.3% Triton X-100 in PBS (3 \times 5 min, pH 7.4). Antigen retrieval was accomplished by immersing the slides in a beaker containing citrate buffer (0.01 M, pH 6.0), followed by heating to a temperature of 95–100°C using a microwave oven for 15 min. After rinsing with PBS (3 \times 5 min), the sections were incubated with 10% normal goat serum (NGS) in PBS containing 0.3% Triton X-100 (PBST) for 1.5 h to block nonspecific binding of the antibodies. The sections

containing the SN were incubated with primary antibodies diluted in 2% NGS and 0.3% Triton X-100 (TH: 1:10,000; Sigma/T1299, mouse monoclonal), and sections containing the DMV were incubated with primary antibodies diluted in 2% NGS and 0.3% Triton X-100 (TH: 1:10,000, ChAT:1:100; Abcam/ab68779, rabbit polyclonal) for 12 h. The sections were then washed with PBS (3×10 min) and incubated with Alexa Fluor 488-labeled goat anti-mouse Ig (H+L) (Beyotime/A0428, 1:100) and Cy3-labeled goat anti-rabbit IgG (H+L) (Beyotime/A0516, 1:1000) for 1-2 h at room temperature. Cell nuclei were stained by incubation with 4', 6-diamidino-2-phenylindole (DAPI) for 3 min at room temperature. After the sections were rinsed with PBS (3×10 min), anti-fade mounting medium was used to mount the specimens with coverslips. Photomicrographs were acquired with a laser scanning confocal microscope (Olympus, FV1000) equipped with Alexa Fluor 488 (to visualize TH-IR), Cy3 (to visualize ChAT-IR and TH-IR), and UV (to visualize DAPI) filters. All sections were processed for visualization of the SN and the DMV.

The sections from the anterograde tracing group were processed for observation of the injection site in the SN and the distribution of BDA-labeled fibers in the diencephalon and brainstem [18]. The sections were incubated in 0.3% Triton X-100 in 0.01 M

PBS (pH 7.6) for 15 min prior to incubation in fluorescent isothiocyanate (TEX RED)-labeled avidin D (1:200, A-2001, Vector Laboratories, Burlingame, CA, USA) at room temperature for 2 h. After incubation, all sections were rinsed in 0.01 M PBS, mounted onto gelatin-coated glass slides, air-dried, and cover-slipped with a mixture of 50% (v/v) glycerin and 2.5% (w/v) triethylene diamine (anti-fading agent) in 0.01 M PBS. The injection sites and distribution of BDA-labeled fibers were examined under a fluorescence microscope.

RESULTS

Distribution of TH-IR in the SN and TH-IR and ChAT-IR in the DMV

TH, a rate-limiting enzyme in catecholamine synthesis, is generally used as a marker of catecholaminergic neurons [6]. The majority of TH-immunoreactive (TH-IR) neurons in the SN are dopaminergic; thus, we reasoned that the TH-IR neurons primarily reflect dopaminergic neurons in the SN. Six weeks after injection of 6-OHDA into the SN, immunofluorescence analysis revealed that the number of TH-IR neurons in the SN was considerably reduced in 6-OHDA rats compared with controls, consistent with our previous report [12]. In control rats (Fig. 1A), a few TH-IR

