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A Nigro-Vagal Pathway Controls Gastric Motility and is Affected in a Rat Model of Parkinsonism

Laura Anselmi, Luca Toti, Cecilia Bove, Jessica Hampton, and R. Alberto Travagli*

Department of Neural and Behavioral Sciences Penn State - College of Medicine, Hershey, PA

Abstract

Background & Aims—In most patients with Parkinson's disease, gastrointestinal (GI) dysfunctions, such as gastroparesis and constipation, are prodromal to the cardinal motor symptoms of the disease. Sporadic Parkinson's disease has been proposed to develop following ingestion of neurotoxins that affect the brain–gut axis via the vagus nerve, and then travel to higher centers compromising the substantia nigra pars compacta (SNpc), and, later, the cerebral cortex. We aimed to identify the pathway that connects the brainstem vagal nuclei and the SNpc, and to determine whether this pathway is compromised in a rat model of Parkinsonism.

Methods—To study this neural pathway in rats, we placed tracers in the dorsal vagal complex (DVC) or SNpc; brainstem and midbrain were examined for tracer distribution and neuronal neurochemical phenotype. Rats were given injections of paraquat once weekly for 3 weeks to induce features of Parkinsonism, or vehicle (control). Gastric tone and motility were recorded following NMDA microinjection in the SNpc and/or optogenetic stimulation of nigro-vagal terminals in the DVC.

Results—Stimulation of the SNpc increased gastric tone and motility via activation of dopamine 1 receptors in the DVC. In the paraquat-induced model of Parkinsonism, this nigro-vagal pathway was compromised during the early stages of motor deficit development.

Conclusions—We identified and characterized a nigro-vagal monosynaptic pathway in rats that controls gastric tone and motility. This pathway might be involved in the prodromal gastric dysmotility observed in patients with early-stage Parkinson's disease.

*Corresponding author: Dr R. Alberto Travagli, Department of Neural and Behavioral Sciences, Penn State - College of Medicine, 500 University Drive, MC H109, Hershey, PA 17033- USA, rtravagli@pennstatehealth.psu.edu.

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Author Contribution: Laura Anselmi: acquisition of data, analysis and interpretation of data, drafting of the manuscript, statistical analysis

Luca Toti: acquisition of data, analysis and interpretation of data, statistical analysis

Cecilia Bove: acquisition of data, analysis and interpretation of data, drafting of the manuscript, statistical analysis

Jessica Hampton: acquisition of data, analysis of data, statistical analysis

R. Alberto Travagli: study conception and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, obtained funding.

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Keywords

neurology; animal model; central nervous system; vagus

The extensive degeneration of dopaminergic neurons in the SNpc, and the presence of cytoplasmic aggregates of misfolded α -synuclein are pathophysiological hallmarks of PD. Movement disorders are the cardinal characteristic of PD, however a plethora of non-motor symptoms increase the overall morbidity significantly¹⁻³. GI dysfunctions, including gastric dysmotility and delayed emptying, as well as severe constipation, are prominent non-motor manifestations of PD; these issues are prodromal to the onset of motor symptoms, reduce significantly the patients' quality of life, and complicate the appropriate absorption of therapeutic agents, including L-DOPA⁴⁻⁷.

The upper GI tract, and the stomach in particular, is influenced heavily by vagal inputs originating from brainstem nuclei located in the DVC (i.e. nucleus tractus solitarius, NTS; dorsal motor nucleus of the vagus, DMV; and area postrema). Motor commands to the upper GI tract arise from the spontaneously active, preganglionic cholinergic parasympathetic motoneurons of the DMV⁸. These neurons integrate signals from higher centers^{9, 9, 10} as well as from the adjacent catecholaminergic neurons of the A2 area¹¹, which provide synaptic modulation via α -adrenoceptors¹². The efferent vagal fibers project to postganglionic myenteric neurons of the enteric nervous system (ENS) that ultimately control the motility response of the GI tract¹⁰.

In the search for the mechanisms that trigger Parkinsonian neurodegeneration, and based on the presence of misfolded α -synuclein aggregates in both ENS and DMV motoneurons, Braak's group postulated that idiopathic PD develops in six stages, beginning with the entry of environmental toxins into the GI tract. From there, the neurotoxins either themselves infiltrate the ENS or induce neuronal damage which spreads, via retrograde transport, through vagal pathways, to the DMV and, ultimately, the SNpc, basal ganglia and cortical areas^{13, 14}. Anatomical or physiological evidence that links the SNpc directly to the control of gastric motility and tone has not been uncovered yet.

Since the aforementioned circumstantial evidence suggests a pathophysiological connection between SNpc and DMV neurons, the aim of our study was to demonstrate the existence of the nigro-vagal pathway, to characterize this connection, to assess if this pathway regulates GI function, and whether it is compromised in a well-recognized rodent model of PD.

