ENVIRONMENTAL PARKINSON’S DISEASE:
PATHOPHYSIOLOGICAL CONSEQUENCES ON THE
FUNCTION OF THE DORSAL MOTOR NUCLEUS OF THE
VAGUS NEURONS

A Dissertation in
Neuroscience
by
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Submitted in Partial Fulfillment
of the Requirements
for the Degree of
Doctor of Philosophy
December 2019
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ABSTRACT

Parkinson’s disease (PD) is a neurodegenerative disorder caused by degeneration of dopaminergic neurons of the Substantia nigra pars compacta (SNpc), and by the presence of intracellular aggregates of α-synuclein. The dysregulated activity of the basal ganglia caused by lack of dopamine (DA) release onto this area results in the cardinal motor symptoms that characterize the disease, such as bradykinesia, tremor at rest and rigidity. Autonomic dysfunctions occur frequently in parkinsonian patients, especially gastrointestinal motility disorders which are prodromal to the clinical manifestation and diagnosis of PD. Chronic constipation and delayed gastric emptying are observed in many parkinsonian patients and impact their quality of life.

Based on the spatio-temporal pattern of α-synuclein aggregation in different neuronal populations, Braak and collaborators have hypothesized that non-genetic forms of PD, i.e. environmental forms of PD, may start in peripheral neurons, such as enteric neurons innervating the GI tract, and reach the central nervous system via vagal pathways. Moreover, the involvement of the vagus nerve in the etiology of this disease has been shown in both humans and experimental models.

The central hypothesis of this dissertation is that environmental toxins disrupt the brain-gut axis through a vagally-dependent enteric nervous system (ENS)- dorsal motor nucleus of the vagus (DMV)-SNpc pathway prior to the development of parkinsonism. This study will provide further evidence of vagal involvement in the onset of PD, and will elucidate the mechanisms underlying disease onset and progression.

Our results show that i) there is a direct pathway connecting the SNpc to the DMV, that this pathway modulates DMV activity through DA release, and it is impaired in PD; ii) DA release onto the DVC of naïve rats mediates a profound gastroinhibition, but a biphasic, i.e. excitatory followed by inhibitory, gastric response following PD induction; iii) subthreshold doses of the herbicide paraquat in combination with dietary lectins recapitulate all features of parkinsonism in the rat, replicate Braak’s spatiotemporal stages of α-synuclein deposition starting in the enteric nervous system and progressing to the DMV and SNpc, and produce prodromal gastric dysfunction; iv) parkinsonism in the rat produces prodromal electrophysiological alterations of DMV neuronal properties; and v) high doses of paraquat treatment alters the synaptic inputs onto DMV neurons.

These results shed more light on the role of the vagus nerve in this disease, and suggest that PD is not solely a neurodegenerative disease confined to basal ganglia and SNpc neurocircuits,
but a circuit-based disorder in which more than one area plays a role in the onset of the autonomic dysfunctions associated.
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ABBREVIATIONS

5-HT: 5-hydroxytryptamine
6-OHDA: 6-hydroxydopamine
ACh: Acetylcholine
ADP: Adenosine diphosphate
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP: Area postrema
ATP: Adenosine triphosphate
CAIP: Cholinergic anti-inflammatory pathway
cAMP: Cyclic adenosine monophosphate
CaV: Calcium channels of the voltage gated family
CCA: Common carotid artery
CCK: Cholecystokinin
CNQX: Cyanquixaline
CNS: Central Nervous System
CTB: Cholera toxin type B
CV: Cresyl violet
DA: Dopamine
DVC: Dorsal vagal complex
EEC: Enteroendocrine cell
EEG: Electroencephalogram
ENS: Enteric Nervous System
DMV: Dorsal Motor Nucleus of the Vagus
EPSC: Excitatory post synaptic current
GABA: γ-aminobutyric acid
GI: Gastrointestinal
GIP: Glucose-dependent insulinotropic peptide
GIRK2: G-protein-regulated inward-rectifier potassium channel 2
GLP-1: Glucagon-like peptide 1
GPe: Globus pallidus external segment
GI: Gastrointestinal
GPi: Globus pallidus internal segment
HCN: Hyperpolarization-activated, cyclic nucleotide-gated cation channel
I_{DR}: Delayed rectifier potassium current
IGLE: Intraganglionic laminar ending
IMA: Intramuscular arrays
IPSP: Inhibitory post synaptic current
K_{V}: Potassium channels of the voltage gated family
L: Lectins (from Pisum sativum)
L-NAME: Nitro-L-arginine methyl ester
LB: Lewy Bodies
LDOPA: Levodopa
LPS: Lipopolysaccharide
mEPSCs: Miniature excitatory postsynaptic currents
mGLUR: Metabotropic glutamate receptor
mIPSCs: Miniature inhibitory postsynaptic currents
NAmb: Nucleus ambiguus
NANC: Non-adrenergic non-cholinergic (pathway)
NMDA: N-methyl-D-aspartate
NTS: Nucleus of the tractus solitarius
P/PQ: Paraquat
PBS: Phosphate buffer saline
PD: Parkinson’s Disease
PFA: Paraformaldehyde
PKA: Protein kinase A
PP: pancreatic polypeptide
PPn: Pedunculopontine nucleus
PYY: peptide YY
ROS: Reactive oxidative species
SNpc: Substantia nigra pars compacta
SNr: Substantia nigra pars reticulata
STN: Subthalamic nucleus
TH: Tyrosine hydroxylase
TNF-α: tumor necrosis factor α
TRH: Thyrotropin-releasing hormone
TRPM2: Transient Receptor Potential Cation Channel Subfamily M Member 2
VNS: vagal nerve stimulation
α-syn: α-synuclein
ACKNOWLEDGEMENTS

I would like to acknowledge my mentor, Dr. R. Alberto Travagli, and everyone in the Brain-Gut laboratory at Penn State that guided me and allowed me to reach my goals during my Ph.D. I would also like to thank my committee members for their support on this project. Lastly, I would like to thank Gabriella Balsorio, Paolo and Eleonora Bove, for their ongoing and undying support throughout my studies, and Ian F. Krizner, for his unconditional encouragement during this last year of my Ph.D.
CHAPTER 1

INTRODUCTION

Cecilia Bove and R Alberto Travagli

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1 Chapter 1 consists of a previously published book chapter and review article. Cecilia Bove was the first author, drafted, edited and revised the completed both manuscripts, which have been reformatted to fit into this thesis. The citation for the book chapter is:
“Vagus nerve” Bove C and Travagli RA. Encyclopedia of Gastroenterology, 2nd Edition by Professor Ernst Kuipers
The citation for the review article is:
“Neurophysiology of the brain stem in Parkinson's disease.” Bove C and Travagli RA.
CHAPTER 1.1 THE VAGUS NERVE

The Xth cranial nerve, or the vagus nerve, is one of the most widely distributed nerves in the body. Vagus, in fact, is Latin for “wandering”, giving an immediate idea of how extensive its anatomical distribution is. In this book chapter, we will focus on the anatomy and physiology of the vagus nerve relevant to the control of gastrointestinal (GI) function.

The vagus nerve, originally named the pneumogastric nerve, originates from the caudal medulla oblongata and, in humans, is composed of 10 terminal branches, including the meningeal, auricular, pharyngeal, carotideal, superior laryngeal, recurrent laryngeal, cardiac, pulmonary, esophageal, and gastrointestinal. The latter branches provide parasympathetic innervation to the abdominal viscera, from the lower third of the esophagus to the colonic flexure. Within the medulla oblongata, the motor branches of the vagus nerve originate from two bilateral nuclei, i.e. the dorsal motor nucleus of the vagus (DMV) and the nucleus ambiguus (NAmb). As it emerges from the ventral medulla oblongata, rootlets of the vagus nerve can be identified between the inferior olive and the inferior cerebellar peduncle, in close proximity to the glossopharyngeal nerve (cranial nerve IX). From here, the rootlets of the vagus assemble into a single trunk that exits the skull through the jugular foramen, and forms the first ganglion, named the jugular or superior ganglion, which gives rise to the cutaneous branch of the nerve. Caudal and adjacent to the jugular ganglion lies the nodose ganglion, which contains the bipolar cell bodies of the vagus nerve (see below). At this level, the vagus nerve gives off the pharyngeal branches that join the glossopharyngeal nerve and the superior cervical sympathetic ganglion to form the pharyngeal plexus. The vagus nerve also sends off laryngeal branches which, when combined, form the vagus nerve proper at this level. The cervical, or superior branch of the vagus descends within the carotid sheath in proximity to the common carotid artery (CCA) and the internal jugular vein, although at times it can also be found anterior to the CCA. The cervical vagal branch divides into upper branches, which work in close relation with the sympathetic cardiac branches to modulate cardiac activity, and into lower branches, which arise from the root of the neck. Here, the right lower branch runs frontal to the innominate artery to innervate the deeper areas of the cardiac plexus, while the left lower branch runs alongside the aortic arch and joins the superficial part of the cardiac plexus. From this point onwards, the left and right vagus nerves have distinct anatomical differences. The right vagus passes in front, or anterior to, the subclavian artery and enters the thoracic cavity where it branches off as the right inferior laryngeal nerve. The inferior cardiac branches arise directly from the trunk of the right vagus lateral to the trachea, and then descend to the posterior aspect of the pulmonary root. Here, numerous vagal branches form the right posterior pulmonary plexus; its lower portion descends
as one branch adjacent to the esophagus, and, together with the corresponding branches from the left vagus, forms the esophageal plexus. Conversely, the left vagus runs along the left common carotid and subclavian arteries into the thoracic cavity posterior to left innominate vein. The left inferior laryngeal nerve emerges from the vagal branch passing anterior to the aortic arch at the lower border, and the inferior cardiac branch winds around the aorta. Then, at the level of the lungs the vagus nerve descends posterior to the root of the left lung, and the numerous rootlets spread to form the posterior pulmonary plexus. As with the right vagus, two branches emerge from the lower part of the pulmonary plexus, and together with branches from the contralateral side, form the esophageal plexus. From the entry point into the abdomen, the left and right vagal branches become the anterior and posterior trunks of the subdiaphragmatic vagus respectively, and are directed towards different target areas within the GI tract. Indeed, the anterior trunk divides in the anterior gastric branch innervating the anterior surface of the stomach, the hepatic branch innervating the pylorus, the proximal part of the duodenum, the liver and the pancreas, and the accessory celiac branch, which innervates the GI tract from the duodenum to the splenic flexure. The posterior trunk instead separates in to the posterior gastric branch, which targets the posterior aspect of the stomach, and the celiac branch which separates towards the spleen and the rest of the GI tract down to the colonic flexure.

Although the parasympathetic functions of the vagus nerve are mostly related to the efferent branches described above, the majority of the vagal fibers are afferent fibers that carry sensory information from the periphery back to the medulla oblongata. Peripheral information is collected from nerve endings embedded in the lamina propria, the thin layer of connective tissue at the base of the mucosa lining the GI tract, and from the muscularis externa, the smooth muscle layer adjacent to the submucosal layer responsible for peristalsis. These fibers receive information from the neuronal network constituting the enteric nervous system (ENS). The point of communication is at varicosities that allow the contact between the afferent vagus nerve and the two layers of the ENS. The vagal afferent fibers have their cell bodies residing in the nodose ganglion. These neurons are pseudounipolar: in fact, the axon branches toward the periphery as well as toward the medulla oblongata, where the information collected from the periphery is relayed\textsuperscript{2,3}.

**CHAPTER 1.2 DEVELOPMENT OF THE VAGUS NERVE**

During embryonic development, the vagus nerve emerges from the fourth and sixth branchial arch. These arches derive from the pharyngeal or visceral arches, evaginations of the mesoderm located on both sides of the developing pharynx, which in humans become apparent during the
fourth week of development. Sensory cell bodies located in the nodose ganglia are instead derived from the second and third epibranchial placodes, two of the neurogenic placodes deriving from the embryonic head ectoderm layer. Since the vagus is a mixed motor-sensory nerve, it comprises A-, B-, as well as C-fibers, which are classified as such according to their degree of myelination and conduction velocity. Specifically, the larger vagal A-fibers are myelinated, B-fibers are relatively smaller and myelinated, while, C-fibers are small and unmyelinated (see below for more details about the different types of sensory information carried by each fiber type). Formation of the myelin sheath starts between 14 and 17 weeks post-conception in humans, and proceeds until 10 weeks after birth, eventually reaching about 40,000 myelinated fibers in adulthood. The different characteristics of these fibers are crucial for determining different responses to vagal nerve stimulation, which will be discussed later.

CHAPTER 1.3 BRAINSTEM NUCLEI ASSOCIATED WITH THE VAGUS NERVE

Several nuclei, located within the medulla oblongata, the caudal-most portion of the brainstem immediately rostral to the spinal cord, are associated with both the afferent and efferent branches of the vagus nerve. In humans, the efferent branches of the vagus originate from the NAmb and the DMV.

The neurons in the NAmb provide innervation to the striated muscle of the upper GI tract, i.e. the esophagus and the lower esophageal sphincter. As the name suggests, the exact location of the NAmb within the brainstem is not defined clearly, however, it is embedded in the brainstem ventrolateral reticular formation, and is located anteromedial to the spinal trigeminal nucleus and posterior to the inferior olivary nuclear complex. In the rat, the NAmb comprises compact and semicompact subdivisions, where the latter includes the motoneurons innervating upper esophagus and pharynx, whereas the neurons in the compact subdivision innervate the esophagus, the pharynx, and the cricothyroid muscles. The viscerotopic organization of the rat NAmb is also present in humans, in fact the rostral portion of the nucleus, also called the rostral compact formation, innervates the esophagus, the intermediate semi-compact portion innervates the pharynx, and the caudal semi-compact portion innervates the larynx. The compact subdivision of the NAmb neurons also has extensive reciprocal projections with the NTS, as well as projections from the semicompact subdivision to the surrounding reticular formation.

The remainder of the GI tract, up to the splenic flexure, is under modulation by the preganglionic parasympathetic neurons of the DMV. This nucleus is located bilaterally alongside
the central canal, and the caudal (in rodents)/anterior (in humans) portion of the IVth ventricle, and dorsally (in rodents)/posterior (in humans) to the nucleus of the hypoglossus nerve (cranial nerve XII). In the rat, the neurons within this nucleus are organized in five nuclear columns running throughout its rostrocaudal extent and from which the five GI branches of the vagus nerve originate. Specifically, the anterior gastric, hepatic, and accessory coeliac branches are located in the left DMV, and the posterior gastric and coeliac branches are located in the right DMV. Neurons within the DMV have been also classified according to cyto- and chemoarchitectonic features in six types, five of which (classified as type I to V) include cholinergic preganglionic motor neurons, and one includes small, non-cholinergic interneurons. Analysis of transverse, sagittal and coronal sections of human medulla oblongata generated a more detailed classification of DMV neurons, subdivided in five types according to their size, shape and subnuclear localization. Specifically, in the DMV, smaller round or oval cells are found in the ventrorostral and ventrointermediate subnuclei; medium sized oval cells are found in the dorsorostral, centrointermediate and caudal subnuclei; medium sized fusiform and multipolar shaped neurons are part of the caudointermediate subnucleus; and larger triangular neurons are found mostly in the dorsointermediate subnucleus. Comparative analysis of the anatomy of human DMV with other species, including the monkey, the rat, the rabbit, and the pigeon, has revealed similar organization of the rostral division of the DMV in both the dorsal and ventral subregions. The intermediate division of the DMV contains the largest and most diverse proportion of cells across species, and it is the one predisposed to the modulation of gastrointestinal and pancreatic function. Interestingly, evidence in the rat suggests that different types of fibers originate from the intermediate division: C-fibers, characterized by their responsiveness to higher frequency stimulation and which innervate pancreatic islets, while A- and B-fibers instead are activated by very low frequency stimulation and innervate the stomach walls. Finally, the caudal division of the DMV revealed a loose neuronal structure in humans, mostly comprised of small interneurons, while in other species, notably the rabbit and pigeon, includes neurons innervating the esophagus. Since they are preganglionic, neurons in the DMV and NAmb utilize acetylcholine (ACh) as their main neurotransmitter.