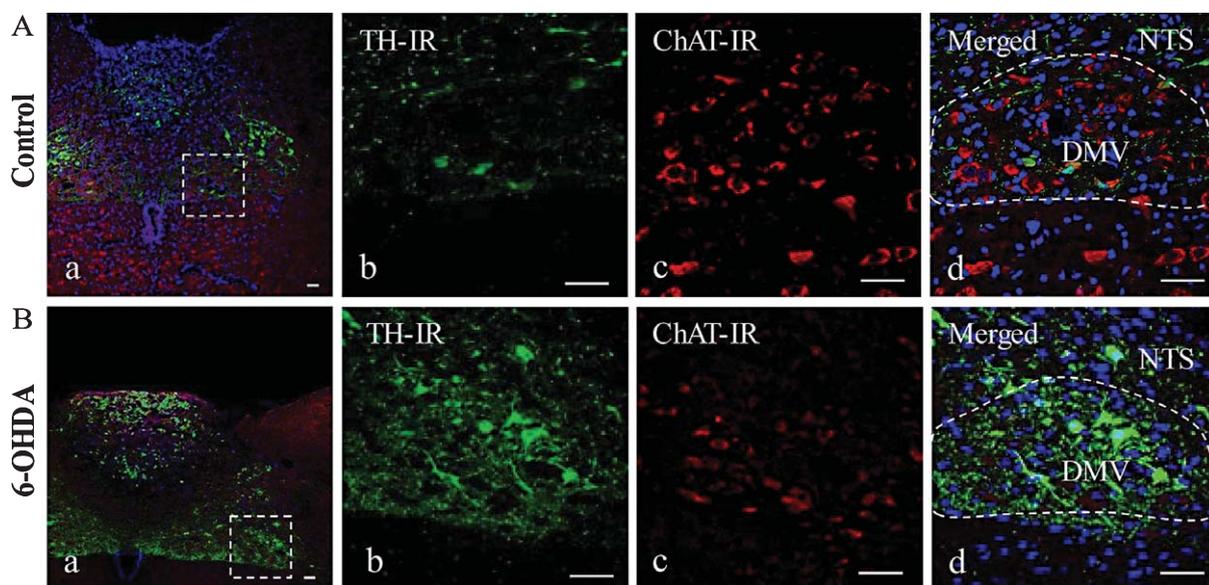


Fig. 1. Distribution of TH-IR and ChAT-IR in the DVC in control and 6-OHDA rats. A. In control rats, TH-IR neurons and fibers (green) were observed in the NTS and DMV. Numerous ChAT-IR neurons (red) were observed in the DMV. B. In 6-OHDA rats, the intensity of TH-IR neurons and fibers (green) was significantly increased in the DMV and NTS. By contrast, the intensity of ChAT-IR neurons (red) was visibly decreased in the DMV. Aa and Ba are low magnification images. Ab-d and Bb-d are high magnification images of the white framed regions in Aa and Ba, respectively. Ad and Bd are the merged results, and the white dashed oval identifies the DMV region. Nuclei were stained with DAPI (blue). Scale bar: 50 μ m.