Preliminary accounts of the present work have been presented at the DDW meeting¹⁵.

Methods

Male Sprague-Dawley rats (250-350g) were housed in an AAALAC accredited animal care facility at 24°C on a 12:12 hour light cycle with food and water *ad libitum*.

Surgical procedures were performed using aseptic techniques and were conducted in accordance with NIH guidelines, with the approval of the Penn State University College of

Medicine Institutional Animal Care and Use Committee, and according to the policies and regulations of journal policy on animal experimentation.

Tracing

To identify SNpc neurons projecting to the DVC, either the retrograde tracer cholera toxin subunit B (CTB, 0.5% w/v, List Biological Laboratories, Campbell, CA) or the anterograde tracer dextran 10,000MW (in either the biotinylated (5% w/v) or the fluorescein (7.5% w/v) formulation, Life Technologies, Grand Island, NY) were dissolved in sterile saline and injected into the DVC or the SNpc, respectively.

Rats were anaesthetized with rodent cocktail (ketamine/xylazine/acepromazine; 80/1.6/5mg ml⁻¹kg⁻¹ i.p.), and placed in a stereotaxic frame. Three-five microinjections of CTB (150nl each) were made into the DVC (in mm: rostro-caudal (RC): 0.0-0.6 from calamus scriptorius; medio-lateral (ML): 0.2-0.4 from midline; dorso-ventral (DV): 0.5-0.65 from the brainstem surface). In another group of rats dextran (1µl/each injection) was microinjected unilaterally in two areas of the SNpc at in mm: (I) RC: 5.0 from bregma, ML: 2.4 from midline, DV: 7.6 from the surface of the dura mater; and, (II) RC: 5.6, ML: 1.6, DV: 7.8.

After 7 days of recovery, rats were anaesthetized, euthanized with a bilateral pneumothorax, perfused-fixed, and the midbrain at the level of the SNpc and medulla were processed for immunohistochemistry¹⁶ (see below).

Gastric studies

Rats were fasted overnight (water *ad libitum*) and anesthetized with thiobutabarbital (Inactin®; Sigma, St Louis, MO; 100-150 mg kg⁻¹ i.p.). Following a laparotomy, two miniature strain gauges (AT Engineering, Hershey, PA) were sutured to the circular smooth muscle of the anterior gastric corpus and antrum. Animals were then placed on a stereotaxic frame and the rat was instrumented for measuring the effects of microinjections in SNpc on gastric tone and motility as described in detail previously¹⁷.

NMDA was microinjected into the SNpc (0.2-10nmol/210nl; when conducting pharmacological experiments NMDA was used at 5nmol/210nl); following 30min of recovery, 2µl of a PBS solution containing either DA1 or DA2 receptors antagonists (SCH23390 or L-741,626 respectively; 45nmol), or α1 or α2 receptors antagonists (prazosin or yohimbine; 100/500pmol, respectively), or the dopamine reuptake inhibitor bntropine (100nmol) were applied to the surface of the 4th ventricle, at the level of obex, followed 2-5min later by a second NMDA microinjection.

In another group of rats the subdiaphragmatic right vagus was sectioned prior to strain gauge apposition¹⁷. NMDA was microinjected in the SNpc and, 30min later, the left cervical vagus was severed, thus attaining a complete vagotomy, and NMDA was microinjected again 45min later.

At the conclusion of the experiments, rats were euthanized, perfused-fixed, and brain and gastric tissues were harvested for immunohistochemistry and localization of microinjection sites¹⁶.

Tissue Processing

The rostrocaudal extent of SNpc and the DVC were sectioned into 50µm transverse sections. The microinjection sites were verified with cresyl-violet counterstain and visualized using a Nikon E400 microscope. The stomach was pinned to a wax-filled petri dish under light tension, and the longitudinal muscle-myenteric plexus was isolated by microdissection.

Immunohistochemistry

The detailed methods were described previously¹⁶⁻¹⁸. Primary antibodies were i) rabbit-α-choline acetyltransferase (ChAT; Chemicon, Temecula, CA; 1:5000); ii) mouse-α-tyrosine hydroxylase (TH; Chemicon; 1:10000); iii) rabbit-α-cFOS (Santa Cruz Technologies, Dallas, TX; 1:1000; in these studies, rats were fixed 90min after NMDA microinjection in SNpc); iv) mouse-α-dopamine-1 receptors (Sigma, St. Louis, MO; 1:2500); v) goat-α-CTB (List Biological Labs, Campbell, CA; 1:100000); vi) rabbit-α-PGP 9.5 (Millipore, Billerica, MA; 1:500); vii) mouse-α-Ser¹²⁹α-synuclein (Abcam, Cambridge, UK; 1:1000). For immunoperoxidase staining, secondary antibodies were biotinylated donkey immunoglobulins optimized for multiple labelling (Jackson Immuno Research Laboratories, West Grove PA; 1:500); the detection complex was ExtrAvidin-horseradish peroxidase (ExtrAvidin-HRP; Sigma; 1:1500).