Peripheral vagal inputs are conveyed to the NTS. In the rat, the NTS is located in the dorsomedial medulla starting at the level of the inferior olive nuclei alongside the central canal and the 4th ventricle and terminating rostral to the area postrema close to the caudal pole of the facial motor nucleus (VII cranial nerve). At the caudal level, below the area postrema, the nucleus fuses along the midline forming the commissural nucleus of Cajal, which can be found dorsal to
the central canal, dorso-lateral to the DMV, and medial to the reticular formation. Neurons within the NTS are of medium size, and can be morphologically described as multipolar/stellate, or bipolar. With rare exceptions, these neurons do not possess pacemaker activity; hence they rely heavily on modulation by synaptic activity and circulating hormones to function.

NTS nuclei have been described as being organized in a topographic manner according to the inputs received. Using the obex as an anatomical landmark, the NTS that lies caudally to the obex is, indeed, defined as the caudal NTS. Specifically, the subpostremal NTS is defined between the obex and the caudal tip of the AP, and the commissural NTS includes the portion ventral to the AP reaching to the caudal end of the nucleus. Both subnuclei receive subdiaphragmatic visceral afferents via the vagus and glossopharyngeal nerves in a topographical distribution. Briefly, esophageal and gastric mechanoreceptors, duodenal glucoreceptors, and hepatic chemo- and osmoreceptors project to the subpostremal subnucleus of the NTS. Conversely, the cecum is represented in the commissural NTS. These areas also receive baro- and chemoreceptors inputs.

The intermediate NTS receives fibers from the lingual-tonsillar branch of cranial nerve IX, which carries additional gustatory as well as somatosensory information. More rostrally, the intermediate NTS also receives afferent fibers from the larynx and the lingual branch of the trigeminal nerve, from the pharynx and the esophagus through the glossopharyngeal nerve, and more fibers that originate from its superior laryngeal branch. At the level of, and immediately rostral to, the area postrema, the NTS is further divided in the subnuclei medialis, centralis and lateral. At this level, the subnucleus centralis receives information from the esophagus, and the subnucleus commissuralis from the small intestine, conversely the subnucleus medialis receives sensory inputs from areas throughout the entire GI tract.

Finally, the rostral aspect of the NTS is devoted mostly to taste perception, receiving inputs from taste receptors located in the anterior tongue and the oral cavity. Within the dorsomedial NTS, two additional subnuclei can be distinguished, the subnucleus gelatinosus, which receives information from the stomach, and the intermediate subnucleus. The NTS also receives projections from the lungs, baro- and chemoreceptor fibers, which detailed description is beyond the scope of this chapter.

Vagal afferent fibers have different characteristics:
1) Special visceral afferent fibers arise from vagal fibers situated on taste buds of the epiglottis.
2) General somatic afferent fibers transmit general sensation from pharynx, larynx, trachea, bronchi, esophagus, concha of the ear, and other anatomical areas involved in gustation. Although the cell bodies that process this information are located in the jugular ganglion, they terminate in the spinal trigeminal nucleus, and pain sensation for these areas is relayed by the sympathetic nervous system.

3) General visceral afferent fibers transmit information from thoracic, abdominal viscera and aortic baro- and chemoreceptors. Cell bodies of these afferents are located within the nodose ganglion which plays a pivotal role in the regulation of respiratory reflexes, as well as in the general regulation of cardiorespiratory and visceral functions.

Based on the inputs from these afferent fibers, as well as from inputs carried by cranial nerves V, VII, and IX, the NTS projects to, and modulates NAmb neurons devoted to the control of swallowing and DMV motoneurons modulating GI functions. The NTS is also source of dense efferent projections targeting the amygdala, the hypothalamus, thalamus, parabrachial nucleus, raphe nucleus, locus coeruleus, and the cerebellum. The main neurotransmitter utilized by the tractus solitarius to relay the peripheral inputs is glutamate, which binds to both ionotropic and metabotropic receptors present on NTS neurons. Some vagal afferent neurons also make direct synaptic connections with the DMV, while others innervate the neighboring area postrema, located medially within the brainstem. NTS neurons receive a variety of additional peripheral sensory information, including information from the spinal cord, gustatory fibers, and glossopharyngeal nerves, and from other brainstem nuclei as well as higher CNS areas including the amygdala.

The activity of NTS and DMV neurons can be modulated by a variety of neurotransmitters and modulators, including circulating hormones that can access the circumventricular areas of the brainstem. Thyrotropin-releasing hormone (TRH), for example, produced by the paraventricular nucleus of the hypothalamus to regulate thyroid function, and by the medullary raphe nuclei in situations of cold stress and anticipation of feeding, depolarizes DMV neurons, increasing gastric acid secretion, tone and motility, that prepare the stomach for the incoming meal, as well as increasing pancreatic exocrine secretion. TRH further activates DMV neurons by acting at the level of the NTS to modulate inhibitory neurotransmission (see below). Indeed, activation of TRH receptors on inhibitory NTS nerve terminals increases cAMP/PKA levels such that GABAergic transmission is modulated by otherwise silent serotonergic inhibitory receptors. This disinhibition, together with direct activation of DMV neurons, results in a sustained activation of
gastric-projecting vagal efferent outputs. Such modulation of cAMP/PKA-dependent signaling by TRH, as well as other Gs-coupled neurotransmitters and modulators, appears to be a common mechanism by which the responsiveness of vago-vagal reflexes to other peptides, such as opioids, neuropeptide Y, and oxytocin, are modulated.

Orexin, also known as hypocretin, produced in the lateral hypothalamic area, is important for the regulation of appetite, as well as arousal and wakefulness. Both the NTS and the DMV perceive changes in glucose levels and, by consequence modulate pancreatic innervation. In fact, changes in peripheral glucose levels activate the lateral hypothalamus to secrete orexin, which in turn activates pancreas-projecting neurons in the DMV\textsuperscript{11}. The NTS-DMV synapse relies mostly on the release of γ-aminobutyric acid (GABA), but several other neurotransmitters are released by these neurons, including glutamate, catecholamines, glycine, and a variety of neuromodulatory peptides such as enkephalins, somatostatins, and glucagon-like peptide 1 (GLP-1)\textsuperscript{6}. Interestingly, the recent discovery of a monosynaptic nigro-vagal pathway highlighted the role of dopaminergic inputs from the substantia nigra pars compacta to the DMV in modulating gastric tone and motility, as well as gastric emptying\textsuperscript{6}. Indeed, dopaminergic inputs into a specific subgroup of DMV neurons appear to mediate an excitation of these neurons, and seem to be impaired in experimental models of Parkinson’s Disease. This could be a partial explanation of the prodromal gastrointestinal dysfunction associated with this disease\textsuperscript{12}.

CHAPTER 1.4 VAGAL CONTROL OF GASTROINTESTINAL FUNCTION

The vagus nerve innervates the GI tract to serve multiple purposes. In fact, in addition to regulating GI function and digestion, the Xth cranial nerve is crucial for the maintenance of the GI barrier, and for the regulation of the immune response in the digestive tract\textsuperscript{2}.

1.4.1 Regulation of GI function and digestion

Vagal control of the digestive tract can be described as two different, but connected processes: the first involving the acquisition of peripheral signals from vagal afferent fibers, elaborated by the NTS and relayed to the DMV; the second involving the activation of DMV neurons which innervate the GI tract directly. In this paragraph we will start by examining the different modalities of peripheral signaling that activate vagal afferents, to then explain the activity of the preganglionic parasympathetic neurons of the DMV.

The mechanical distention of the walls of the GI tract activates two distinct mecanoceptors: the intraganglionic laminar ending (IGLE) and the intramuscular arrays (IMAs). IGLEs are
expressed for the most part in the stomach, and are organized in close association with myenteric neurons and the surrounding connective tissue. Based on their location, IGLEs act as low threshold tension receptors detecting the mechanic transduction between the orthogonal muscle layers. Conversely, IMAs are arranged in parallel to muscle bundles in the longitudinal and circular muscle layers, in proximity of the interstitial cells of Cajal. IMAs are expressed densely in the lower esophageal sphincter, in the upper stomach, and in the pylorus. Although their exact functionality is still poorly understood, IMAs have been described as stretch receptors activated by shearing forces along their long axis.

Nutrient sensing is also a very important mechanism of vagal regulation of GI functions. When nutrients are detected in the lumen of the GI tract, enteroendocrine cells (EECs) respond by secreting, among other peptides, leptin from the stomach and cholecystokinin (CCK) from the small intestine. EECs are strategically placed nearby vagal afferent terminals, such that the first response to leptin and CCK is paracrine. In fact, the afferent vagal fibers express the leptin receptor Ob-R and the CCK receptor CCK-A. The result of this localized signaling is activation of the neurons within the NTS to mediate meal termination. CCK has also been shown to have direct effects on the stomach by inhibiting gastric emptying, and promoting biliary and pancreatic exocrine secretion. Moreover, circulating CCK acts centrally by modulating the activity of nodose ganglia, as well as DMV and NTS neurons directly. Interestingly, direct microinjection of CCK in the dorsal vagal complex decreases gastric tone and motility, reduces the gastric relaxation evoked by esophageal distention, i.e. the receptive relaxation reflex, and increases pancreatic secretions in the rat (for more details about the receptive relaxation reflex, and the vagal control of pancreatic function see below).

Several other neuropeptides are also important modulators of vagal activity. Like CCK, glucagon like peptide 1 (GLP-1) and ghrelin have been shown activate vagal afferent fibers, as well as neurons within the dorsal vagal complex. Specifically, GLP-1 is secreted by L-type EECs within the distal ileum and colon in response to glucose ingestion. Because of its ability to regulate glucose levels via insulin release, together with the glucose-dependent insulinotropic peptide (GIP), GLP-1 is defined as an incretin hormone. Originally identified as a gastrointestinal hormone, an effect which is observed at pharmacological doses only, GIP is released by K-type EECs of the small intestine in response to both glucose and fat ingestion. Although the vagus nerve is apparently not directly responsive to GIP, several lines of evidence indicate that GIP modulates GLP-1 activity following meal ingestion.
Ghrelin, a peptide secreted by oxyntic glands of the stomach, proximal small intestine, and pancreas when the stomach is empty, induces a powerful orexogenic function. In laboratory animals, ghrelin is secreted following the circadian feeding rhythm and it is secreted with the timing of scheduled meals in humans. Ghrelin acts by binding to the growth hormone secretagogue receptor expressed ubiquitously in the body, with a higher density in brain areas such as hypothalamus, pituitary, the AP, the DMV and the NTS, and brain regions connected with stress and emotional response.\textsuperscript{13}

Peptide YY (PYY) is released by enteroendocrine L-cells in the distal small intestine and proximal colon in response to ingestion of fats. In particular, its release is part of the ileal brake reflex mechanism (see below), and precedes the actual arrival of dietary lipids in the small intestine. Its action is believed to occur through vagal pathways, possibly through activation of vagal motoneurons of the DMV, to induce gastric relaxation and delayed gastric emptying.\textsuperscript{6}

Lastly, 5-hydroxytryptamine (5-HT; serotonin) is also capable of activating vagal afferent fibers. The activation of 5HT3 and 5HT4 receptors present on vagal afferent fibers is an important defense mechanism which results in emesis and diarrhea to dilute and expel harmful gastrointestinal contents.\textsuperscript{4} Moreover, serotonin inhibits pancreatic exocrine secretion (see below) by activating presynaptic receptors on cholinergic neurons, and modulates this activity by stimulation of the vagal afferent fibers.\textsuperscript{11}

As mentioned earlier in the chapter, given that DMV neurons are preganglionic parasympathetic they release acetylcholine, together with other neuroactive substances, including nitric oxide and monoamines. DMV cholinergic neurons send projections via general visceral efferent fibers to the postganglionic neurons of the myenteric plexus located between the longitudinal and the circular smooth muscle layers of the GI tract.\textsuperscript{6} Preganglionic parasympathetic neurons are also present in the NAmb, specifically in the external portion of the nucleus. These neurons play a major role in the parasympathetic control of esophageal and cardiac functions. Neurons from the dorsal division of the NAmb, on the other hand, innervate muscles of the soft palate, part of the pharynx, larynx and the upper portion of the esophagus, and carry special visceral efferent information related to swallowing, gagging, coughing, and vomiting.\textsuperscript{3}
The postganglionic parasympathetic neurons constitute two distinct pathways that ultimately modulate GI motility. The excitatory pathway releases acetylcholine to activate the smooth muscle/interstitial cells of Cajal by binding to excitatory muscarinic M3 and M1 receptors; the other pathway utilizes non-adrenergic non-cholinergic (NANC) neurotransmitters that induce muscle relaxation through release of nitric oxide, vasoactive intestinal polypeptide, or adenosine triphosphate. In contrast to the tonic release of acetylcholine, NANC transmission is phasic, hence activated “on demand” to counteract the cholinergic input. It is important to highlight that the ENS per se is independent, and as such is able to generate contractile activity autonomously. Therefore, both the vagal efferent cholinergic and the NANC pathways serve to modulate ENS activity, and provide the fine tuning for both this intrinsic activity, as well for the vagal reflexes described earlier in the chapter. The final GI output depends on the type of neurotransmitter released by the enteric neurons.

The vagus nerve serves a crucial role in the regulation of GI function through multiple vagovagal reflexes. While serving distinct functions, all vago-vagal reflexes share some basic core principles, and most act to mediate gastric relaxation of the stomach reservoir (a functional subdivision of this organ including the fundus and the proximal corpus). The starting point of all vago-vagal reflexes are the mechano- and chemo-receptors present within the walls of the GI tract. These signals stimulate vagal afferent fibers to excite NTS neurons which, in turn, regulate the activity of DMV neurons, hence vagal outflow, to adjust and match secretomotor functions in the appropriate portions of the GI tract.

The esophago-gastric reflex, also known as receptive relaxation reflex is triggered by mechanical distention of the lower third of the esophagus to reduce gastric tone. The resulting increase in gastric compliance allows the food bolus to enter the fundus with minimal increase in intragastric pressure; in other words, this reflex increases gastric volume and reduces gastric pressure such that food can be transported efficiently in the stomach. This apparently simple reflex relies on a specific microcircuit involving several key players starting with the mechanoreceptors in the esophagus. The vagal afferents involved have a low activation threshold, and activate specifically the catecholaminergic neurons of the subnucleus centralis of the NTS, which innervate the compact formation of the NAmb to control the esophagus, and the DMV, to induce gastric relaxation. Interestingly, some studies have shown that following esophageal distension DMV neurons respond by increasing or decreasing their firing rate. This suggests that following esophageal distension two different vagal pathways are engaged: the excitatory
cholinergic pathway is inhibited while the NANC pathway is activated, resulting in gastroinhibition\textsuperscript{6,14,15}.