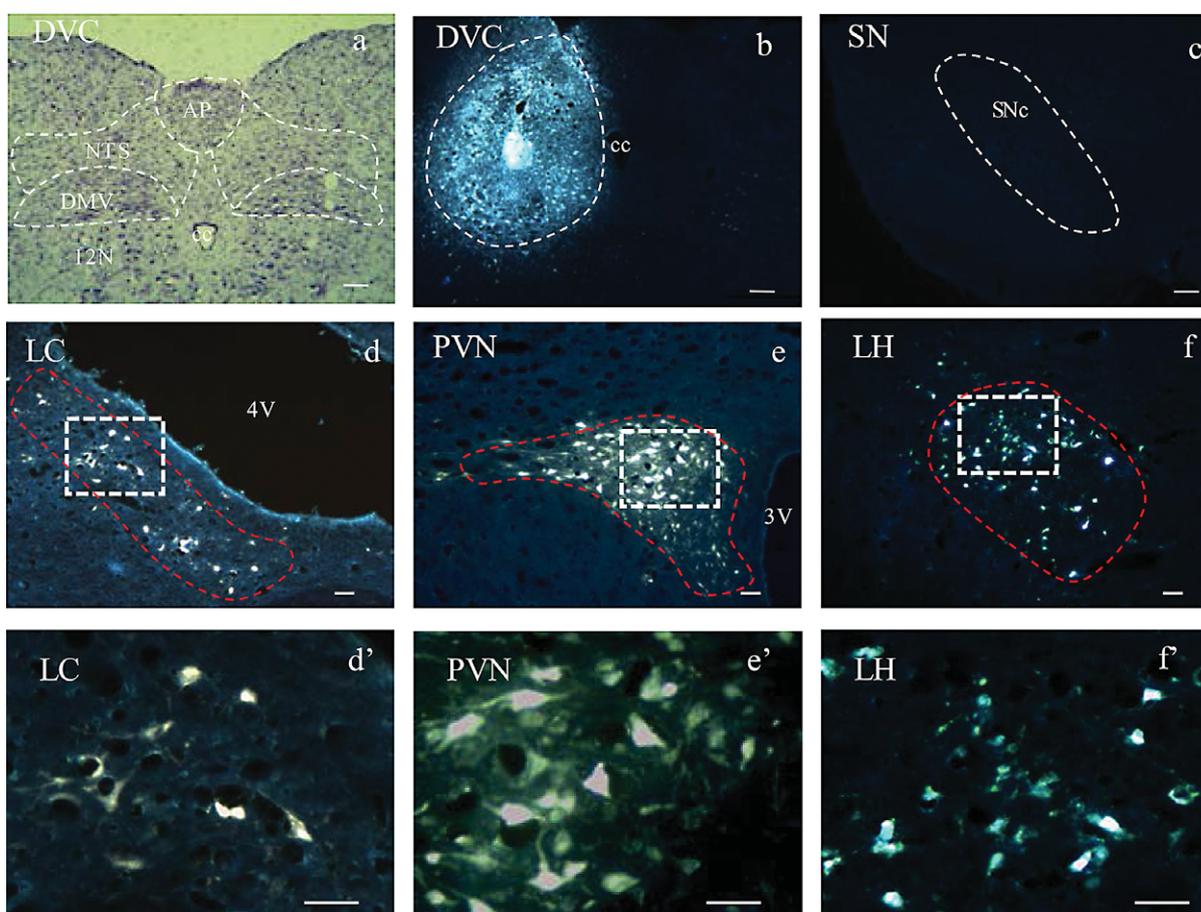


Fig. 2. Retrograde tracing results after injection of FG into the DVC. a. Nissl staining shows the adjacent position of the DMV, NTS, area postrema (AP), and hypoglossal nucleus (12N) in the brainstem. b. 4% FG injection site (white dotted lines) in the left DVC. c. No FG-labeled neurons were observed on either side of the SN. The white dotted lines indicate the left side of the SN pars compacta (SNc). Scale bar (a-c): 100 μ m. d-f: FG-stained retrograde neurons (red dotted lines) in the LC (d), PVN (e), and LH (f). d', e', and f' are the enlarged areas of the white dotted boxes in d, e, and f, respectively. cc: central canal; 4V: Fourth Ventricle, 3V: Third ventricle. Scale bar (d-f'): 50 μ m.

neurons and fibers (green) were observed in the NTS and DMV. Numerous ChAT-IR neurons (red) were observed in the DMV. The TH- and ChAT-IR neurons were medium-sized (approximately 20 μ m), with a round or oval shape. In 6-OHDA rats (Fig. 1B), the intensity of TH-IR neurons and fibers (green) was markedly increased in the DMV and NTS. By contrast, the intensity of ChAT-IR neurons (red) was visibly decreased in the DMV. And ChAT-IR neurons were surrounded by many TH-IR fibers.

FG Retrograde tracing results

To determine if the DVC was directly innervated by neurons of the SN, the retrograde tracer FG was microinjected into the left DVC. After 3 days, a heavy-

ly stained FG area was observed in the injected area (Fig. 2b), including the DMV and NTS. No FG-stained neurons were observed on either side of the SN (Fig. 2c). However, FG-labeled neurons were scattered on both sides of the LC (Fig. 2d) and lateral hypothalamus (LH) (Fig. 2f). The FG-labeled neurons in the LC and LH were medium-sized (approximately 20 μ m) with oval or triangular soma (Fig. 2d', f'). In addition, a cluster of FG-labeled neurons was observed on both sides of the hypothalamic paraventricular nucleus (PVN) (Fig. 2e). Many of these neurons were larger in size (approximately 25 μ m), wedge-shaped, and densely distributed in the area near the third ventricle (Fig. 2e'). In the LC, PVN, and LH, the number of FG-labeled neurons on the ipsilateral side and contralateral side were 280 ± 25 and 206 ± 17 ($n = 10$, $p < 0.05$),