For immunofluorescent staining, the secondary antibodies were: donkey anti-mouse-Alexafluor 488 or 568 and donkey anti-goat-Alexafluor 568 or 647 (ThermoScientific, Waltham, MA; 1:500).

Optogenetic studies

Following anesthesia with rodent cocktail, rAAV2-hSyn-eNpHR 3.0-eYFP or rAAV2-hSyn-eYFP (UNC vector core, 600nl of 10×10¹¹ titer) or saline were injected into the left SNpc at a rate of ~30nl/min. Animals recovered for 3-4 weeks and were then instrumented for *in vivo* gastric recordings as described above; photostimulation of the DVC was performed using a green laser (532nm, CrystaLaser, Reno, NV) controlled by TTL-pulses from a Grass S-48 stimulator (Warwick, RI). The light output at the tip of the fiber optic (200µm diameter, 0.37 optical aperture) was adjusted to provide a final output of 12-15mW before being placed stereotactically on the surface of the brainstem at 0.1-0.3mm RC from cc. The light stimulus was 10ms-long pulses at 20Hz until a plateau effect was obtained, for no longer than 3min.

Parkinson models

Following anesthesia with rodent cocktail and exposure of the SNpc, 3µl of the selective dopaminergic neurotoxin, 6-hydroxy-dopamine (6-OHDA; 4µg/µl) dissolved in saline with 0.02% ascorbic acid was unilaterally microinjected in left SNpc as described above¹⁶. Experiments were conducted 4-5 weeks later.

Rats received i.p. injections of 10mg/kg of paraquat, once a week for three consecutive weeks¹⁹ followed either by 2days or 2weeks of recovery. The motor performance (stepping test)²⁰ was assessed prior to treatment, every week thereafter, and on the day prior to the

gastric studies. Rats were held with one hanging forelimb at a time and the total number of steps was counted during a 1m/5s-long horizontal swift movement.

Data Collection, Analysis and Preparation of Figures

For immunoperoxidase staining, images were captured with a SPOT-RT color camera mounted on a Nikon E400 microscope. Immunoreactive neurons were counted every fourth slice. Cell count values are given as mean $\pm$ SEM neurons/section.

Immunofluorescent images were captured with an Olympus Fluoview FV1000 confocal laser scanning microscope with the FV10-ASV 2.0 viewer software (Center Valley, PA). Gastric tone and motility traces were analyzed with Clampfit® software (Molecular Devices).

All data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test, or t-test with GraphPad Prism (GraphPad Software Inc., LaJolla, CA). Significance was set at $p < 0.05$ with a two-tail test.

Results

Anatomical and immunohistochemical characterization of the nigro-vagal connection

Upon microinjection of the CTB in the DVC, CTB-positive neurons were found scattered throughout the SNpc. These CTB-positive neurons were TH immunoreactive (TH-IR) indicating their catecholaminergic, possibly dopaminergic, neurochemical phenotype (fig. 1Ab-d). Verification of the direct nature of these nigro-vagal projections was obtained following microinjection of the anterograde tracer dextran in the SNpc, which labeled fibers throughout the rostrocaudal extent of the DVC. A high density of fibers apposed both cholinergic neurons of the DMV (fig. 1Bb-g) as well as catecholaminergic neurons of the A2 area (fig. 1Bf). Conversely, in rats pretreated 3-4 weeks earlier with 6-OHDA to cause degeneration of SNpc neurons, dextran-IR fibers were not observed in the DVC (data not shown). Since neither CTB nor dextran cross synapses, these data suggest that the SNpc and the DVC are connected by a monosynaptic pathway.

Microinjection of the ionotropic glutamate receptor agonist NMDA in the left SNpc increased expression of the early gene cFOS-IR in ChAT-IR DMV (fig. 2Ba-b) and TH-IR (fig. 2Bd-e) neurons but not in neurons of XII, n. ambiguus or A1/C1 areas. This, in addition to the limited spread of tracers to adjacent nuclei such as XII or SNreticulata (see fig. 1), supports a direct and discrete nigro-vagal pathway. The data, summarized in Fig. 2C, suggest that an excitatory projection from SNpc impinges selectively onto both cholinergic neurons of the DMV as well as on catecholaminergic neurons of the A2 area.

Physiological role and pharmacological characterization of the nigro-vagal pathway in the regulation of gastric tone and motility

Microinjection of NMDA in the SNpc increased both the tone and motility of the gastric corpus and antrum in a dose-dependent manner (fig. 3A), but had no significant effects on heart rate (not shown).