The function of the gastro-gastric reflex, also known as adaptive relaxation or accommodation reflex, is to provide a means by which storage of the ingested food can be prolonged until it reaches an optimal level of digestion before its passage into the duodenum. This reflex also provides the co-ordination necessary for the gastric pump and reservoir to work appropriately. When the gastric reservoir fills and distends, excitatory reflexes stimulate contractions at the level of the antrum, such that the antral pump is immediately activated when food enters the stomach. Conversely, distention of the antrum produces inhibitory reflexes enhancing and prolonging relaxation of the gastric reservoir. Similarly, distention of the duodenal walls mediates gastric relaxation through the duodeno-gastric reflex, also known as the feedback-relaxation reflex, which allows digestion and absorption of nutrients in the small intestine. This feedback mechanism involves also other portions of the small intestine such as the jejunum and the ileum (i.e. jejunal and ileal breaks), for example, when nutrients, hydrochloric acid, or variations in the chyme osmolality, enter the small intestine. Activation of this reflex ultimately reduces the rate of gastric emptying. Although the vagus nerve has a predominant role in the regulation of this reflex, circulating hormones such as CCK, PYY, and GLP-1 regulate this feedback reflex, especially the ileal break portion of this reflex\textsuperscript{14,16}. Specifically, PYY seems to have a critical role in the “ileal brake” mechanism in which the presence of fatty acids in the ileum decreases gastric transit. Interestingly, PYY receptors are expressed on DMV neuronal membrane, indicating that this brainstem nucleus may play an important role in this physiological reflex. Moreover, PYY influences DMV activity indirectly by binding to its receptors expressed by NTS neurons\textsuperscript{17}.

1.4.2 Maintenance of GI barrier

Maintenance of the intestinal barrier is crucial to avoid unfavorable inflammatory processes and gastrointestinal injuries. Physiologically, the intestinal barrier is maintained by the expression of tight junctions, composed of protein complexes that form a narrow network sealing adjacent epithelial cells together. The main components of this network are the transmembrane proteins, claudins and occludins. Several conditions, including hemorrhagic shock and traumatic brain injuries can impair the intestinal barrier by reducing tight junction expression. Interestingly, vagal nerve stimulation seems to improve barrier integrity indirectly by activating the enteric glial
network. The activation of glial cells mediates the release of S-nitroglutathione, which has been shown to increase the gene expression of tight junction proteins and, overall, mucosal integrity.

Vagal nerve stimulation (VNS) is a procedure that gained increasing interest for the treatment of different peripheral and central conditions. VNS requires implantation of a bipolar electrode connected to a stimulation generator on the vagus nerve. Given that the left vagus innervates the atrioventricular node of the heart, while the right vagus innervates the sinoatrial node, the bipolar electrode is placed on the left vagus nerve such that the intervention does not influence heart rate significantly. The configuration of the bipolar electrode makes it possible to propagate the action potential from the periphery to the central nervous system by positioning the cathode at the proximal lead and the anode at the distant lead, which creates an anodal block in the latter. As mentioned earlier, the vagus is composed of different types of fibers, which are activated differentially according to the frequency chosen for the treatment. In particular, A-fibers have the lowest amplitude-duration threshold (0.02-0.2 mA), B-fibers are at an intermediate level (0.04-0.6 mA), and C-fibers are activated only at higher thresholds (over 2 mA). Activation of A- and B-fibers only by a weaker vagal stimulation has been shown to cause electroencephalogram (EEG) synchronization, while if the fiber recruitment includes C-fibers as well, the EEG is desynchronized. Generally speaking, clinically relevant VNS utilizes frequencies ranging from 20 to 30 Hz (which is subthreshold for vagal C-fibers), with 50 Hz being recognized to cause irreversible vagal damage.

In addition to the beneficial effects on intestinal permeability described above, VNS activates different brain areas including the NTS, the paraventricular nucleus of the hypothalamus, the parabrachial nucleus, the bed nucleus of the stria terminalis, and the locus ceruleus and, upon longer treatment, a more complex brain network including the peripeduncular nucleus, the frontal and cingulate cortex, the hippocampus, the basolateral amygdala, the nucleus accumbens and the dorsal raphe nucleus. Indeed, stimulation of the vagus has been of interest for a variety of diseases including epilepsy and treatment-resistant depression. The ability of vagal nerve stimulation to stimulate brain areas that are apparently unrelated to GI function suggest that the connectome associated with the NTS especially might be more complex than currently known and described, and highlight the importance of these brainstem areas.
1.4.3 Regulation of the immune response

Systemic inflammation is sensed in the brain through the highly permeable vasculature of circumventricular organs. A relevant circumventricular organ that influences greatly GI functions is the AP, which is in very close proximity to the dorsal vagal complex, itself with zones of high permeability. Indeed, both areas are activated by the presence of circulating endotoxins. Localized inflammation also activates the vagus, specifically vagal afferent fibers responsive to cytokines and chemokines. Intravenous and intraperitoneal administration of endotoxins induce activation of the nodose ganglia, NTS, DMV as well as an increase in splenic activity. Interestingly, subdiaphragmatic vagotomy prevents the activation of NTS and DMV neurons following intraperitoneal endotoxin administration, suggesting strongly that the vagus is playing a crucial role in the modulation of the spleen-mediated immune response by detecting localized and systemic inflammation. The splenic immune response is crucial in cases of systemic inflammation (i.e. sepsis), since its activation leads to release of TNF-α during the acute phase of reaction to systemic inflammation. VNS, as well as pharmacological and nutritional stimulation of the vagus, modulates the splenic immune response by suppressing strongly TNF-α release in a model of systemic inflammation. This effect is mediated by the cholinergic anti-inflammatory pathway (CAIP). As mentioned earlier in the chapter, vagal afferents act by releasing acetylcholine, which in this case binds to α7 nicotinic receptors (α7nAChR) expressed on splenic macrophages. Evidences suggest that α7nAChR-positive macrophages do not interact directly with vagal fibers, but rather modulate cholinergic enteric neurons. Other cell population express the α7nAChR and may contribute to the vagally-mediated CAIP, including dendritic cells, mast cells, and lymphocytes. A confirmation of the role of the vagus in modulating the immune splenic response comes from different experimental models. Vagotomized mice are more susceptible to develop colitis in response to dextran sulphate sodium administration compared to animals with an intact vagus; more severe colitis is associated to reduced levels of acetylcholine in the mucosa in a model of depression; lastly, an increase in the nitric oxide synthase-immunoreactive neuronal population over the acetylcholine-immunoreactive neurons has been recently described in the myenteric plexus of rats in an experimental model of necrotizing enterocolitis. Activation of CAIP has relevance in a variety of conditions. Indeed, one mechanism by which VNS might be beneficial in patients with treatment-resistant depression is by reducing the levels of proinflammatory cytokines, usually measured in the serum or cerebrospinal fluid of depressed patients, or by increasing the levels of antiinflammatory cytokines. CAIP prevented surgical induced inflammation, and reduced the immune response in diabetic-induced gastroparesis, colitis, and LPS-induced septic ileus.
1.4.4 Neural control of pancreas

Vagal inputs represent the major excitatory input to the pancreas. Here, the parasympathetic post-ganglionic neurons express nicotinic acetylcholine receptors as well as other neurotransmitter-specific receptors that are important in the modulation of the vagal efferent signals from the brainstem. Given the organization of the DMV mentioned earlier in the chapter, it is not surprising that DMV neurons projecting to the pancreas have a specific location in the left DMV, in an area comprising the hepatic and anterior gastric branches of this nerve. Some pancreas-projecting neurons could also be found scattered in the areas that correspond to the celiac branches of the vagus nerve. Moreover, the morphology and the electrophysiological properties of this subgroup of neurons makes them distinguishable from other DMV neurons. Indeed, pancreas-projecting neurons have a smaller soma area and larger diameter; they display a longer action potential duration, a slow apamin-insensitive afterhyperpolarization, and longer afterhyperpolarization decay time, together with a smaller afterhyperpolarization amplitude and higher firing rate. In addition to the inputs provided from the DMV, second order neurons involved in the neural regulation of pancreatic activity include the NTS, the AP, the accessory nucleus of the spinal trigeminal nerve, the raphe pallidus and obscurus, the substantia reticulata, the ventrolateral medulla and neurons of the A5 area, as well as specific nuclei within the hypothalamic region including the paraventricular, lateral, dorsomedial, and arcuate nuclei, the medial preoptic and retrochiasmatic area, the subfornical organ, and the bed nucleus of the stria terminalis. Higher order neurons also seem to have an involvement in the neural control of pancreas including those within the prefrontal, piriform and gustatory cortices, which are important in the cephalic phase of exocrine secretion, where the release of gut hormones and digestive enzymes happens before nutrients reach the digestive tract and can induce a physiological response.

The nodose ganglia neurons that innervate the pancreas are sensitive to capsaicin and contain both substance P and calcitonin gene-related peptide. Interestingly, the left and right nodose ganglion innervate different areas of this organ, the left ganglion innervating the splenic lobe, while the right ganglion innervates the duodenal pancreatic lobe. Regardless of the side-specificity, the vagal innervation shows an anatomical gradient favoring the head of the pancreas compared to the tail, where the final targets include blood vessels, pancreatic ducts, acini and islets, and only rarely the pancreatic ganglia. It is important to note, however, that there are important species-specific differences in the innervation of the pancreas. Indeed, while parasympathetic neurons innervate α and β cells in the mouse, these inputs are scarcer in humans.
The physiological consequence of pancreas stimulation from the DMV is the increase of both endocrine and exocrine secretion by distinct and specialized vagal circuits. Indeed, the vagus nerve modulates the intrinsic pacemaker activity of the pancreas, which is important for the daily pulsatile insulin secretion. DMV neurons that regulate the pancreatic endocrine secretion have been shown to respond to GLP-1, but not CCK nor pancreatic polypeptide (PP), to which neurons that regulate pancreatic exocrine secretions are responsive. Moreover, these two populations can be distinguished based on their responsiveness to metabotropic glutamate receptors: specifically, DMV neurons that regulate insulin secretion are modulated by the binding of glutamate to mGluRII, while neurons specialized in the control of exocrine secretion from the pancreas express mGluRIII. Peptides that modulate pancreas exocrine secretion, such as CCK, somatostatin, calcitonin gene-related peptide, and PP act through vagally-mediated pathways. These evidences are supported by experiments in which a) vagotomy almost completely abolished pharmacologically or electrically induced pancreatic exocrine secretions, and b) disinhibition of the DMV by blockade of the NTS-mediated GABAergic inputs increase pancreatic exocrine secretion.

CHAPTER 1.5. PARKINSON’S DISEASE

Parkinson’s disease (PD) is a neurodegenerative disease characterized by loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the formation of Lewy Bodies (LB), intracellular aggregates composed mainly of misfolded α-synuclein. Dopaminergic neurons of the SNpc are intrinsic pacemakers, although the excitability and firing rate of these neurons is modulated by GABAergic projections coming from the striatum, globus pallidus, and substantia nigra pars reticulate, and by ionotropic glutamatergic inputs arising in the subthalamic and in the pedunculopontine nuclei. SNpc neurons themselves project to the striatum, globus pallidus, subthalamic nucleus, SNpr and thalamus (Fig.1). PD-induced neuronal degeneration causes progressive loss of dopaminergic neurons in SNpc and dopaminergic inputs into the striatum that results in the emergence of oscillatory patterns of burst firing in output neurons of the basal ganglia, increased synchrony of the discharge of the neighboring basal ganglia, and an overall increase in basal ganglia output. These neurophysiological changes alter the motor thalamus, and diminish motor cortical activity leading to the key motor, as well as many non-motor, signs of PD.

The cardinal findings of PD, i.e. resting tremor, rigidity, and bradykinesia, often include a wide range of non-motor symptoms, including cognitive impairment, sleep and mood disorders, and gastrointestinal (GI) disturbances, such as esophageal dysfunctions, delayed gastric...
emptying, and constipation, which may appear up to 20 years prior to the clinical diagnosis of PD
25,26. Indeed, the occurrence of GI symptoms in otherwise healthy people has been associated with
an increased PD risk25,27-32.

While rare genetic mutations are certainly triggering factors in ~10% of sporadic PD cases33-
36, the vast majority of PD is idiopathic in origin, and increasing evidence indicates a triggering
role of ingested environmental neurotoxicants33,37-44. The involvement of the GI tract in PD
pathology is substantiated by the findings that, in PD patients, LB are present in myenteric
neurons of the enteric nervous system (ENS)45 as well as in the preganglionic motor neurons of
the dorsal motor nucleus of the vagus (DMV)6,46. Based on this distinct distribution pattern of LB
in the ENS and DMV, together with the prodromal GI symptoms, Braak et al. suggested that
idiopathic PD begins with absorption of an environmental “unknown pathogen” into the ENS.
The pathogen is then transported to the central nervous system (CNS) via the vagus nerve, and
spreads from the DMV to higher CNS areas34,38,47. Support for the involvement of the DMV in
the etiology of PD comes from the recent description of a monosynaptic nigro-vagal pathway in
the rat, which connects the SNpc to the DMV12.
Figure 1- Summary of the PD-induced alterations in the basal ganglia and nigro-vagal pathways.

Graphic representation of the pathophysiological basal ganglia and nigro-vagal neurocircuitry in PD. Regardless of the etiology, in PD there are increased glutamate inputs from the subthalamic (STN) and the pedunculopontine nuclei (PPn) to the substantia nigra pars compacta (SNpc), and from STN to the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr). Striatal alterations are determined by the decreased/lack of dopamine (DA) modulation, which results in increased inhibition of the external segment of the globus pallidus (GPe) and disinhibition of the GPi/SNr, resulting in increased inhibition of the thalamus. Similarly, the loss of dopaminergic inputs from the SNpc might impact the functions of the dorsal motor nucleus of the vagus (DMV) significantly, inducing vagally-mediated gastrointestinal dysfunctions associated with PD. The glutamate excitotoxicity observed commonly in PD may also occur within Nucleus of the tractus solitarius (NTS) to DMV synapse, providing an additional level of dysregulation of vagal control of gastrointestinal function. Thicker arrows represent increased neurotransmission. Conversely dotted lines indicate decreased neurotransmission. The black arrow between NTS and DMV indicates a mixed, excitatory and inhibitory neurotransmission.

Indeed, SNpc neurons innervate the brainstem dorsal vagal complex (DVC) including the DMV and the A2 area\textsuperscript{12}. When pharmacologically activated by SNpc excitation, this monosynaptic pathway increases gastric tone and motility, while optogenetic experiments have demonstrated that its tonically active nature\textsuperscript{12}. Moreover, the nigro-vagal pathway is impaired in
a paraquat (PQ)-induced rodent model of PD since in PQ-treated rats SNpc stimulation evoked significantly smaller gastric motor responses in treated animals. In a more recent publication from our group, Braak’s staging of idiopathic PD was modeled in the rat through gastric administration of subthreshold doses of a combination of PQ and lectins. Oral administration of paraquat and lectins resulted in the formation of LB in the ENS first, then in the DMV, and later in the SNpc, and recapitulated both the prodromal GI dysfunctions as well as the motor features of PD. Such disease progression was dependent on the integrity of the vagus nerve since vagotomy prevented both the appearance of α-synuclein in the DMV and SNpc as well as the motor dysfunctions, further highlighting the importance of the vagus and the DMV in PD etiology. In addition, since synaptic inputs from the nucleus tractus solitarius (NTS) finely regulate the spontaneous firing of DMV neurons, the possibility of alterations in NTS inputs to the DMV cannot be excluded.

Additional support for Braak’s theory derives from the evidence of the spread of α-synucleinopathy from the ENS to the CNS through the DMV as described in the rotenone model of PD and from a genetic mouse model expressing a mutated form of α-synuclein. Interestingly, misfolded α-synuclein was also detected in the DMV after injection of human post-mortem brain lysate from PD-affected individuals, following administration of recombinant α-synuclein in the ENS, directly in the vagus nerve, as well as in the peripheral neurons connected to centripetal vagal neurocircuits.