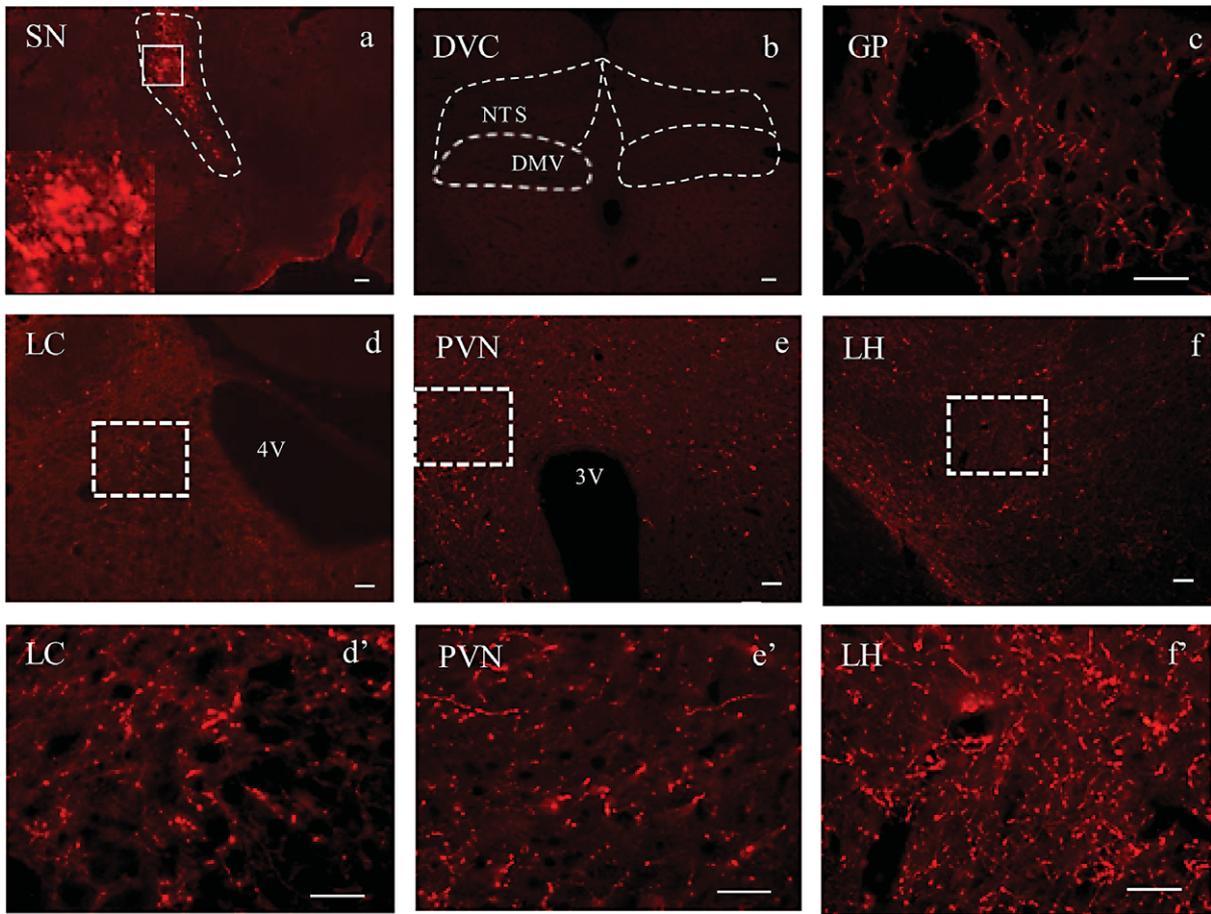


Fig. 3. Anterograde tracing results after injection of BDA into the SN. a. Injection site (white dotted lines) of BDA (heavy dots) in the SN. The area surrounded by a solid white line is magnified in the bottom left corner. b. No BDA-labeled fibers were observed in the bilateral DVC (including the DMV and NTS). c. Dense puncta and irregular curved BDA-positive nerve fibers were observed in the globus pallidus (GP). d-f: The distribution of BDA-stained anterograde fibers in the LC (d), PVN (e), and LH (f). d', e', and f' are the magnified areas of the white dotted boxes in d, e, and f, respectively. In the magnified pictures, the positive fibers are red puncta and curved irregular dotted lines. cc: central canal. Scale bar: 50 μ m.

695 \pm 25 and 620 \pm 34 ($n = 10$, $p < 0.05$), 340 \pm 25 and 325 \pm 23 ($n = 10$, $p > 0.05$), respectively. Moreover, FG-labeled neurons were also observed in the medial part of the bilateral inferior olive (IO) (data not shown).

Anterograde tracing results for BDA

The anterograde tracer BDA was microinjected into the left SN to determine if BDA-labeled fibers could be observed in the DVC. After 7 days, a dense BDA-stained area was located in the injection area, the left SN pars compacta (Fig. 3a). However, no BDA-positive fibers were observed in the DVC (Fig. 3b). Dense puncta and irregular curved BDA-positive nerve fibers were observed in the ipsilateral striatum and globus pallidus (GP) (Fig. 3c) and in the bilateral LC

(Fig. 3d, d'), PVN (Fig. 3e, e'), and LH (Fig. 3f, f'). BDA-positive fibers were also observed in the medial nucleus, parafascicular nucleus, ventromedial nucleus, and lateral nucleus of the thalamus on both sides (data not shown).