In contrast, NMDA had no effects on gastric tone and motility when microinjected in the left SNpc of rats treated previously with 6-OHDA; Conversely, in the same rats, microinjections of NMDA in the right, i.e. untreated, SNpc increased corpus and antrum tone and motility similar to naïve rats (fig.3B). These data suggest that the pathway involves neurons of SNpc but not GABAergic neurons of SNreticulata.

Complete vagotomy abolished the increase in gastric tone and motility induced by NMDA microinjection in the SNpc, suggesting that not only this pathway is vagally-dependent, but is also physiologically and functionally relevant (fig.3C).

To elucidate the neurotransmitters released in the DVC upon SNpc stimulation, NMDA microinjections were carried out before and after application of SCH23390 or L-741,626 to the floor of the 4th ventricle. Perfusion with these dopaminergic antagonists did not change baseline tone and motility significantly (not shown, see fig. 4A). Representative trace and summary data (fig.4A and C) indicate that application of SCH23390 diminishes the NMDA-induced increase in corpus and antrum tone and motility significantly, whereas application of L-741,626 was without significant effect. Furthermore, dextran-labeled fibers from the SNpc appose DMV neurons with DA1 receptor-like immunoreactivity (fig.4B).

Upon application of benztropine on the 4th ventricle an increase of gastric tone and motility was observed. This excitatory effect, mediated by local increase in dopamine, was reduced by application of the DA1 receptor antagonist to the fourth ventricle (fig.4E-F).

Since fibers originating in the SNpc make contact with noradrenergic, TH-IR neurons of the A2 area (fig.1Bf), in different group of rats we investigated whether administration of α -adrenoceptor antagonists blocks the increase in gastric tone and motility induced by NMDA microinjection in the SNpc. In these rats, NMDA microinjections were made before and after application of prazosin or yohimbine to the floor of the 4th ventricle. Neither antagonist altered the NMDA induced increase in gastric tone, whereas the increase in gastric motility was reduced significantly by pretreatment with yohimbine (fig.4D).

Overall these data indicate that excitation of the nigro-vagal pathway increases gastric tone and motility via activation of DA1 receptors on cholinergic DMV neurons as well as noradrenergic neurons of the A2 area, that subsequently modulates vagal efferent control of gastric motility in an α 2 adrenoceptor-dependent manner.

Optogenetic stimulation of DVC

To further confirm the physiological relevance of the monosynaptic nigro-vagal pathway, a group of rats received halorhodopsin microinjections in the SNpc prior to measurement of gastric tone and motility. Inhibition of nigro-vagal terminals, via green light applied at the level of the DVC, decreased both basal (fig.5B-C) as well as the NMDA-induced increase in tone and motility (fig.5D-E, green bars). Application of SCH23390 to the floor of the 4th ventricle reduced the inhibitory effects of green light stimulation (not shown), further confirming a tonic dopaminergic input from SNpc. Conversely, in control animals that were either not transfected, or that received the virus without halorhodopsin, green light stimulation of the DVC had no effect (not shown).

Since the opsins used do not cross synapses, and the green light stimulation was applied at the level of the DVC, the gastric response obtained provides strong evidence to suggest that opsin-containing terminals from the SNpc make synaptic contacts with DMV neurons via a monosynaptic nigro-vagal connection.

The nigro-vagal connection is impaired in paraquat-treated animals

Paraquat administration induces experimental PD, and motor symptoms are tested typically after four or more weeks¹⁹. Motor performance tests, i.e. a stepping test, revealed a mild, albeit significant, decrease in motor performance 2 days after the 3rd and final injection of paraquat; the motor performance deteriorated further when animals were tested 2 weeks later (fig.6B). Optogenetic stimulation of the DVC in halorhodopsin-injected rats decreased baseline as well as NMDA-stimulated gastric tone and motility, a decrease that was significantly lower than that obtained in naïve, control, animals (fig.6C, vs. fig.5D-E). These animals show also a reduction of ChAT- and TH-immunoreactivity in the DMV and SNpc, respectively (fig.6D-E) as well as immunostaining for α -synuclein aggregates (fig.6Dc-Ec).

In paraquat-treated rats, microinjection of NMDA in the SNpc induced an increase in gastric tone and motility which was significantly lower than the increase obtained in naïve, control, littermates (fig.7A). Similarly, the impairment of the NMDA-mediated increase of gastric tone and motility was similar in rats tested 2 weeks after the last injection of paraquat (fig. 7A).

To investigate whether the reduced response in paraquat-treated rats was due to impairment of neural pathways vs. gastric smooth muscle, the response of rats to a i.v. injection of bethanecol, a muscarinic agonist that does not cross the blood brain barrier and excites the gastric smooth muscle directly, was examined. In these rats, the increase in gastric tone and motility was similar to that of controls (fig.7B). Likewise, the number (PGP9.5-IR) and neurochemical phenotype (ChAT-IR) of gastric myenteric neurons was similar in control and paraquat-treated rats (fig.7C-D).