In this chapter, we will describe and summarize briefly the current knowledge on the electrophysiology of two of the areas involved in PD pathology, i.e. the SNpc and the DMV.

CHAPTER 1.6 SNpc

1.6.1. Basic properties and ionic currents

Adult SNpc neurons are characterized by spontaneous firing activity (2-4 pulses per second, p.p.s.) in the absence of synaptic input; such pacemaking activity relies mainly on Cav1.3 subunit-containing L-type calcium channels (I\textsubscript{Ca,L}), which are encoded by the gene Cacna1d. Following genetic deletion of Cav1.3, adult SNpc neurons can still fire action potentials due to the presence of hyperpolarization-activated, cyclic nucleotide-gated cation (HCN) and Na\textsuperscript{+} channels. The resulting phenotype is similar to that observed in juvenile SNpc neurons, where pacemaking activity is not dependent upon Cav1.3 but relies on the presence of HCN channels only. The transition from the juvenile to the adult phenotype is determined by, among other mechanisms, the insertion of the Cav1.3 subunit in the neuronal membrane, and a concomitant
A decline in the importance of HCN channels\textsuperscript{60}. Such changes in the protein expression profile have a profound impact for the understanding and treatment of PD. In fact, this developmental switch in channel-dependency for the pacemaking activity (occurring between P21 and P28 in rodents) might explain why adult brains are more susceptible to the development of PD compared to young brains that do not carry any genetic predisposition to the disease. Indeed, historically, an increase in intracellular Ca\textsuperscript{2+} levels and glutamate excitotoxicity have been associated with SNpc metabolic stress and neuronal death (see below)\textsuperscript{61,62}. Furthermore, the dependence on the Cav1.3 subunit, which has a more limited distribution in the brain compared to other subunits (i.e. Cav1.2) might explain why dopaminergic neurons of the SNpc are more susceptible to genetic or environmental insults than other neuronal subtypes that do not express the Cav1.3. This evidence supports the idea that calcium channel blockers such as isradipine, currently in Phase III clinical trial for use in PD, could be more beneficial in treating the disease compared to other currently available pharmacological therapies\textsuperscript{63-65}.

Other ion channels might play a role in the etiology of SNpc neuronal dysfunction, however. Potassium channels of the voltage gated family (K\textsubscript{V}) are responsible for the repolarization phase of the action potential, and regulate other important cellular functions, including proliferation, migration and programmed cell death\textsuperscript{66,67}. Acute application of rotenone, a neurotoxin able to induce PD via both inhibition of Complex I in the mitochondrial electron transport chain and increased oxidative damage\textsuperscript{68,69}, inhibits the potassium delayed rectifier, I\textsubscript{DR}, but not the fast-transient I\textsubscript{A}, current in ventral mesencephalic neurons\textsuperscript{70}. The inhibition of IDR may be one of the mechanisms responsible for rotenone toxicity and nigral cellular death in PD since, in certain cellular types, inhibition of K\textsuperscript{+} channels is associated with apoptosis\textsuperscript{71,72}. For example, in a transgenic mouse model of mutated \(\alpha\)-synuclein, an increase in the intrinsic firing rate of dopaminergic SNpc neurons, associated with a glutathione-sensitive dysfunction of the A-type K\textsubscript{V} 4.3 channels\textsuperscript{73}, is the first indicative event of dopaminergic dysfunction. The aforementioned evidence supports the “stressful pacemaker theory” and provides a possible explanation of why dopaminergic SNpc neurons, but not other neuronal populations, are more susceptible to the toxicity and cellular death induced by \(\alpha\)-synuclein aggregates\textsuperscript{74}. The exact mechanism by which accumulated \(\alpha\)-synuclein affects K\textsubscript{V} 4.3 channels selectively is not understood fully, but oxidation of the protein structure might be involved\textsuperscript{75-77}.

Rotenone has been shown to open mitochondrial K\textsuperscript{+}ATP channels and increases mitochondrial reactive oxidative species (ROS), eventually leading to accumulation of both Na\textsuperscript{+} and Ca\textsuperscript{2+}\textsuperscript{78}. These intracellular effects, specific to SNpc neurons, lead to the activation of membrane-bound Transient Receptor Potential Cation Channel Subfamily M Member 2
(TRPM2), non-selective cationic, and ADP-ribose-gated channels, all of which are highly sensitive to ROS levels, and have been linked to neuronal damage and other PD-like pathologies\textsuperscript{70-81}. Surprisingly, in a rodent model of α-synuclein overexpression, G-protein-regulated inward-rectifier potassium channel 2 (GIRK2) channels, which are expressed on DA neurons cell membrane and are affected in pathological conditions including chronic inflammation\textsuperscript{82} and metabolic dysfunction\textsuperscript{83}, did not show any impairment\textsuperscript{84} suggesting some degree of specificity in ion channel pathology, rather than a broad non-selective dysfunction.

Indeed, in the same model of α-synuclein overexpression, alterations of the intrinsic membrane properties of SNpc neurons were not observed even in the presence of neurological symptoms. Analysis of the amplitude and density of the I\textsubscript{H} current revealed a reduction of both parameters, leading the authors to suggest that α-synuclein overexpression and accumulation reduce the number of functional I\textsubscript{H} channels in this model\textsuperscript{84}. Similarly, in a rodent model of human α-synuclein overexpression, in which the striatal level of α-synuclein matched those of patients with both familial and sporadic PD, extracellular recordings of SNpc revealed a significant reduction in the firing rate only in aged mice, suggesting an age-dependency in the α-synuclein overexpression-mediated hyperpolarization\textsuperscript{82}. Interestingly this model displays disruption only in dopaminergic neurotransmission from SNpc, leaving other neurotransmitters and other DA inputs intact\textsuperscript{82}. These data suggest that dysfunction of specific ion channels might not be sufficient to disrupt SNpc neuronal function, and that other factors, e.g. age and, more broadly, environmental factors, might be necessary for the alterations to become apparent.

1.6.2. Glutamate

Glutamate neurotoxicity has been described as a possible mechanism underlying various diseases state, including PD\textsuperscript{85-88}. Alterations in glutamatergic transmission at the level of the SNpc have been observed in different models of PD, including genetic and neurotoxin-dependent rodent models. Dopaminergic neurons in the SNpc receive excitatory glutamatergic inputs mediated by activation of both NMDA and non-NMDA receptors\textsuperscript{89}. This glutamatergic drive originates in neighboring areas, including the subthalamic nucleus\textsuperscript{90}, the pedunculopontine nuclei\textsuperscript{91,92}, as well as excitatory neurons within the substantia nigra itself\textsuperscript{89}.

Perfusion of isolated midbrain slices with rotenone induces a significant increase in NMDA-dependent currents in dopaminergic neurons of the SNpc\textsuperscript{93} dependent on Mg\textsuperscript{2+} block removal and mediated by a tyrosine kinase-dependent receptor phosphorylation, similar to that described in other neuronal populations\textsuperscript{94-96}. In fact, low levels of Mg\textsuperscript{2+} in the perfusion solution replicated rotenone effects\textsuperscript{97}. In line with its molecular mechanism of toxicity, rotenone effects were
prevented by pretreatment with the antioxidant N-acetyl-cysteine, showing a direct link between glutamatergic excitotoxicity and oxidative stress. Moreover, DA seems to be necessary for the increase in NMDA currents, since DA depletion by treatment with α-metyl-p-tyrosine, an inhibitor of tyrosine hydroxylase, prevented the alteration in NMDA-mediated glutamatergic currents. Wu and Johnson also suggested that the molecular mechanism underlying this potentiation might involve a tyrosine kinase-dependent phosphorylation process most likely recruited by the increase in ROS, rather than a direct effect of ROS on NMDA receptors. Kress and Reynolds further demonstrated that, in organotypic cultures of substantia nigra-striatum-cortex, perfusion with 6-hydroxydopamine (6-OHDA), a selective catecholaminergic toxin, and 1-methyl-4-phenylpyridinium, which interferes with the complex I of the mitochondrial respiratory chain, do not induce DA neurons loss in the absence of NMDA currents, inferring a major role of glutamate excitotoxicity in the pathophysiology of PD. Similar results were obtained in cultured mesencephalic neurons after exposure to a combination of rotenone and glutamate, in which both AMPA and NMDA receptors played a role in determining rotenone toxicity. In particular, NMDA receptor activation induced a stronger effect, which was dependent on the influx of sodium ions through the open channel.

Acute application of the herbicide PQ to midbrain slices of rodent suppressed AMPA-mediated evoked excitatory post synaptic currents (EPSCs) and reduced the amplitude of miniature EPSCs in the SNpc. These data suggest that PQ, regardless of the mechanism of absorption in the brain, inhibits postsynaptic AMPA receptors, ultimately reducing the glutamatergic input and the excitability of dopaminergic neurons of the SNpc. The consequence of the reduced glutamatergic drive has been postulated to result in abnormal DA release, which might explain the motor symptoms of PD. Indeed, PD patients appear to have lower glutamate content than age-matched non-PD individuals. Moreover, it is possible that the vulnerability of SNpc is determined by the expression of different subunits of ionotropic glutamate receptors. Indeed, areas such as the hippocampus and thalamus are unaffected in PD and express ionotropic glutamate receptors with different subunit composition that may prevent their vulnerability.

In an animal model of spontaneous α-synuclein overexpression, a dramatic reduction in the frequency, but not in the amplitude, of spontaneous EPSCs was reported. These data imply that over-expression of α-synuclein results in a pathological presynaptic effect at the mesencephalic level that reduces the normal glutamatergic drive onto the dopaminergic neurons in the SNpc. Such spontaneous excitatory events, moreover, seemed to be relying solely on AMPA receptors, since they were blocked by the selective AMPA-receptor antagonist, CNQX. In the same over-
expression model, α-synuclein accumulation was also observed in the frontal cortex. Stimulation of cortico-striatal glutamatergic fibers projecting onto striatal GABAergic medium spiny neurons increased the paired pulse ratio, without altering the AMPA/NMDA ratio, suggesting that α-synuclein over-expression impairs cortico-striatal glutamatergic transmission. Since the loss of dopaminergic neurons is significant, albeit not dramatic with this model, the motor deficit observed by Stoica et al. appears to derive from impairment of the glutamatergic transmission from the striatum, rather than from SNpc neuronal loss.

It is important that to bear in mind, however, that glutamate receptors are also modulated by other PD-related neuroactive agents, including levodopa (L-DOPA) and its major oxidation product, DOPA quinone. In SNpc, L-DOPA administration targets mainly D2-receptors to induce a sulpiride-sensitive hyperpolarization. In the presence of sulpiride, however, L-DOPA administration depolarizes the membrane via activation of AMPA/kainate receptors, and, in part, by NMDA receptors. This glutamate-mediated effect of L-DOPA was, however, also observed in a model of ischemia, where endogenous DOPA stimulates striatal glutamate release and induces delayed cellular death. Analysis of spontaneous EPSCs recorded from DA neurons in the SNpc, however, revealed only postsynaptic actions of L-DOPA, implying that the observed effects may not be due to increased glutamate release. These controversial data suggest that, in addition to the beneficial increase in DA levels, L-DOPA may induce excitotoxicity that could worsen the progression of PD. Contrary to the these potentially toxic effects, in a 6-OHDA model of PD, acetylcholine release was inhibited following injections of L-DOPA, but not dopamine, in the area encompassing the median forebrain bundle. Thus, together with actions to facilitate dopamine release and activate postsynaptic D2 receptors, these observations might account for the efficacy of exogenous L-DOPA in alleviating PD symptoms in the earlier stages of PD. Such actions, however, may lead to glutamate-driven L-DOPA auto-oxidation, amplifying the excitotoxicity observed with glutamate alone, making L-DOPA therapy actually detrimental at later PD stages. A caveat should be borne in mind, however, that these data may apply to in vitro studies only, since they do not appear to be supported by clinical literature.

1.6.3. Dopamine and L-DOPA

Administration of L-DOPA inhibits the firing of dopaminergic neurons through a membrane hyperpolarization determined by the activation of D2 autoreceptors. Physiologically, DA also enhances GABAergic-mediated inhibitory post synaptic potentials (IPSPs) by acting on D1-like receptors expressed on presynaptic GABAergic terminals in the midbrain. DA can also enhance glutamate metabotropic IPSPs by modulation of postsynaptic noradrenergic α1
receptors\textsuperscript{122,123} or inhibit excitatory inputs by activation of presynaptic D2-like or 5-HT receptors\textsuperscript{124}. However, Federici et al. have demonstrated that, in the midbrain, DA reduces GABA\textsubscript{B}-mediated post synaptic potentials without involvement of D1-like or D2-like receptors, actions that can be attenuated by prior application of serotonin, suggesting that DA acts via activation of 5-HT receptors, in particular the 5-HT\textsubscript{1B} receptors, likely located on GABAergic terminals. The mechanism by which DA, hence L-DOPA, modulates GABAergic signaling may be critical in the context of PD, where a sustained inhibition of the GABA synapses in the ventral midbrain may underlie some of the beneficial effects of L-DOPA\textsuperscript{125}.

In summary, then, in addition to its widely described, and therapeutically beneficial, effects in PD, dopamine has also been implicated in some forms of neurotoxicity\textsuperscript{126}. Treatment with the dopamine precursor L-DOPA, certainly increases the efficacy of dopaminergic transmission, improves the health status of the non-degenerated DA neurons in the SNpc, and alleviates parkinsonian motor symptoms by providing the cellular machinery that leads to DA production. This is an energetically demanding process, however, which increases the production of reactive oxygen species and toxic byproducts, such as DA-derived quinones \textsuperscript{127,128}. This observation has led to the controversial hypothesis that long-term treatment with L-DOPA may potentially lead to increased stress in vitro but not in vivo\textsuperscript{56,115-119}.

CHAPTER 1.7 DMV

Cholinergic neurons of the DMV have been studied extensively, and their basic properties have been described thoroughly, especially in relation to GI-related functions\textsuperscript{6,129-132}. These neurons are spontaneously active with a firing rate of approximately 1p.p.s. \textsuperscript{133}, generated by HCN cationic channels and modulated by a large array of synaptic inputs\textsuperscript{6,134}. The spontaneous firing activity is the main source of calcium influx into DMV neurons, with minimal dependence on the activation of the I\textsubscript{Ca-L} voltage gated calcium channels expressed on their membrane. Although remarkably resilient to different types of stressful environments\textsuperscript{135-139}, this membrane phenotype makes DMV neurons prone to mitochondrial stress, potentially to similar extent as dopaminergic neurons of SNpc. Moreover, intracellular calcium is buffered weakly by DMV neurons, which do not express calcium binding proteins such as calbindin and parvalbumin, but rather rely on ATP-dependent pumps and other intracellular organelles for calcium buffering \textsuperscript{140}. Other studies, however, suggest that DMV neurons are remarkably resilient to stress, giving a potential explanation for the differences in neuronal survival between DMV and SNpc neurons \textsuperscript{141}.  

1.7.1 Basic properties and ionic currents
Few electrophysiological studies of DMV neurons in experimental models of PD have been carried out. One recent study examined the effects of α-synuclein over-expression in mice\(^{142}\). In the presence of a cocktail of glutamatergic, GABAergic, and cholinergic receptor antagonists, α-synuclein over-expression had minimal effects on the firing rate of DMV neurons\(^{142}\) which, in this model, appears to be driven by a persistent sodium current. α-synuclein over-expression in DMV neurons resulted in a small, albeit significant, decrease of the maximal rate of change of voltage during a train of evoked action potentials.