DISCUSSION

In this study, we demonstrate for the first time that neurons in the SN do not directly innervate the DVC; no FG-positive neurons were observed in the SN after injection of the retrograde tracer FG into the DVC, and no BDA-positive fibers were observed in the DVC after injection of the anterograde tracer BDA into the SN. However, FG-labeled neurons and BDA-labeled fibers

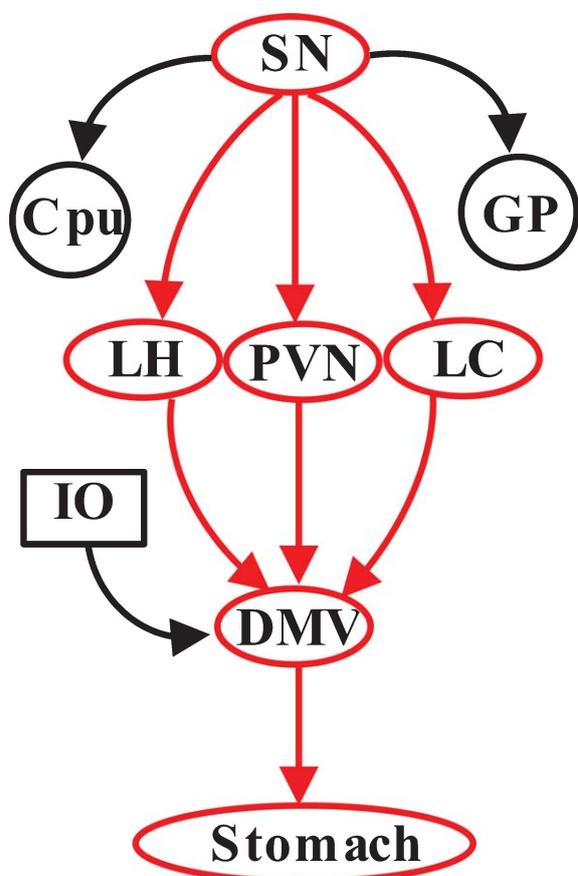


Fig. 4. Diagram of possible neuronal networks between SN and DMV. Red arrowheads represent the pathways from SN to DMV. The neurons in SN are also projected to the Cpu and GP, whereas the neurons in DMV also receive the innervation from IO. SN: substantia nigra; Cpu: caudate putamen; LH: lateral hypothalamus; LC: locus coeruleus; PVN: paraventricular nucleus; IO: inferior olive; GP: globus pallidus; DMV: dorsal motor nucleus of the vagus.

were observed in the hypothalamus and LC, respectively, which suggests that the DVC could be indirectly regulated by SN neurons through the hypothalamus and/or LC. Thus, the DMV pathology induced by the damaged SN in PD might be mediated by the hypothalamus and/or LC (Fig. 4).

Gastric dysmotility is common in PD patients, but the associated pathology has been poorly characterized in PD animal models. The DMV and NTS are the visceral motor and sensory nuclei, respectively, and the NTS is localized in the dorsolateral area of the DMV. In addition to their close localization, nerve fiber connections also exist between the DMV and NTS. The sensory fibers of the vagus nerve send sensory signals to the NTS, which integrates sensory information and

sends motor signals to the DMV. The vagal parasympathetic motor fibers originating in the DMV regulate GI motility.

Our previous data suggest that gastric motor function is impaired in both digestive and interdigestive phases in 6-OHDA rats [12], consistent with reports from clinical studies [19]. Cholinergic neurons in the DMV promote gastric emptying via the vagus nerve. However, catecholaminergic neurons (releasing NE or DA) inhibit gastric motility. In 6-OHDA rats, the decreased ChAT and increased TH expression in the DMV signify reduced Ach and enhanced NE or DA; this may contribute to the weakened gastric motility in 6-OHDA rats. Electrophysiological studies report that 71% of gastric-projecting motor neurons in the DMV respond to DA with either excitation (28%) or inhibition (43%) of the membrane via D1- or D2-like receptors, respectively [6]. The distribution of DA receptor-1 and receptor-2 on cholinergic neurons in the DMV provides further morphological evidence [20].