These data indicate that the reduced gastric tone and motility response in paraquat-treated rats is due to impairment of the nigro-vagal pathway, rather than impairment of the gastric smooth muscle or the ENS.

Discussion

In the present study we have provided evidence for the existence of a novel, anatomically defined and physiologically functional, mono-synaptic pathway that connects the SNpc to brainstem nuclei of the DVC. By activating DA1 receptors on DMV cholinergic neurons as well as on catecholaminergic neurons of the A2 area, inputs from the SNpc modulate vagal efferent output regulating gastric functions. Using a well-recognized rodent model of PD, we also demonstrate that this nigro-vagal pathway is compromised prior to the full development of motor dysfunctions induced by paraquat.

The physiological relevance of our data is highlighted by the demonstration that pharmacological stimulation of SNpc neurons increases gastric tone and motility via a

dopaminergic, vagally-dependent pathway. Indeed the gastro-excitatory effects of SNpc stimulation are abolished by either subdiaphragmatic vagotomy, by 6-OHDA mediated ablation of the SNpc, or by DVC application of a selective DA1 antagonist. Increase of DA at the level of the DVC by application of benztropine induces the same DA1-mediated increase in gastric function. Likewise, the gastro-excitatory effects of SNpc stimulation are attenuated significantly by optogenetic inhibition of nigro-vagal fiber terminals impinging onto vagal motoneurons. In this regard it is important to highlight that photoactivation of halorhodopsins expressed in, and transported from, SNpc neurons to their fiber terminals in the DVC also decreases basal gastric tone and motility. These data support further the monosynaptic nature and tonic excitatory influence on DMV neurons, vagal efferent outflow, and gastric motor functions of this pathway. Interestingly, while microinjection of dopamine in the DVC is gastroinhibitory via DA2-mediated actions²², here we show that the response to benztropine is mediated via DA1 receptors. These data suggest strongly that the nigro-vagal fibers target selective subpopulations of DMV neurons dedicated to modulation of specific neurocircuits; the observation is supported also by the differential responses on tone vs motility obtained with yohimbine pretreatment. Indeed, the concept of distinct vagal neurocircuits comprising subsets of DMV neurons which modulate GI functions differentially was put forward several years ago²³ and has been supported by a wealth of evidence since¹⁰.

Given the pivotal role played by the degeneration of dopaminergic neurons in the SNpc in Parkinsonian pathology, and that functional GI disturbances affect the vast majority of PD patients prior to appearance of the cardinal motor symptoms^{1,3,24,25}, this newly identified nigro-vagal connection may occupy a central role in the etiology and the extrapyramidal dysfunctions observed in PD. The presence of tonically active dopaminergic inputs originating in the SNpc that modulate vagal efferent outflow may explain the previously reported intriguing experimental observation that gastrointestinal functions, as well as the neurochemical phenotype of both DMV and ENS, are altered as a consequence of 6-OHDA administration in the SNpc^{16,26-28,33,47,48}. Indeed, given the direct anatomical connection between the SNpc and DMV neurons innervating the gut, it is likely that this nigro-vagal pathway may serve as the means by which pathological alterations in gut functions arise from SNpc degeneration.

Apart from this “top-down” hypothesis, the nigro-vagal pathway may also serve as a means to permit the “bottom-up” pathogenesis of PD. The prodromal GI pathologies in Parkinsonian patients, and the presence of misfolded α -synuclein aggregates in both ENS and vagal motoneurons¹³ suggests that the etiology of idiopathic PD, at least in some cases, may indeed start in the GI tract, as postulated by Braak^{14,29}. According to this hypothesis, α -synuclein pathology begins in the ENS and spreads to the DMV through the vagus nerve, and then travels retrogradely to the SNpc and higher centers^{13,29}. The involvement of the vagal efferent fibers and the DMV at such an early stage of PD may account for the GI dysfunctions that affect Parkinsonian patients up to 20 years prior to clinical diagnosis. It is notable that a retrospective analysis of patients who received truncal vagotomy, thereby severing the gastric-vagal-DMV connection, showed a clear, and significant, reduction in the incidence of PD³⁰. Furthermore, the disrupted electromyogastrography recordings from

Parkinsonian patients closely resemble those recordings obtained from patients in the immediate, acute phase after a vagotomy^{31,32}.

While it is accepted that experimental and clinical PD alters the physiology and neurochemical phenotype of DMV neurons^{16,33-36}, in the absence of clear means by which the DMV pathology affects the SNpc, Braak's hypothesis is still open to debate³⁷⁻⁴⁰. Based on our data, it is tempting to speculate that the transport of α -synuclein pathology from DMV to SNpc neurons occurs by means of this newly-discovered nigro-vagal pathway. This would involve prion-like retrograde propagation of misfolded α -synuclein, which by crossing synapses, ultimately induces degeneration of pathway-specific neurocircuits. Indeed, several lines of evidence indicate that, in both humans and experimental animals, misfolded α -synuclein spreads between resident and grafted neurons as well as from neurons to astroglia^{21,29,39,41-46}.