In contrast to observations in SNpc neurons of the same mouse model, overexpression of α-synuclein did not mediate any oxidative dysfunction of Kv4.3 channels suggesting that, despite many intrinsic biophysical similarities, DMV neurons are more resistant than SNpc neurons to oxidative stress\(^73\). This increased oxidative stress resistance might be due to a downregulation of Cav currents, reducing activity-dependent calcium levels in α-synuclein over-expressing DMV neurons\(^{140,142}\). Indeed, levels of Cav1.2 and Cav2.3α subunits appear reduced in DMV neurons of this model, and might provide a mechanism of oxidative stress relief in this neuronal population.

1.7.2. Dopamine and L-DOPA

Catecholamines released from neurons of the A2 area play an important role in the modulation of the activity of DMV neurons\(^{143}\). Indeed, within the NTS, the majority of A2 area neurons are noradrenergic/adrenergic, while only 10% of these neurons express tyrosine hydroxylase but not dopamine-β-hydroxylase, indicating that they are purely dopaminergic\(^{143}\). Catecholaminergic neurons of the A2 area also reside within the DMV itself, however, they represent less than 5% of the total number of neurons and these DMV neurons express TH only\(^{143,144}\). The role of DA appears to be modulatory of the cholinergic DMV output, indeed a great proportion of DMV neurons are able to respond to DA with a D2-like receptor dependent hyperpolarization, while a smaller proportion responds with a D1-like receptor dependent depolarization\(^{143}\). Given the role of DMV neurons, to provide parasympathetic motor output to visceral organs, the impairment of dopaminergic neurotransmission within brainstem vagal circuits might play a role in the sialorrhea, dysphagia and gastrointestinal dysfunction reported in Parkinson’s disease patients; indeed, dopamine and catecholamines in general, are involved in the modulation of the synaptic inputs onto those DMV neurons comprising vago-vagal reflexes as well as mood and cognitive disorders affected by vagal stimulation\(^3,6,10,145-148\). Goshima and collaborators suggested that, following electrical field stimulation of hypothalamic slices, L-DOPA modulates the local release of catecholamines via presynaptic regulatory mechanisms that involve both dopamine and norepinephrine receptors, indicating once more that L-DOPA effects
are not exclusively mediated by its conversion to DA\textsuperscript{149}. Following microinjections of 6-OHDA in SNpc, Toti and Travagli, reported a significant increase in dopamine-\(\beta\)-hydroxylase immunoreactive neurons in the A2 area\textsuperscript{150}. These paradoxical data may be the result of decreased dopamine/DOPA production occurring within the SNpc but not in the A2 area, since significant reductions in the number of tyrosine hydroxylase-immunoreactive neurons was not observed\textsuperscript{150}. DAergic neurons appear to be also present at the level of the NTS\textsuperscript{151}, a key center of regulation of several autonomic functions\textsuperscript{6,145,152,153}. Despite the potential implications in PD-related autonomic dysfunctions, to the best of our knowledge no studies have been conducted to investigate the electrophysiological responses of NTS neurons in models of PD.

CHAPTER 1.8 CONCLUSIONS AND HYPOTHESIS

The experimental evidence presented in this mini-review highlights two main key points for the understanding of the electrophysiological alterations accompanying PD:

1) The increase in Ca\textsuperscript{2+} currents in the SNpc is linked tightly to additional intracellular stress, which could be one of the causes of dopaminergic loss in this area.

2) Cholinergic neurons of the DMV are more resilient to the same stressors than SNpc neurons.

Taken together, these data show that regardless of the experimental model studied, genetic or environmental, DA neurons in the SNpc respond poorly to oxidative damage caused by either malignant expression of \(\alpha\)-synuclein or mitochondrial toxins such as pesticides and herbicides. These agents, per se, do not completely impact the electrophysiological behavior of these neurons, but may rather affect the surrounding environment. Specifically, glutamatergic inputs impinging on dopaminergic SNpc neurons seem to play a crucial role in determining the motor dysfunctions observed in PD patients, underscoring how this disease is a circuit-based disorder, rather than a neuronal depletion-related, disease (Fig.1). With this perspective in mind, some of the side effects of L-DOPA administration might also be explained, given that in vitro, but not in vivo, this drug is capable of modulating the glutamatergic drive coming from the basal ganglia\textsuperscript{56,115-119}.

The ability of DMV neurons to initiate an anti-oxidative response by reducing the “stressful pacemaking” activity that damages SNpc neurons seems to be the “secret” weapon conferring resilience to this neuronal population. The evidence provided in this mini-review may provide a different perspective from Braak’s original hypothesis, and put forward the idea that the vagus nerve and DMV neurons may be less affected by the presence of LBs, but, rather, are simply conduits for the passage of pathological, misfolded \(\alpha\)-synuclein. Indeed, the DMV might be a
“dormant” area, and the disease becomes evident only when α-synucleinopathy hits a vulnerable territory, i.e. the SNpc.

The electrophysiological data presented herein appear to support this interpretation of PD etiology. A large array of data, however, indicate a prominent role of the vagus nerve in the spread of α-synucleinopathy and PD etiology, and the prodromal GI issues observed in the majority of PD patients support its involvement.

As in many instances, both the “Braak’s” and “Stress” theories have both strong supporting evidence, are not mutually exclusive, and, since PD is a multisystem and very complex disorder, are likely valid under different circumstances. In any case, more studies must be conducted to verify the hypothesis that, as in the basal ganglia, the vagal microcircuitry is also altered in PD.

Given the information presented herein, this thesis will test the hypothesis that environmental toxins disrupt the brain-gut axis through a vagally-dependent ENS-DMV-SNpc pathway prior to the development of parkinsonism. Results from these studies will provide important information about the mechanisms behind PD etiology which could be used to prevent the onset of the disease, and target gastric dysfunctions in Parkinsonian patients specifically. Our studies have a high translational potential and could be beneficial in the identification of vulnerable patients prior to the development of the motor symptoms of PD.
CHAPTER 1.9 REFERENCES


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CHAPTER 2

A NIGRO-VAGAL PATHWAY CONTROLS GASTRIC MOTILITY
AND IS AFFECTED IN A RAT MODEL OF PARKINSONISM

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¹ Chapter 2 consists of a previously published research article. Cecilia Bove drafted, edited and revised the completed manuscript. The research article has been reformatted to fit into this thesis. The citation for the research article is:

CHAPTER 2.1 SIGNIFICANCE

Studies from other laboratories have demonstrated previously the involvement of the dorsal motor nucleus vagus (DMV) in Parkinson’s disease (PD) in experimental models as well as in human patient cohorts. Indeed, a clear correlation between gastrointestinal disorders and incidence of PD has been found in several longitudinal studies. Moreover, the DMV is considered the first brain region to show the appearance of Lewy bodies, intracellular aggregates of α-synuclein, which eventually spread to higher brainstem areas, the substantia nigra pars compacta (SNpc), and higher cortical centers. Previous studies from our laboratory have suggested that in the 6-hydroxydopamine (6-OHDA) model of SNpc lesion, a classical and well-established model of PD induction, gastric emptying and motility were affected, confirming functional and anatomical alterations of the brain-gut axis neurocircuitry controlling the stomach that reproduce symptoms similar to those observed in patients. Although many studies support the involvement of the vagus nerve in this disease, there was no demonstration of a direct anatomical and functionally relevant connection between the DMV and the SNpc that would explain i) why SNpc neurodegeneration affects gastrointestinal function, implying a top-down spread of GI-related issues in PD, without explaining the prodromal observations of GI dysmotility, and ii) how Lewy bodies first seen in the DMV progress in the midbrain. In the present study we have provided evidence of a monosynaptic nigro-vagal pathway that connects DA neurons of the SNpc to cholinergic neurons of the DMV as well as to catecholaminergic neurons of the A2 area embedded in the dorsal vagal complex. Here we suggest that DA acts tonically via DA1-like receptors to increase gastric tone and motility and that, upon PD induction through paraquat administration, the SNpc-mediated modulation of gastric functions is impaired. This data led us to investigate how DA release onto the DMV modulates gastric function as presented in the next chapter.
CHAPTER 2.2 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 neurotoxicants 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.

KEYWORDS: Neurology, animal model, central nervous system, vagus.
CHAPTER 2.3 INTRODUCTION

The extensive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and the presence of cytoplasmic aggregates of misfolded α-synuclein are pathophysiological hallmarks of Parkinson’s disease (PD). Movement disorders are the cardinal characteristic of PD, however a plethora of non-motor symptoms increase the overall morbidity significantly\(^1\)\(^-\)\(^3\). Gastrointestinal (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-DOPA\(^4\)\(^-\)\(^7\).

The upper GI tract, and the stomach in particular, is influenced heavily by vagal inputs originating from brainstem nuclei located in the dorsal vagal complex (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 DMV\(^8\). These neurons integrate signals from higher centers\(^6\)\(^-\)\(^10\) as well as from the adjacent catecholaminergic neurons of the A2 area\(^11\), which provide synaptic modulation via α-adrenoceptors\(^12\). The efferent vagal fibers project to postganglionic myenteric neurons of the enteric nervous system (ENS) that ultimately control the motility response of the GI tract\(^10\).

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\(^13\)\(^,\)\(^14\). From there, the neurotoxicants 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. 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 meeting\(^15\).
CHAPTER 2.4 MATERIALS AND 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.

2.4.1 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 immunohistochemistry (see Immunohistochemistry section).

2.4.2 Gastric studies

Rats were fasted overnight (water ad libitum) and anesthetized with thiobutabarbitol (Inactin; Sigma, St Louis, MO; 100-150 mg/kg intraperitoneally). After 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 previously.

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

In another group of rats the subdiaphragmatic right vagus was sectioned prior to strain gauge apposition\textsuperscript{17}. NMDA was microinjected in the SNpc and, thirty min later, the left cervical vagus was severed, thus attaining a complete vagotomy, and NMDA was microinjected again forty five min 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 sites\textsuperscript{16}.

### 2.4.3 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.

### 3.4.4 Immunohistochemistry

The detailed methods were described previously\textsuperscript{16-18}. 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 ninety min 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-Ser\textsuperscript{129}α-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).
2.4.5 Optogenetic studies

Following anesthesia with rodent cocktail, rAAV2-hSyn-eNpHR 3.0-eYFP or rAAV2-hSyn-eYFP (UNC vector core, 600nl of 10x10¹¹ titer) or saline were injected into the left SNpc at a rate of ~30nl/min. Animals recovered for three-four 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 stereotaxically 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 three min.

2.4.6 Parkinson models

After 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 here¹⁶. Experiments were conducted four-five weeks later.

Rats received i.p. injections of 10mg/kg of paraquat, once a week for three consecutive weeks¹⁹ followed either by two days or two weeks 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.

2.4.7 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±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.
CHAPTER 2.5 RESULTS

2.5.1 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 after 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 three-four 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. ambiguous, 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.
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.
2.5.2 Physiologic role and pharmacologic 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, that is, 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 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 benztrapine 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).

Because 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 α2 adrenoceptor-dependent manner.
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.
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 SCH 23390 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 juxtapes 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 SCH 23390 (gray bars) but not by pretreatment with the DA2 antagonist L741,626 (white bars). SCH 23390, Tone: corpus, n=6; antrum, N=5. Motility: corpus: n=5; antrum, n=4 L-741,626, Tone: corpus and antrum, n=7. Motility: corpus: n=6; antrum, n=7. In all panels *p<0.05 vs control.
(Figure 4 continued) (D) Graphic summaries showing that pretreatment with the α1 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 α2 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.

2.5.3 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).

Because 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.
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 parquat-treated (blue bars) rats respectively. Motility: corpus: n=3,7 in controls (black and white bars), and opsin (green bars) rats respectively; antrum, n=3,7 in controls (black and white bars), and opsin (green bars) rats respectively. *p<0.05 vs control.
2.5.4 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 weeks\(^\text{19}\). Motor performance tests, i.e. a stepping test, revealed a mild, albeit significant, decrease in motor performance two days after the 3rd and final injection of paraquat; the motor performance deteriorated further when animals were tested two 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 two 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.
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.
(Figure 6 continued) *p<0.05 vs opsins in naive rats (figure 5). (D) Representative micrographs showing the same area of the DMV of a control (continued) (upper) and a paraquat-treated (middle) rat; red=ChAT and green=Ser α-synuclein. Lower panels show ChAT-IR (left), Ser α-synuclein (middle) and merged (right) images of the dotted area in the middle panels. Arrows indicate DMV neurons with 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=Ser α-synuclein. Lower panels show TH-IR (left), Ser α-synuclein- (middle) and merged (right) images of the dotted area in the middle panels. Arrows indicate SNpc neurons with Ser α-synuclein deposits. Note that in both panels D and E control animals did not show any 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.

![Graph showing changes in tone and motility](image)

![Micrographs showing ChAT and Ser α-synuclein](image)
(Figure 7 continued) 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 bethaneol 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.

CHAPTER 2.6 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 before 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
actions\textsuperscript{21}, 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 ago\textsuperscript{22} and has been supported by a wealth of evidence since\textsuperscript{10}.

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\textsuperscript{1,3,23,24}, 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\textsuperscript{16,25-30}. 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 motoneurons\textsuperscript{13} suggests that the etiology of idiopathic PD, at least in some cases, may indeed start in the GI tract, as postulated by Braak\textsuperscript{14,31}. 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\textsuperscript{13,31}. 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 PD\textsuperscript{32}. Furthermore, the disrupted electromyogastrography recordings from Parkinsonian patients closely resemble those recordings obtained from patients in the immediate, acute phase after a vagotomy\textsuperscript{33,34}.

While it is accepted that experimental and clinical PD alters the physiology and neurochemical phenotype of DMV neurons\textsuperscript{16,28,35-37}, in the absence of clear means by which the DMV pathology affects the SNpc, Braak’s hypothesis is still open to debate\textsuperscript{38-41}. 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\textsuperscript{31,40,42-48}.

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 neurotoxicants from DMV to SNpc neurons, thus triggering the prodromal GI dysfunction observed in PD\textsuperscript{1,3,13,42}. 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.

Acknowledgments, funding, and disclosures: Supported by a RRIA grant from the Michael J. Fox Foundation for Parkinson’s Disease, a grant from the PA Tobacco settlement fund and a NIH grant NIDDK DK-55530; Jessica Hampton was supported by UGSRF grant from the Am. Physiol. Society. The authors would also like to thank Cesare M. and Zoraide Travagli for support and encouragement, and Dr. K.N. Browning for critical comments on earlier versions of the manuscript.