The DMV receives innervation from multiple nuclei [21, 22], and the NTS receives sensory signals from the stomach and sends motor signals to the DMV to regulate gastric motility [23]. TH-IR fibers originating in the NTS project to the DMV [24]. The DVC receives signals from both sides of the PVN, LH, LC, raphe nucleus, and IO [25]. For SN-LC-DMV pathway: It has been reported that SN neurons innervate LC, a major site of noradrenergic neurons in central nervous system [26], and NE is able to hyperpolarize the membrane potential and decrease the neurons excitability by binding to α_2 -adrenoceptors in DMV [27]. Except SN and DMV, the degeneration of LC neurons has also been reported in PD patients [28]. Therefore, the degeneration of SN neurons may affect the noradrenergic neurons in LC, in turn the DMV neurons through the SN-LC-DMV pathway. For SN-LH-DMV pathway: LH is one of the functional zones in hypothalamus regulating feeding, sleep and wakefulness [29]. LH contains orexins-A-IR neurons, and loss of the orexins-A-IR neurons has been reported in the progression of PD in human [30]. Orexin-A is able to excite DMV neurons via binding to the orexin-1 receptors of DMV in rats [31]. The stimulatory effect of orexin-A on rat gastric emptying has also been reported [32, 33]. Based on the physiological functions ascribed to the LC and LH, impaired function of LC and LH in PD has been associated with affective disorders, cognitive disturbances, sleep disorders, sensory impairment and autonomic dysfunction [34–36]. Degeneration of noradrenergic neurons in LC and the loss of orexins-A-IR neurons in LH might

lead to gastric dysmotility via the above hypothesized pathways in PD. For SN-PVN-DMV pathway: Rogers et al. found that stimulation of the PVN reduced gastric acid secretion [37] and inhibited gastric motility [38]. Immunohistochemistry studies have shown both vasopressinergic (VP) and oxytocinergic (OT) neurons located in the PVN [39]. Sofroniew et al. [40] confirmed there were direct projections from OT and VP neurons in the PVN to the DMV. All the three types of VP receptors (V1a, V1b and V2) and OT receptor (only one type) have been found in NTS and DMV [41]. OT and VP neurons in the PVN may regulate gastric motility via respective receptors in the DMV.

In PD patients, multiple neuronal populations in the brain were involved. We agree that in some PD alpha-synuclein (aSyn) pathology spreads according to Braak's theory. It is well known that PD includes idiopathic and sporadic PD. The progressive aSyn pathology associated with sporadic PD may advance according to a predictable caudo-rostral direction [42]. However, Kalaitzakis reported that the most affected regions in PD were the SN in 100% of cases, but in 7% of cases, no aSyn pathology was found in the DMV, in which the progressive aSyn pathology did not spread in a caudo-rostral pattern [43]. Therefore, Braak's theory could not explain the aSyn spreading process in all PD patients. It is possible that in the idiopathic PD (initial degeneration occurred in the SN), progressive aSyn pathology may spread through our postulated pathways (SN-LC/ hypothalamus-DMV).

For the hypothalamus, Langston and Forno in 1978 had reported that the expression of Lewy body exists in the lateral hypothalamus in PD [10]; Although aSyn pathology is not reported in the PVN and IO in PD, the aSyn pathology in the IO occurs in diffuse Lewy body disease [44]. It was reported that a long distance aSyn spreading must be triggered by overexpression of the protein in a concentration-, time- and connectivity-dependent fashion [42]. It is possible that the aSyn pathology may not be seen in these sites in PD because of the low concentration of aSyn protein.

Positive nerve fibers are observed in the LC upon injection of the retrograde tracers Alexa Fluor 555-conjugated cholera toxin B subunit or 1,1'-diiodo-3,3',3'-tetramethylindocarbocyanine perchlorate into the DVC of rats [45]. Injection of horseradish peroxidase (HRP) into the cat DVC induces positively stained neurons in the PVN [46], indicating that there are projection fibers from the PVN to the DVC [47]. This study is consistent with our results using the retrograde tracer FG. Anatomical data reveal that neurons in the LH project directly

to the DVC and receive ascending information from the NTS [45, 48]. These data indicate mutual interconnections between the LH and NTS, which were also observed in the present study. Many BDA- or HRP-stained nerve fibers are observed in the striatum and GP after injection of the respective anterograde tracer into the SN [49]; this is similar to our data using BDA, but no BDA-stained nerve endings were observed in the DVC. Taken together, the results of anterograde or retrograde tracing methods indicate an indirect projection pathway from the SN to the DVC.

In summary, the present study demonstrates increased catecholaminergic expression and decreased cholinergic expression in the DMV of rats after bilateral destruction of the SN by 6-OHDA. The DVC, including the DMV and NTS, is not directly regulated by SN neurons, but indirect regulation through the LC and/or hypothalamus might be involved. The present study provides morphological evidence for the regulation of the DMV and gastric motility by dopaminergic neurons in the SN.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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