In summary, we report the discovery of a novel monosynaptic nigro-vagal pathway which, by virtue of its susceptibility to administration of 6-OHDA and paraquat, may explain the dysfunction of brainstem vagal neurocircuits observed as a consequence of nigral degeneration. Likewise, this pathway may serve as a conduit allowing the direct access of ingested neurotoxins from DMV to SNpc neurons, thus triggering the prodromal GI dysfunction observed in PD^{1,3,13,21}. The identification and characterization of this neurocircuit opens unexpected avenues for the advancement of experimental investigations into both the etiology of environmentally-triggered Parkinson's disease, as well as the prodromal gastric dysmotility that adversely affects the quality of life of most Parkinsonian patients.

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Figure 1. SNpc provides a monosynaptic input to cholinergic and catecholaminergic neurons of the dorsal motor nucleus of the vagus (DMV) and A2 area

A. Representative micrographs showing: a) The location of the retrograde tracer cholera toxin-B (red) microinjection in the DMV, green=ChAT-IR, N=9. b) Low magnification image showing TH-IR (red, digital color) neurons in the SNpc of the animal shown in (a). c) Same panel as in (b) using filters for identification of the retrograde tracer (white, digital color). d) Enlargement of the dotted area in (c) showing TH-IR neurons of the SNpc (red) co-localized with retrograde tracer (white, arrows).

B. Representative micrographs showing: a) The location of the anterograde tracer biotinylated-dextran in the SNpc (red, green=TH-IR, N=5). b) low magnification of the DMV of an animal which received microinjection of the anterograde tracer biotinylated-dextran in SNpc, brown=ChAT-IR. c,d,e) enlargement of the dotted areas in (b) showing labeled fibers (black) apposing ChAT-IR positive DMV neurons. f) TH-IR (red) neurons in the DMV. Following microinjection of the anterograde tracer dextran-fluorescein in the SNpc (N=4), labeled fibers (green) can be seen apposing TH-IR DMV neurons of the A2 area. g) Neurolucida® reconstruction of the biotinylated-dextran labeled fibers shown in (b). DMV: dorsal motor nucleus of the vagus; NTS: nucleus tractus solitarius; XII: hypoglossal nucleus; cc: central canal; SNpc: substantia nigra pars compacta.

Figure 2. Chemical stimulation of SNpc increases c-FOS-IR in the DMV

A. Low magnification micrographs showing the location of ChAT- (brown, left panel) and TH-IR (brown, right panel) in the brainstem.

B. Representative micrographs of the intermediate DVC showing cFOS-IR in response to SNpc microinjections of saline (left panel, a) or NMDA (right panel, a). Note that following SNpc microinjection of NMDA, but not saline, cFOS-IR (blue) is increased in ChAT-IR neurons in the DMV (panel b, high magnification of the dotted area in the right panel a) but not in the nucleus ambiguus (c). Similarly, NMDA microinjection in the SNpc increases c-FOS in the A2 area (panel e, high magnification of the dotted area in the right panel, d) but not in neurons of the A1/C1 area (panel f).

C. Graphic summaries showing the co-localization of cFOS-IR in ChAT-IR neurons of the DMV, hypoglossus nucleus or nucleus ambiguus ($n=6$ for saline and NMDA; number of neurons/slice that co-localize cFOS-ChAT, left panel), and in TH-IR neurons of the A2 or A1/C1 areas ($n=5-6$ for saline and NMDA, respectively; right panel).

The cFOS-IR increase occurred bilaterally (cFOS-ChAT co-localization in DMV, right: from 1.9 ± 0.3 to 9.6 ± 1.3 , left: 2.3 ± 0.2 to 9.3 ± 1.1 ; cFOS-TH co-localization in A2 area, right: from 2.6 ± 0.4 to 5.2 ± 0.8 , left: from 3 ± 0.5 to 5.6 ± 1.2 ; $p < 0.05$ vs control for all).

* $p < 0.05$.

Figure 3. Chemical stimulation of SNpc modulates gastric tone and motility via vagal pathways

A. Left panel: representative recording from the anterior gastric corpus showing that microinjection of NMDA in the left SNpc increases tone and motility.

Right panels: Graphic summaries showing the dose-dependent increase in corpus (n=5-15 per each dose) and antrum tone (n=5-13 per each dose) or motility index in corpus (n=3-13 per each dose) and antrum (n=5-14 per each dose).