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CHAPTER 2.7 REFERENCES


CHAPTER 3

VAGALLY MEDIATED EFFECTS OF BRAIN STEM DOPAMINE ON GASTRIC TONE AND PHASIC CONTRACTIONS OF THE RAT

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1 Chapter 3 consists of a previously published research article. Cecilia Bove drafted, edited and revised the completed manuscript. The research article has been reformatted to fit into this thesis. The citation for the research article is:

CHAPTER 3.1 SIGNIFICANCE

Previous data in our laboratory has demonstrated that neurons from the dorsal motor nucleus of the vagus (DMV) are responsive to dopaminergic (DA) inputs. Specifically, whole-cell patch clamp recordings of DMV neurons have shown that perfusion of DA is able to both depolarize and hyperpolarize their membrane through activation of the DA1-like and DA2-like receptors respectively. As presented in chapter 2, we have demonstrated that DMV neurons receive a tonic dopaminergic input from the substantia nigra pars compacta (SNpc) which activates DA1-like receptors: indeed, stimulation of the SNpc mediates an increase in both gastric tone and motility. In the present chapter, the in vivo effects of DA application to the dorsal vagal complex (DVC) were assessed. While the SNpc-mediated stimulation of DMV neurons2-like receptor-dependent gastroinhibition. The data presented herein confirm previous data from other laboratories showing the differential distribution of DA receptors in the DVC, and suggest the presence of two distinct neuronal populations within this area. Specifically, a smaller proportion of DMV neurons receive nigro-vagal dopaminergic projections and express DA1-like receptors, while the vast majority of DMV neurons express DA2-like receptors. This evidence prompted us to investigate in the following chapter the response of DVC neurons to DA microinjection in a model of PD, and to investigate the membrane properties of DMV neurons in chapters 6 and 7.
CHAPTER 3.2 ABSTRACT

Dopamine (DA)-containing fibers and neurons are embedded within the brain stem dorsal vagal complex (DVC); we have shown previously that DA modulates the membrane properties of neurons of the dorsal motor nucleus of the vagus (DMV) via DA1 and DA2 receptors. The vagally dependent modulation of gastric tone and phasic contractions, i.e., motility, by DA, however, has not been characterized. With the use of microinjections of DA in the DVC while recording gastric tone and motility, the aims of the present study were 1) assess the gastric effects of brain stem DA application, 2) identify the DA receptor subtype, and, 3) identify the postganglionic pathway(s) activated. Dopamine microinjection in the DVC decreased gastric tone and motility in both corpus and antrum in 29 of 34 rats, and the effects were abolished by ipsilateral vagotomy and fourth ventricular treatment with the selective DA2 receptor antagonist L-741,626 but not by application of the selective DA1 receptor antagonist SCH 23,390. Systemic administration of the cholinergic antagonist atropine attenuated the inhibition of corpus and antrum tone in response to DA microinjection in the DVC. Conversely, systemic administration of the nitric oxide synthase inhibitor nitro-L-arginine methyl ester did not alter the DA-induced decrease in gastric tone and motility. Our data provide evidence of a dopaminergic modulation of a brain stem vagal neurocircuit that controls gastric tone and motility.

KEYWORDS: brain stem; gastric motility; vagus

NEW & NOTEWORTHY: Dopamine administration in the brain stem decreases gastric tone and phasic contractions. The gastric effects of dopamine are mediated via dopamine 2 receptors on neurons of the dorsal motor nucleus of the vagus. The inhibitory effects of dopamine are mediated via inhibition of the postganglionic cholinergic pathway.
CHAPTER 3.3 INTRODUCTION

Functions of the upper gastrointestinal (GI) tract are influenced and modulated by vagovagal neurocircuits originating within brain stem nuclei located in the dorsal vagal complex (DVC; i.e., nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus (DMV), and area postrema); vagal motor fibers projecting to the GI tract from the lower third of the esophagus to the splenic flexure derive from the preganglionic neurons of the DMV. A vast array of synaptic inputs impinge upon DVC neurons and contribute to the fine tuning of selected circuits devoted to specific functions, including inputs originating in the adjacent catecholaminergic A2 area. The majority of neurons in the A2 area are dopamine-β-hydroxylase (DβH) but not phenylethanolamine N-methyltransferase immunoreactive (IR), thus making them noradrenergic rather than dopaminergic or adrenergic. A small subgroup of NTS neurons in the A2 area, however, are exclusively tyrosine hydroxylase (TH)-IR; hence, they are dopaminergic. These A2 area neurons are the likely source of dopaminergic inputs to DMV neurons; however, other areas such as the locus coeruleus, the pontine tegmentum, the ventrolateral medulla, the substantia nigra, and/or the hypothalamus may also provide catecholaminergic, possibly dopaminergic, inputs to DMV neurons.

Using immunohistochemical techniques, Kitahama et al. described a dense network of varicose dopaminergic fibers in the dorsal medullary area, including the DMV, although the source of such inputs was not defined. In association with this robust dopaminergic fiber network, several reports have described a wide distribution of DA2 receptors in the DMV; conversely, DA1 receptor immunoreactivity in the DMV is less prominent. These observations were supported by electrophysiological data that reported a larger percentage (i.e., ~40%) of DMV neurons responding to DA with a DA2-mediated inhibition and a smaller subgroup (i.e., ~25%) that were excited by DA via a DA1-mediated action. Thus the overwhelming majority of DMV neurons respond to exogenous application of DA; however these neurons appear to express either DA1 or DA2 receptors but not both. Furthermore, despite the likely overlapping sources of dopaminergic and noradrenergic fibers, the response of vagal motoneurons to DA or to norepinephrine was not always similar. Indeed, DMV neurons depolarized by DA were more likely to be inhibited by norepinephrine, whereas neurons unresponsive to DA were more likely hyperpolarized by norepinephrine, suggesting different physiological roles for these catecholamines.

Overall there is robust evidence that suggests DA plays a role in the regulation of GI-related brain stem vagal circuits; however, little evidence is available about the effects of brain stem DA to modulate gastric tone and phasic contractions, henceforth referred to as motility.
The aims of the present study were, therefore, to 1) assess the effects of DA application on gastric tone and motility, 2) identify the DA receptor subtype in the DMV responsible for gastric modulation, and 3) identify the postganglionic myenteric pathway(s) activated by brain stem administration of DA.

Preliminary accounts of this study were presented at the 2015 Digestive Disease Week.

CHAPTER 3.4 MATERIALS AND METHODS

Male Sprague-Dawley rats were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited Animal Care Facility maintained at 24°C on a 12-h:12-h light-dark cycle with food and water provided ad libitum.

All procedures were conducted in accordance with the National Institutes for Health guidelines, with the approval of the Penn State University College of Medicine Institutional Animal Care and Use Committee and according to journal policies and regulations on animal experimentation.

In vivo recordings of corpus and antrum tone and motility were conducted in 34 anaesthetized rats; neither microinjections of PBS in the DVC (60 nl) nor administration of PBS on the surface of the fourth ventricle (2 μl) had any significant effect (data not shown).

3.4.1 Gastric studies.

Gastric tone and motility recordings were performed as described previously. Briefly, male Sprague-Dawley rats weighing 200–350 g were fasted overnight (water ad libitum) before being anaesthetized deeply with thiobutabarbitral (Inactin; Sigma, St Louis, MO; 100–150 mg/kg ip). Rats were intubated with a tracheal catheter and, following a midline laparotomy, two custommade 6 x 8 mm strain gauges (AT Engineering, Hershey, PA) were sutured to the serosal surface of the anterior gastric corpus and antrum in alignment with the circular smooth muscle; the abdominal incision and the wound margins were sutured with the leads exteriorized. The signals were acquired with a Wheatstone bridge, filtered (low-pass filter cutoff = 0.5 Hz; AT Engineering), amplified (EXP CLSG-2; QuantaMetrics, Newton, PA), and recorded on a computer using Axotape 10 software (Molecular Devices, Sunnydale, CA). Animals were then placed in a stereotaxic frame, and rectal temperature was monitored and maintained at 37 ± 1°C with a heating pad (TCAT 2LV; Physi temp Instruments, Clifton, NJ).
The fourth ventricle was exposed via midline incision and the meningeal membranes were dissected before the exposed brain stem was covered with prewarmed saline during a 60- to 90-min period of stabilization.

The gastric tone and motility traces were monitored throughout the duration of the experiment, and baseline measures were extracted from 5 min before and 15–20 min after drug application; the drug induced effects on tone were calculated through average value of the calibration measures. Although the basal gastric tone was not preset to a fixed value, the strain gauge transducer was sewn to provide a baseline tension of ~0.5 g; the data reported are thus absolute values of tone displacement over baseline of the 30- to 60-s period centered around the peak effect. Note that the recorded magnitude of the drug-induced effects on tone and motility can be influenced by such factors as the size of the animal and variations in the strain gauge placement. Because these variations may lead to slight differences in responses between individual animals, each animal served as its own control, and motility data were measured as percent changes over baseline. The drug-induced effects on gastric motility were measured in percentage against the normalized value of gastric motility before microinjection (baseline = 100%).

Gastric motility was calculated using the following formula, as described previously23:

\[
\text{Motility index percent} = \frac{[(N1 \times 1) + (N2 \times 2) + (N3 \times 4) + (N4 \times 8)]}{t} \times 100
\]

where \( N \) = number of peaks in a particular force range and \( t \) = interval time in which the gastric motility is measured. \( N1 = 20–59 \) mg, \( N2 = 60–100 \) mg, \( N3 = 101–200 \) mg, \( N4 \geq 201 \) mg.

When the response to DA was measured after either atropine or nitro-L-arginine methyl ester (L-NAME) pretreatment, the effect of DA on gastric motility was calculated as the variation in arbitrary units of the motility index from either baseline or from atropine/L-NAME alone.

### 4.4.2. Experiment design/drugs administration

A micropipette (25- to 30-μm-tip diameter) was lowered into the left DVC (in mm: +0.4–0.6 rostrocaudal from calamus scriptorius, 0.2–0.4 mediolateral from midline, and -0.5–0.65 dorsoventral from the brain stem surface). Drugs were microinjected in 60-nl volumes using a picospritzer (Toohey, Fairfield, NJ) or applied to the surface of the fourth ventricle (2 μl). All drugs were dissolved in isotonic PBS (in mM: 115 NaCl, 75 Na2HPO4, and 7.5 KH2PO4, pH 7.4). A first group of rats was microinjected with DA into the DVC in a dose-dependent manner (0.3–3 nmol/60 nl). A second group of rats underwent vagotomy as described previously23.
Briefly, after the subdiaphragmatic posterior vagus was sectioned, DA (1 nmol/60 nl) was microinjected in the DVC and, 30 min later, the left cervical vagus was severed, disconnecting the remaining vagal outflow to the stomach. DA was microinjected again ~45 min after the cervical vagotomy.

In the third group of rats, 30–45 min after DA microinjection (1 nmol/60 nl) into the DMV, 2 μl of a PBS solution containing either the DA1 or the DA2 dopamine receptor antagonists SCH 23,390 and L-741,626 (both at 45 nmol) were applied to the surface of the fourth ventricle, at the level of obex, followed 2–5 min later by a second DA microinjection (1 nmol/60 nl).

The last group of rats, 30–45 min after the first DA microinjection (1 nmol/60 nl), received intravenous drug treatments: 1) atropine methyl nitrate (100 μg/kg iv; nonselective muscarinic antagonist), or 2) L-NAME (10 mg/kg iv, nitric oxide synthase inhibitor) followed 2–5 min later by a second DA microinjection (1 nmol/60 nl) in the DVC.

At the conclusion of the experiment, rats were euthanized with a bilateral pneumothorax and perfused transcardially with 200 ml of saline followed by 200 ml of 4% paraformaldehyde in PBS. The brain stem was removed and postfixed in 4% paraformaldehyde and 20% sucrose for 24–48 h at 4°C and then transferred in a solution containing PBS and 20% sucrose for at least 1 day. The brain stem was then frozen, sliced in 50-μm-thick coronal sections throughout the rostrocaudal extent of the DVC, and counterstained with cresyl violet. Injection sites were identified on a Nikon E400 microscope.

3.4.3 Statistical analysis
Data were evaluated by comparing the change in response between pre- and posttreatment values within each group using one-way ANOVA followed by post hoc Tukey's multiple comparison test or paired t-test (Graph Pad Prism; Graph Pad Software, La Jolla, CA) and are reported as means ± SE. In all instances, significance was set at P < 0.05.

CHAPTER 3.5 RESULTS
3.5.1 Microinjection of dopamine in the DVC decreases gastric motility in a dose-dependent manner.

Microinjections of DA (0.3–3 nmol) in the left DVC decreased gastric tone and motility in 29 of 34 rats (i.e., 85.3%) and increased gastric tone and motility in 5 of 34 rats (i.e., 14.7%). Since it appeared that there were no significant topographic differences in the microinjection site location in rats that responded with inhibition or excitation of gastric tone and motility (Fig. 1), we focused our study on the investigation of the inhibitory effects.
Microinjections of DA in the left DVC decreased antrum and corpus motility in a dose-dependent manner; both the frequency as well as the amplitude of the phasic contractions were decreased (0.3–3 nmol; n = 8 for each dose); however, the DA-induced inhibition of gastric tone was not dose dependent and appeared maximal even at the lowest dose tested (Fig. 1).

The inhibitory effects of dopamine microinjection lasted up to 25 min; however, there were no significant differences in the duration of the inhibition among the different DA doses or between corpus and antrum responses (Fig. 1).
Fig. 1 Microinjection of dopamine in dorsal vagal complex (DVC) decreases gastric tone and motility.

(A) Left: representative micrograph showing the site of dopamine (DA) microinjection (arrow) in the intermediate DVC. A, right: schematic map of the DMV showing microinjection localization; note that for clarity not all the injection sites have been included. (B) Representative traces from gastric corpus showing the decrease in tone and motility following DA microinjection (arrows). Oblique bars indicate a 5- to 10-min break in the recording. (C) graphic summary showing the decrease in gastric tone and motility in corpus (white bars) and antrum (black bars). Note that the decrease in gastric tone was already maximal at the lower DA dose. N = 8 for each dose. *P < 0.05 vs DA at 0.3 nmol. AP, area postrema; DMV, dorsal motor nucleus of the vagus; CC, central canal; XII, hypoglossal nucleus. (D) Left: representative traces from gastric corpus (top) and antrum (bottom) showing the duration of the DA-mediated inhibition. Arrows indicate the time of DA (3 nmol/60 nl) microinjection. D, right: graphic summary showing the duration of the decrease in gastric tone following DA microinjection in the DMV.
3.5.2 Dopamine modulation of DMV motoneurons is vagally dependent.

Five rats received a posterior subdiaphragmatic vagotomy, thus severing the vagal motor pathway originating in the right DMV (36). In these animals, microinjection of 1 nmol/60 nl of DA in the left DVC decreased gastric tone (186 ± 36 and 146 ± 11.1 mg in corpus and antrum, respectively) and motility (32 ± 15.1 and 40 ± 2.5% in corpus and antrum, respectively) in a manner that is comparable to the decrease observed in vagally-intact rats (tone: 158 ± 13.5 and 199 ± 17.2 mg, in corpus and antrum, respectively; motility: 40 ± 8.4 and 48 ± 6.1% in corpus and antrum, respectively; P > 0.05 for all). The effects of DA microinjection in the left DVC of these rats were abolished completely after the left cervical trunk of the vagus was severed, thus achieving a complete vagotomy (tone: 20 ± 20 and 0 ± 0 mg in corpus and antrum, respectively; motility: 5 ± 31 and 9 ± 5.8% in corpus and antrum, respectively; P < 0.05 for all vs DA in rats with posterior subdiaphragmatic vagotomy; Fig. 2).

These data indicate that the effects of DA are mediated by an effect on DMV neurons. Henceforth, the site of microinjection will be referred to as DMV rather than DVC.

Fig. 2 Dopaminergic inhibition of gastric tone and motility is vagally dependent. (A) Representative recording from the anterior antrum showing that microinjection of DA (arrow, 1 nmol/60 nl) in the left DVC decreased tone and motility (top trace). In the same animal, following complete vagotomy, a second DA microinjection (arrow) did not affect gastric tone or motility (bottom trace). Oblique bars indicate a 5- to 10-min break in the recording. (B) Graphic summary showing the decrease in corpus and antrum tone (top) and motility (bottom) pre- and postvagotomy (white and gray bars, respectively; n = 5 for all; *P <0.05 vs. first DA microinjection); vgtx = after vagotomy.
3.5.3 The dopamine-induced inhibition of gastric tone and motility is mediated by DA2 receptor activation in the DMV.