B. Left panel: representative recording from the anterior corpus of a rat that received 6-OHDA in the left SNpc. Following NMDA microinjection in the left SNpc, the gastric excitatory effects of NMDA were negligible indicating that they were mediated by catecholaminergic neurons of the SNpc (upper trace). Conversely, microinjection of NMDA in the right SNpc (untreated side) of the same animal increased gastric tone and motility (lower trace).

Right panels: graphic summaries showing the decreased response to NMDA microinjection in the left SNpc in corpus and antrum tone and motility in rats pretreated with 6-OHDA or saline in the SNpc. Tone: corpus, n=11; antrum, n=13. Motility: corpus and antrum, n=13. Conversely, microinjection of NMDA in the right SNpc (untreated side) increased corpus and antrum tone and motility. Tone: corpus, n=7; antrum, n=8. Motility: corpus and antrum, n=8. * p<0.05 vs untreated side.

C. Left panel: Representative recording from the anterior corpus of a rat that received a posterior gastric branch vagotomy. Microinjection of NMDA in the left SNpc increased tone and motility (upper trace); after the complete vagotomy, the NMDA microinjection was repeated (lower trace); the excitatory effects of NMDA were prevented.

Right panels: Graphic summaries showing the decreased response to NMDA microinjection in the left SNpc in corpus (black bars) and antrum (gray bars) tone and motility upon complete vagotomy. Tone: corpus and antrum, n=5. Motility: corpus and antrum, n=5. * $p<0.05$ vs control.

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Figure 4. The NMDA-induced increase in gastric tone and motility is mediated via DA1 receptors

A. Representative recording from the anterior corpus showing that the increase in gastric motility and tone following microinjection of NMDA in the left SNpc (left trace). Upon recovery, the DA1 antagonist SCH23390 was applied to the floor of the 4th ventricle and the NMDA microinjection was repeated (right trace). The excitatory effects of NMDA were attenuated significantly indicating that they were mediated by activation of DA1-like receptors.

B. Representative confocal micrograph showing that, upon microinjection of dextran-fluorescein in SNpc, a labeled fiber apposes a DMV neuron immunoreactive for DA1 receptors (arrow).

C. Graphic summaries showing the decreased response to NMDA microinjection in the left SNpc in corpus and antrum tone and motility upon pretreatment with SCH23390 (gray bars) but not by pretreatment with the DA2 antagonist L741,626 (white bars).

SCH23390, Tone: corpus, n=6; antrum, N=5. Motility: corpus: n=5; antrum, n=4 L741,626, Tone: corpus and antrum, n=7. Motility: corpus: n=6; antrum, n=7.

In all panels *p<0.05 vs control.

D. Graphic summaries showing that pretreatment with the α_1 adrenoceptor antagonist, prazosin (white bars), did not affect the increase in gastric tone or motility in response to left SNpc NMDA microinjection. Conversely, pretreatment with the α_2 adrenoceptor antagonist

yohimbine (gray bars) reduced the increase in corpus or antrum motility in response to left SNpc microinjection.

Prazosin, Tone and motility: corpus and antrum, n=5.

Yohimbine, Tone and motility: corpus and antrum, n=5.

In all panels *p<0.05 vs control.

E. Representative traces of gastric tone and motility from the anterior corpus showing that application of the dopamine reuptake inhibitor benztropine on the 4th ventricle increases gastric tone and motility (upper trace). Following application of the DA1 receptor antagonist SCH23390, the excitatory effects of benztropine were significantly reduced (lower trace).

F. Graphic summaries showing the decreased response to benztropine application on the 4th ventricle in corpus and antrum tone and motility upon pretreatment of DA1 antagonist SCH23390 (gray bar)

Tone and motility: corpus and antrum, n=5. In all panels *p<0.05 vs control.

Figure 5. Optogenetic inhibition of the DVC decreases gastric tone and motility, and attenuates the effects of SNpc stimulation

A. Microinjection of rAAV2/hSyn-eNpHR3.0-EYFP (green) in the SNpc does not alter the neurochemical phenotype of TH-IR (red) neurons in the SNpc.

B. Representative recording from the anterior corpus showing that optogenetic inhibition of the DVC with green light decreases baseline tone and motility.

C. Representative recording from the anterior corpus showing that optogenetic inhibition of the DVC with green light attenuates the increase in tone and motility resulting from NMDA-mediated (arrow) stimulation of the SNpc.

D. Graphic summaries showing the decreased baseline response to green light stimulation in the DVC in corpus (black bars) and antrum (white bars) tone and motility.

Tone: corpus, n=3,6 in controls (black and white bars), and opsin (green bars) rats respectively; antrum, n=3,8 in controls (black and white bars), and opsin (green bars) rats respectively.

Motility: corpus: n=3,6 in controls (black and white bars), and opsin (green bars) rats respectively; antrum, n=3,7 in controls (black and white bars), and opsin (green bars) rats respectively.