To investigate the dopamine receptor responsible for the decreased gastric tone and motility, we conducted a series of experiments in which DA microinjections (1 nmol/60 nl) were performed before and after application of the DA1 or DA2 receptor antagonists, SCH 23,390 and L-741,626 (45 nmol/2 μl), respectively, to the floor of the fourth ventricle (n = 5–11). Application of either L-741,626 or SCH 23,390 alone did not alter baseline gastric tone or motility (data not shown, but see traces in Fig. 3). DMV microinjection of DA decreased gastric tone and motility in the corpus and antrum. Following return to baseline values and a minimum recovery period of 30 min, the DA2 antagonist L-741,626 was applied to the floor of the fourth ventricle 2–5 min before a second DMV microinjection of DA; the DA2 antagonist attenuated the DA-induced decrease in corpus and antrum tone (from 181 ± 24 to 109 ± 16 and from 156 ± 22.2 to 102 ± 35.3 mg in corpus, n = 7, and antrum, n = 6, respectively; P < 0.05 vs DA alone) and motility (from 59 ± 8 to 36 ± 10.6 and from 47 ± 12 to 22 ± 9.5% of baseline in corpus, n = 11, and antrum, n = 5, respectively; P < 0.05 vs DA alone). Conversely, administration of the DA1 antagonist SCH 23,390 did not antagonize the DA-induced inhibition of gastric tone and motility (n = 7–11; P > 0.05 for all). Representative traces and summary data are shown in Fig. 3.

These data indicate that the DA-induced inhibition of gastric tone and motility are mediated by activation of DA2-like receptors in the DMV.
3.5.4 The dopamine-induced inhibition of gastric tone and motility is mediated by withdrawal of cholinergic tone.

To investigate the vagal postganglionic pathway affected by DMV microinjection of DA, we conducted a series of experiments in which DA microinjections (1 nmol/60 nl) were performed before and after application of either the muscarinic receptor antagonist atropine (100 μg/kg iv) or the nitric oxide synthase inhibitor L-NAME (10 mg/kg iv). DMV microinjection of DA decreased gastric tone (173 ± 16.3 and 245 ± 44 mg in corpus, n = 4, and antrum, n = 4,
respectively) and motility [from 100 ± 38.5 to 24 ± 6.5 arbitrary units (AU) and from 167 ± 28.5 to 42 ± 7.3 AU in corpus, n = 4, and antrum, n = 8, respectively]. Following return to baseline values and a minimum recovery period of 30 min, the muscarinic antagonist atropine was administered and, 2–5 min later, a second DVC microinjection of DA induced a significantly attenuated inhibition of corpus and antrum tone (66 ± 32.8 and 60 ± 20 mg in corpus and antrum, respectively; P < 0.05 vs DA alone; n = 4) and motility (from 50 ± 6.3 to 28 ± 5.1 AU and from 54 ± 9.3 to 33 ± 6.8 AU in corpus and antrum, respectively; P < 0.05 vs DA alone; n = 4–8). Representative traces and summary data are shown in Fig. 4.

Conversely, administration of the nitric oxide synthase inhibitor L-NAME did not antagonize the DA-induced inhibition of gastric tone and motility (n = 3–4; P > 0.05 for all). Summary data are shown in Fig. 4.

These data indicate that the DA-mediated gastric inhibition is the result of tonic cholinergic withdrawal.
DA microinjections reduce gastric tone and motility through inhibition of the vagal cholinergic pathway.

**Fig. 4** (A) representative traces from the anterior antrum showing that DA (1 nmol/60 nl) microinjection decreases tone and motility (top trace). Following a ~45-min recovery period, application of atropine (100 µg/kg iv) reduced significantly the inhibitory effects of a 2nd DA microinjection (bottom trace). Oblique bars indicate a 5- to 10-min break in the recording. (B) graphic summary showing the effect of administration of atropine (left) and nitro-L-arginine methyl ester (L-NAME; right; 10 mg/kg iv) on gastric tone following DA microinjection in the DVC. The DA-induced decrease in gastric tone was reduced significantly by atropine, whereas intravenous administration of L-NAME did not prevent the inhibitory effects of DA. White bars: DA; black: DA + atropine n = 4; gray bars: DA + L-NAME; n = 3. *P < 0.05 vs. baseline. (C) scatterplot showing the effects of atropine (left) and L-NAME (right) on gastric motility following DA microinjection in the DVC. The reduction in motility observed after microinjection of DA is prevented by intravenous administration of atropine (n = 4–8), whereas L-NAME (n = 3–4) had no effect. *P < 0.05 vs baseline.

**CHAPTER 3.6 DISCUSSION**

In the present study we report that application of exogenous DA on the DVC decreases gastric tone and motility via inhibition of vagal pathways. The inhibitory effects of DA microinjection in the DVC are mediated via activation of DA2 receptors in the DMV, which results in the inhibition of the cholinergic postganglionic pathway.

Our evidence is the following: 1) microinjection of DA in the DVC decreased gastric motility in the vast majority (i.e., 85%) of the rats; 2) the DA-induced decrease in gastric motility was dose dependent; however the decrease in gastric tone was not and appeared to be maximal at the
tested doses (0.3–3 nmol); furthermore, the DA-induced decrease in both gastric tone and motility was 3) vagally mediated, since it was prevented by complete vagotomy, and 4) mediated by activation of DA2 receptors in the DMV, since it was attenuated significantly by pretreatment with the DA2 selective antagonist L-741,676, but not by the DA1 antagonist SCH 23,390; and 5) DA2 receptor-mediated inhibition of DMV neurons resulted in the withdrawal of the tonically active cholinergic postganglionic pathway, since the gastroinhibition was attenuated significantly by pretreatment with a submaximal dose of the muscarinic selective antagonist atropine but not the nitric oxide synthase inhibitor L-NAME.

Our data thus support a role for dopaminergic neurotransmission in the brain stem vagal circuits that control gastric tone and motility.

As mentioned previously, the dopaminergic input to DMV neurons that modulate gastric tone and motility most likely originates from TH-positive neurons of the adjacent A2 area, although fibers originating from areas such as A6, A5, A2, A1/C1, A9, and/or the A13 cannot be excluded. While the large majority of the catecholaminergic neurons of the A2 area are also DβH-IR, suggesting that these neurons are noradrenergic or adrenergic, ~10% of the A2 neurons contain TH-IR only, indicating that this subpopulation is exclusively dopaminergic. Furthermore, a dense network of dopaminergic-IR fibers innervates both the NTS as well as the DMV, suggesting that dopamine may play a prominent role in the modulation of vagal activity.

Investigation of the role played by dopaminergic innervation of vagal neurocircuits regulating gastrointestinal functions, however, is limited.

Brain stem dopamine has been hypothesized to play a role in the control of ingestive as well as reflexive behavior. Given the close proximity of brain stem dopamine receptors with N-methyl-D-aspartate and cholecystokinin receptors, Södersten and colleagues put forward the hypothesis that cholecystokinin interacts with DA and glutamate to decrease food intake. This inhibitory action of DA was proposed to occur via activation of both DA1 and DA2 receptors. A further role of DA in ingestive behavior was proposed by the same group, which showed that the ingestive responses of sucrose by decerebrate rats were reduced by administration of the nonselective dopaminergic agonist apomorphine, thus suggesting that dopaminergic transmission in the brain stem is sufficient for this particular ingestive reflex.

A dopamine-mediated modulation of brain stem neurocircuits was also proposed in relation to nausea and vomiting, esophageal motility, and small intestine secretions. In fact, dopamine was shown to induce the gastric relaxation and decrease in gastric pressure that precedes nausea and vomiting in dogs, possibly via an effect on the chemoreceptive trigger zone. Likewise, several groups have reported that mechanical distention of the esophagus or the stomach activates c-FOS
in TH-IR, including dopaminergic, brain stem neurons of the A2 area\textsuperscript{4,10,28}. Furthermore, application of the centrally acting DA1/DA2 antagonist haloperidol increased duodenal secretion of bicarbonate, possibly via brain stem vagal pathways\textsuperscript{29}.

Brain stem dopaminergic neurocircuits may also play a role in pathological conditions. For example, in the 6-OHDA animal model of Parkinson’s disease, concomitant with the decreased number of dopaminergic neurons in the substantia nigra pars compacta, there is an increase in number of catecholaminergic neurons in the A2 area\textsuperscript{24,30}, suggesting a potential upregulation of brain stem catecholaminergic neurotransmission from A2 to vagal motoneurons. Interestingly, neurons of the A2 area are involved in swallowing reflexes\textsuperscript{31-33}, these observations may provide a mechanistic explanation to the sialorrhea and dysphagia that affect Parkinsonian patients\textsuperscript{34-37}. Indeed, microinjections of either DA or the nonselective DA1-DA2 receptor agonist apomorphine in the NTS inhibits the swallowing reflex induced by stimulation of the superior laryngeal nerves\textsuperscript{38}.

Vagal efferent fibers originating in the preganglionic motoneurons of the DMV modulate gastric tone and motility via tonic projections to excitatory cholinergic, or projections to inhibitory nonadrenergic-noncholinergic (NANC) postganglionic myenteric neurons; hence, gastric relaxation results from vagal modulatory inputs that either inhibit the cholinergic or excite the NANC pathway\textsuperscript{1}. The loss of effects of DA on gastric tone and motility following complete (i.e., bilateral) vagotomy suggest strongly that the effects of DA in the DVC are vagally-mediated. Furthermore, since the inhibition of gastric tone and motility observed upon DA microinjection in vagally intact animals is similar to that obtained in hemivagotomized animals, these data suggest that the DA effects are mediated by direct activation of ipsilateral-projecting DMV neurons, rather than a combined effect of DA on both DMV neurons and contralateral-projecting NTS neurons.

In the present study, we focused on the DA2-mediated decrease in gastric tone and motility, since the gastric effects were attenuated by pretreatment with the DA2 receptor antagonist L-741,626. Our results confirm previous studies of the inhibitory actions of DA being mediated by DA2 receptor activation\textsuperscript{39}, and our previous data showing a DA2 receptor-mediated hyperpolarization of DMV neurons suggest that inhibition of these neurons is the likely brain stem target that decreases gastric tone and motility. Indeed, a dense presence of DA2 receptors in the DVC has been reported\textsuperscript{19}; furthermore, electrophysiological studies have shown that the glutamatergic inputs onto cardiorespiratory vagal neurons are inhibited significantly by DA2 receptor activation\textsuperscript{40} and microinjections of DA2 agonists in the nucleus tractus solitarius induced a pressure response\textsuperscript{41}.
Given the fact that DA2-mediated effects are inhibitory to DMV neurons\textsuperscript{11}, the only logical vagal mechanism that would ultimately induce gastric relaxation is via inhibition of the cholinergic postganglionic pathway. In fact, DMV neurons are pacemakers\textsuperscript{42} and provide a tonic vagal input to excitatory cholinergic and inhibitory NANC postganglionic myenteric neurons that ultimately shapes gastric tone and motility\textsuperscript{1}. DA2-mediated inhibition of DMV reduces vagal output to myenteric neurons; hence, a DA2-mediated inhibition of vagal projections impinging upon cholinergic myenteric neurons would result in a decrease in gastric tone and motility. Conversely, a DA2-mediated inhibition of vagal projections impinging upon NANC myenteric neurons would result in a limited increase in gastric tone and motility, given the weak influence that tonic NANC pathways have on gastric tone and motility\textsuperscript{43}. Since pretreatment with the selective acetylcholine muscarinic receptor antagonist atropine, but not the nitric oxide synthase inhibitor L-NAME, attenuated the DA2-mediated gastric inhibition, our data indicate that the gastroinhibitory effects of DA microinjection in the DVC are mediated via inhibition of the excitatory cholinergic pathway in a manner that involves activation of DA2 receptors located on DMV neurons.

Using an electrophysiological approach, we also showed that perfusion with DA1 or DA2 antagonists did not alter the membrane potential of DMV neurons\textsuperscript{11}. Our present results confirmed that application of either DA1 or DA2 antagonists on the floor of the fourth ventricle per se did not modulate gastric tone or motility significantly, indicating either that the DA released under our experimental conditions is not sufficient to induce measurable alterations of gastric tone or motility or that there are no tonic dopaminergic inputs impinging on gastric-projecting DMV neurons. The first explanation, although, appears to be more likely since preliminary data indicate the presence of a tonic dopaminergic input to DMV neurons arising from neurons of the A9 area\textsuperscript{12}.

Finally, although the gastric excitatory response to dopamine was limited to ~15\% of the animals tested may argue against a prominent role of the excitatory inputs, one has to keep in mind that dopamine has an affinity toward DA2 receptors that is one order of magnitude higher than the affinity toward DA1 receptors\textsuperscript{44}. Furthermore, one has to consider that brain stem vagal neurocircuits are likely organized along lines of specificity\textsuperscript{45,46}, so that sets of neurons, although adjacent, may control different and diverse physiological responses.

In conclusion, our study reports a potentially important role of brain stem vagal DA2 receptors in the modulation of gastric tone and motility; these dopaminergic inputs are likely relevant in the fine tuning of gastrointestinal vagovagal reflexes.
Acknowledgments, funding, and disclosures: We thank Cesare M. Travagli and Zoraide Travagli for support and encouragement. We also gratefully acknowledge the discussion, suggestions, and editing of Dr. Kirsteen N. Browning.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-55530 and DK-99350 and Michael J. Fox Foundation Grant for Parkinson’s Disease (to R. A. Travagli).

No conflicts of interest, financial or otherwise, are declared by the authors.

CHAPTER 3.7 REFERENCES


CHAPTER 4

ALTERED GASTRIC TONE AND MOTILITY RESPONSE TO BRAINSTEM DOPAMINE IN A RAT MODEL OF PARKINSONISM

Cecilia Bove, Laura Anselmi, and R Alberto Travagli

1 Chapter 4 consists of a previously published research article. Cecilia Bove drafted, edited and revised the completed manuscript. The research article has been reformatted to fit into this thesis. The citation for the research article is: “Altered gastric tone and motility response to brainstem dopamine in a rat model of parkinsonism.” Bove C, Anselmi L., Travaglì RA. Am J Physiol Gastrointest Liver Physiol. 2019 May 1. doi: 10.1152/ajpgi.00076.2019. PMID: 31042398
CHAPTER 4.1 SIGNIFICANCE

Previous data in our laboratory has shown the presence of a monosynaptic nigro-vagal pathway connecting the substantia nigra pars compacta (SNpc) to the dorsal motor nucleus of the vagus (DMV) and catecholaminergic neurons of the A2 area (chapter 2). The release of dopamine (DA) from these terminals provide a stimulation for the dorsal vagal complex (DVC) that results in an increase in gastric tone and motility via activation of DA1-like receptors. We have also shown that direct microinjection of DA in the DVC produces the opposite effect (chapter 3), and that in a model of Parkinson’s disease (PD) the nigrovagal pathway is impaired (chapter 2). The aim of the present study was to assess the response to direct microinjection of DA into the DVC in a rodent model of PD. The data presented herein suggests that, instead of mediating a gastroinhibitory response as observed in naïve animals, microinjection of DA in the DVC of parkinsonian animals mediates a biphasic response, where the gastroinhibition follows an initial increase in both tone and motility. While in naïve animals only DA2-like receptors are responsible for the gastroinhibition, following PD induction there is the emergence of a mixed DA1- and DA2-like receptor-dependent response. These data suggest the possibility of a maladaptive plastic mechanism to compensate, unsuccessfully, for the loss of DA inputs from the nigrovagal pathway in an effort to prevent the dysmotility described in chapters 2; such maladaptive plasticity will also be described in a different model of PD in chapter 5. Such maladaptive plasticity might also be due to an increase in reflex-mediated activity in response to the decreased motility of the GI tract which tries to compensate by increasing inputs from the SNpc, amongst other mechanisms. Such responses might also be the reason why other studies, including our previous study by Toti and Travagli, show alterations in the neurochemical phenotype of DVC neurons. Indeed, Toti & Travagli showed an increase in the number of dopamine-β-hydroxylase (DβH)-, but not tyrosine hydroxylase (TH)- immunoreactive (IR) neurons in the A2 area after SNpc lesion with the toxin 6-hydroxydopamine (6-OHDA), which also caused gastric dysfunction. Furthermore, Zheng et al. showed a decrease in both TH and choline acetyltransferase (ChAT) in DMV neurons leading to similar degree of gastric dysfunction.