E. Graphic summaries showing the decreased response to green laser stimulus in the DVC in corpus (black bars) and antrum (white bars) tone and motility following NMDA-mediated stimulation of the SNpc.

Tone: corpus, n=3,5 in controls (black and white bars), and opsin (green bars) rats respectively; antrum, n=3,6,5 in controls (black and white bars), and opsin (green bars) or paraquat-treated (blue bars) rats respectively.

Motility: corpus: n=3,7 in controls (black and white bars), and opsin (green bars) rats respectively; antrum, n=3,7in controls (black and white bars), and opsin (green bars) rats respectively.

*p<0.05 vs control.

Figure 6. Paraquat treatment impairs motor activity, reduces the opsin-mediated inhibition of gastric tone and motility, and promotes α -synuclein formation in DMV and SNpc

A. Schematic representation of the paraquat-treatment schedule of administration and experiments.

B. Graphic summary showing the decrease in motor performance of rats treated with paraquat. Note that motor activity was already reduced 2 days after the last injection of paraquat, but continued to decline for at least 2 weeks after paraquat injection. n=10,7,3 in control, 2 days after last injection and 2 weeks after last injection, respectively. *p<0.05 vs baseline.

C. Left panel: graphic summary showing the decreased baseline response to green light stimulation in the DVC in corpus (striped bars) and antrum (blue bars) tone and motility. Tone: corpus, n=5; antrum, n=5. Motility: corpus: n=4; antrum, n=5 in controls.

Right panel: Graphic summary showing the decreased response to green laser stimulus in the DVC in corpus (striped bars) and antrum (blue bars) tone and motility following NMDA-mediated stimulation of the SNpc. Tone: corpus, n=4; antrum, n=5. Motility: corpus: n=4; antrum, n=5 in controls.

*p<0.05 vs opsins in naive rats (figure 5).

D. Representative micrographs showing the same area of the DMV of a control (upper) and a paraquat-treated (middle) rat; red=ChAT and green=¹²⁹Ser α -synuclein. Lower panels

show ChAT-IR (left), ^{129}Ser α -synuclein (middle) and merged (right) images of the dotted area in the middle panels. Arrows indicate DMV neurons with ^{129}Ser α -synuclein deposits. E. Representative micrographs showing the same area of the SNpc of a control (upper) and a paraquat-treated (middle) rat; red=TH- and green= ^{129}Ser α -synuclein. Lower panels show TH-IR (left), ^{129}Ser α -synuclein- (middle) and merged (right) images of the dotted area in the middle panels. Arrows indicate SNpc neurons with ^{129}Ser α -synuclein deposits. Note that in both panels D and E control animals did not show any ^{129}Ser α -synuclein-IR neurons.

Figure 7. The nigro-vagal pathway is impaired in paraquat-treated animals

A. Graphic summary showing that in paraquat-treated animals microinjection of NMDA in the left SNpc increased gastric tone and motility significantly less than in controls.

Tone: corpus, n=6,7,5 in control (white bars) and paraquat-treated animals 2 days (light blue bars) and 2 weeks (blue bars) after the last injection of paraquat respectively; antrum, N=6,6,5 in control (white bars) and paraquat-treated animals 2 days (light blue bars) and 2 weeks (blue bars) after the last injection of paraquat respectively.

Motility: corpus: n=6,7,6 in control (white bars) and paraquat-treated animals 2 days (light blue bars) and 2 weeks (blue bars) after the last injection of paraquat respectively; antrum, n=5,7,5 in control (white bars) and paraquat-treated animals 2 days (light blue bars) and 2 weeks (blue bars) after the last injection of paraquat respectively.

In all panels *p<0.05 vs control.

B. Graphic summaries showing that stimulation of the gastric smooth muscle via i.v.administration of bethanecol induced similar increase in gastric tone and motility in paraquat-treated rats.

Tone: corpus, n=5,7,4 in control (white bars) and paraquat-treated animals 2 days (light blue bars) and 2 weeks (blue bars) after the last injection of paraquat respectively; antrum, n=5,6,5 in control (white bars) and paraquat-treated animals 2 days (light blue bars) and 2 weeks (blue bars) after the last injection of paraquat respectively.

Motility: corpus: n=3,6,4 in control (white bars) and paraquat-treated animals 2 days (light blue bars) and 2 weeks (blue bars) after the last injection of paraquat respectively; antrum, n=3,6,4 in control (white bars) and paraquat-treated animals 2 days (light blue bars) and 2 weeks (blue bars) after the last injection of paraquat respectively.

C. Representative micrographs of gastric myenteric ganglia in a control (left) and a paraquat-treated (right) rat. Red=PGP9.5-IR and green=ChAT-IR.

D. Graphic summary showing that paraquat treatment did not reduce the proportion of cholinergic neurons in gastric myenteric ganglia.