The possible molecular basis of this maladaptive plasticity will be described by the electrophysiological experiments described in chapters 6 and 7.
CHAPTER 4.2 ABSTRACT

The majority of Parkinson’s disease (PD) patients experience gastrointestinal (GI) dysfunction. Recently, we described a nigro-vagal pathway, which uses dopaminergic (DA) inputs to dorsal motor nucleus of the vagus (DMV) and A2 area neurons to modulate gastric motility and tone. This pathway is disrupted in a rodent model of PD.

The aim of the present study was to test the hypothesis that brainstem dopaminergic modulation of gastric tone and motility is altered in a rodent model of PD.

Male Sprague Dawley rats received 3 weekly intraperitoneal injections of 10mg/kg paraquat (PQ) or saline (control).

In naive conditions, DA microinjection in the DMV induced a gastroinhibitory response in 100% of animals. In 19/28 of the paraquat-treated animals, however, microinjection of DA in the DVC induced a biphasic response with an initial increase in gastric tone and motility followed by a profound gastroinhibition. The excitatory response to DA microinjection was attenuated by a combination of DA1- and DA2-like receptor antagonists. Conversely, the inhibitory response was reduced by the DA2-like antagonist only. Pretreatment with the α2-adrenoceptor antagonist, yohimbine, did not modulate the response to DA, thus excluding the involvement of the A2 area.

At the end of the experiments, the induction of the parkinsonian phenotype was confirmed by the presence of α-synuclein immunoreactivity in the DMV and substantia nigra pars compacta.

These data suggest a maladaptive neural plasticity in brainstem vagal circuits regulating gastric motility in paraquat-treated rats, which may be responsible for the gastric dysfunction observed in PD models.

KEYWORDS: Vagus, brainstem, dorsal motor nucleus of the vagus, in vivo.

NEW & NOTEWORTHY: Following paraquat treatment and PD-induction, brainstem dopamine application induces a biphasic gastric response in the majority of rats, with an initial increase in tone and motility followed by gastroinhibition. The initial increase in gastric tone and motility is mediated via a combined activation of DA1- and DA2-like receptors. The inhibitory effects of dopamine are mediated by DA2-like receptors, and are not affected by blockade of adrenergic inputs mediated by α2-adrenoceptors.
CHAPTER 4.3 INTRODUCTION

Parkinson’s disease (PD) is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and by its pathological hallmark of Lewy bodies (LB), which are composed primarily of misfolded α-synuclein (α-syn) protein aggregates. SNpc neuronal degeneration causes progressive loss of dopaminergic inputs into the striatum that results in the emergence of oscillatory patterns of burst firing of basal ganglia output neurons; these neurophysiological changes alter the functional output from the motor thalamus and diminish motor cortical activity leading to the key motor, as well as many non-motor, symptoms of PD. A range of non-motor symptoms precedes the motor phase of PD. Gastrointestinal (GI) symptoms, such as esophageal dysfunctions, delayed gastric emptying, and constipation, are prodromal to the onset of motor symptoms of PD, and GI symptoms in otherwise healthy individuals have been associated with an increased PD risk.

The coordinated digestive and reflexive processes in the upper GI tract, from the lower esophagus to the colonic splenic flexure, are under vagal modulatory control. The afferent vagus conveys visceral sensory information to the dorsal vagal complex (DVC; i.e. neurons of the nucleus tractus solitarius, NTS, dorsal motor nucleus of the vagus, DMV, and area postrema) where it is integrated with information from other central nervous system (CNS) centers involved in the regulation of autonomic and homeostatic functions. The DVC is known to receive inputs also from the SNpc, via a newly described nigro-vagal pathway, which modulates gastric tone and motility via activation of excitatory dopaminergic DA-1 like receptors on both DMV and the adjacent A2 area.

In vitro and in vivo studies in naïve rats indicate that dopamine (DA) modulates vagal outputs to the stomach. Both inhibitory DA-2 like receptors as well as DA-1 like receptors are present on subgroups of DMV neurons. Our recent study, however, reported that application of exogenous DA to the DVC of naïve rats decreases gastric tone and motility via activation of DA-2 like receptors only.

Paraquat (PQ) was once the most widely used herbicide worldwide, and evidence shows a strong positive correlation between its use and the incidence of PD. Indeed, PQ administration is used frequently to induce experimental parkinsonism. Using a classical model of PD, i.e. intraperitoneal administration of PQ, we demonstrated recently that the increased gastric tone and motility obtained upon stimulation of SNpc was reduced significantly. Furthermore, using both pharmacologic and optogenetic approaches we showed that, in both this PQ rodent model as well as in a novel model of PD, the dopaminergic projections of the nigro-vagal pathway impinging on DVC inputs were impaired.
Given the relevance of the vagal brainstem circuits in the modulation of gastric tone and motility, and the role played by DA in both the tuning of gastric functions as well in PD pathology, the aim of the present study was to test the hypothesis that brainstem dopaminergic modulation of gastric tone and motility is altered in a rodent model of PD.

CHAPTER 4.4 MATERIALS AND METHODS

All procedures were conducted in accordance with the National Institutes for Health guidelines, with the approval of the Penn State University College of Medicine Institutional Animal Care and Use Committee and according to journal policies and regulations on animal experimentation.

4.4.1 Animals

Male Sprague-Dawley rats (Charles River, Kingston, NY, USA), between 50-75 g at the beginning of the experiment, were housed under a standard 12-hour light/dark cycle at 24°C and had ad libitum access to food and water. As described previously, after weaning (post-natal day 21) rats received weekly i.p. injections of 10mg/kg of paraquat, for three consecutive weeks followed by 2 days or 2 weeks of recovery before the experimental procedures described below were conducted.

4.4.2 Gastric motility recordings

Detailed methodologies to record from the anterior surface of the stomach were described previously. Briefly, rats were anesthetized deeply with thiobutabarbital (Inactin®; 100-150 mg/kg i.p, Sigma-Aldrich, St. Louis, MO) and, once a deep level of anaesthesia was reached (i.e. lack of foot-pinck reflex), the stomach was exposed. Miniaturized strain gauges were sutured to the serosal surface of the anterior stomach at the level of the corpus and the antrum in alignment with the circular smooth muscle, and the leads were exteriorized following abdominal closure. Rats were then placed on a stereotaxic frame, neck muscles were blunt dissected, and the brainstem was exposed following removal of meningeal membranes above the fourth ventricle. Rats were allowed to recover for at least 45 minutes before beginning the experiment. Rat body temperature was monitored and kept at 37°C throughout the duration of the experiment, and 5ml of pre-warmed saline were supplemented subcutaneously before the experiment. To verify the location of the pipette, vagal motoneurons were activated by injecting thyrotropin-releasing hormone (TRH, 1pmol/60nl, Sigma-Aldrich, St. Louis, MO) into the left DVC (in mm, RC: 0.0-0.6 from calamus scriptorius; ML: 0.2-0.4 from midline; DV: 0.5-0.65 from the brainstem.
After recovery (>30 minutes) the response to brainstem DA (1nmol/60nl, Sigma-Aldrich, St. Louis, MO) was assessed. Approximately 60 minutes later, 2μL phosphate-buffered saline solution containing either DA1-, DA2-like, and/or the α2 adrenoceptors antagonists SCH 23390, L-741,626 (45nmoles, Sigma-Aldrich, St. Louis, MO), and yohimbine (500pmoles, Research Biochemicals International, Natick, MA) were applied to the surface of the fourth ventricle at the level of calamus scriptorius, followed 2-5 minutes later by a second DA microinjection. All drugs were dissolved in isotonic phosphate buffered saline (PBS, in mM: 115 NaCl, 75 Na2HPO4, 7.5 KH2PO4; pH=7.4). Data from both corpus and antrum were not collected from all the animals due to technical limitations, including the occasional failure of strain gauges during the course of the experiment unfortunately. At the end of the experiment, rats were euthanized via administration of bilateral pneumothorax, and perfused fixed with 4%PFA. Brains were then collected, post-fixed in 4% PFA + 20% sucrose, and maintained in a PBS + 20% sucrose solution until sectioned using a freezing microtome at 50 μm.

4.4.3 Immunohistochemistry

Detailed methods were as described previously. Briefly, 50 μm brainstem coronal slices of DVC, and midbrain slices of SNpc were incubated for 72 hours with primary antibody (mouse α-Ser129α-synuclein, RRID:AB_869973, Abcam, Cambridge, UK; 1:1000). Following a 24 hour incubation period in secondary antibody, the first antibody was detected and the chromogen developed. A second round of incubation with primary antibody was performed to detect cholinergic neurons of the DMV in the dorsal vagal complex (rabbit-α-choline acetyltransferase, ChAT; RRID:AB_91650, Chemicon, Temecula, CA; 1:5000) or dopaminergic neurons of the SNpc (mouse-α-tyrosine hydroxylase, TH; RRID:AB_572268, 1:10,000 Immunostar, Hudson, WI). Secondary antibodies were biotinylated donkey immunoglobulins (IgGs) for multiple labelling (Jackson ImmunoResearch Laboratories, West Grove PA) diluted 1:1000. The detection complex was ExtrAvidin-horseradish peroxidase (ExtrAvidin-HRP; 1:1500, Millipore Sigma, Germany). Images were captured with a Nikon E400 brightfield microscope. The specificity of all antibodies used in this study were validated previously.

4.4.4 Statistical analysis

Data were analyzed by using paired or unpaired t-test (Graph Pad Prism, Graph Pad Software Inc., La Jolla, CA) and are reported as mean±SEM. Specifically, the paired t-test was used when comparing the gastric tone and motility response of an individual rat to microinjection of DA in
the DVC before and after application of either the DA1- or the DA2-like receptor antagonists. In all instances, significance was set at p<0.05.

CHAPTER 4.5 RESULTS

To confirm our previous studies, a short series of experiments was conducted with the purpose of confirming our previous data\textsuperscript{23}.

As reported previously\textsuperscript{17}, PQ-treated rats display the presence of misfolded α-synuclein in the DMV and SNpc (figure 1). Since our previously published data\textsuperscript{17} suggests that gastrointestinal dysfunction become apparent already 2 days after the final injection of PQ, all the experiments described below were conducted at that same time point.

![Figure 1](image_url)

**Figure 1. Paraquat injections induce motor deficits and deposition of misfolded α-synuclein in the dorsal vagal complex as well as in the substantia nigra pars compacta.**

Top panels: representative micrographs of the dorsal vagal complex taken at approximately the same rostro-caudal level showing the co-localization of $^{129}$Ser-α-synuclein (blue) with acetylcholine transferase (ACh, brown) and tyrosine hydroxylase (TH) in the DVC and SNpc respectively in control (i), PQ 2 days (ii) and 2 weeks (iii) after the final PQ injection. Bottom panels: representative micrographs of the substantia nigra pars compacta taken at approximately the same rostro-caudal level showing the co-localization of $^{129}$Ser-α-synuclein (blue) with tyrosine hydroxylase (TH) in the DVC and SNpc respectively in control (iv), PQ 2 days (v) and 2 weeks (vi) after the final PQ injection. Calibration bars: 100 µm. Insets are higher magnifications of their respective panels.
In naïve rats, DA microinjection in the DVC induced a similar inhibitory gastric response as reported previously with a reduction of tone of \(-315\pm127.2\) and \(-115\pm22.8\) mg in antrum and corpus respectively, and a reduction in motility to \(62\pm18\) and \(44\pm11.9\%\) of baseline in antrum and corpus respectively (N=3; p<0.05 vs baseline for all; figure 2A-B).

**Figure 2.** In paraquat-treated rats, microinjection of DA in the DVC induces a biphasic gastric tone and motility response.
(A) Representative traces from gastric antrum showing that in male control (CTL) rats microinjection of DA (arrows) reduces both tone and phasic contractions (top trace). In paraquat treated animals, microinjection of DA induces an initial increase in gastric response that is followed by a profound gastroinhibition (bottom trace). Oblique bars indicate a 5- to 10-min break in the recording. (B) Graphic summary showing the effects of DA microinjection in tone (left panel; antrum N=3 and 19 in CTL and PQ, respectively; corpus N=3 and 16 in CTL and PQ, respectively) and net percent change in motility (right panel; antrum N=3 and 14 in CTL and PQ, respectively, corpus N=3 and 13 in CTL and PQ, respectively). Black bars: CTL; red bars: PQ. * p<0.05 unpaired t-test.

In the 28 PQ-treated rats, only a minority of rats showed a monophasic, either inhibitory or excitatory, response to DA microinjection. Specifically, tone was reduced in 8-9 rats, and increased in 1-2 rats, respectively. Data are summarized in Table 1.

Following DA microinjection in the DVC of PQ-treated rats, however, the majority of the responses were biphasic in which the inhibition was preceded by a prominent increase in gastric
tone and/or motility. We thus focused our attention on the population of PQ-treated rats that showed this novel biphasic response because it was not observed in naïve rats.

<table>
<thead>
<tr>
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<th>Excitation only</th>
<th>Inhibition only</th>
<th>Both excitation and inhibition</th>
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<tbody>
<tr>
<td><strong>Antrum tone (mg)</strong></td>
<td>427 (N=1)</td>
<td>-224±58.9 (N=8)</td>
<td>See text (N=19)</td>
</tr>
<tr>
<td><strong>Corpus tone (mg)</strong></td>
<td>649±362.5 (N=2)</td>
<td>-237±38.0 (N=9)</td>
<td>See text (N=16)</td>
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<tr>
<td><strong>Antrum motility</strong></td>
<td>344±84.6 (N=5)</td>
<td>47±13.8 (N=6)</td>
<td>See text (N=14)</td>
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<td>(% of baseline)</td>
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<tr>
<td><strong>Corpus motility</strong></td>
<td>391±68.1 (N=5)</td>
<td>57±12.1 (N=6)</td>
<td>See text (N=13)</td>
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<td>(% of baseline)</td>
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Table 1. Gastric tone and motility variation following dopamine microinjection in the dorsal vagal complex in animals that responded with either an excitation or an inhibition only.

Specifically, following DA microinjection in the DVC, the biphasic response was +368±74 and -192±26 mg in antrum (N=19), and 295±60 and -146±20 mg in corpus (N=16), respectively. Similarly, following DA microinjection in the DVC, motility variations from baseline were +441±86 and +49±6% of baseline in antrum (N=14), and 399±51 and 54±6% of baseline in corpus (N=13), respectively. Figure 2A-B. These data indicate that PQ treatment induces a gastroexcitation followed by a gastroinhibition in response to DA microinjection.

Since the majority of PQ-treated rats responded to DVC microinjection of DA with a biphasic response, we focused our studies on these rats.

The receptors involved in this biphasic response were investigated upon microinjection of DA prior to and following application to the floor of the 4th ventricle of either the DA1- or the DA2-like receptor antagonists, SCH23,390 and L741,626 (both at 45nmoles/2µl), respectively.

Application of either antagonist alone had no effect upon baseline gastric tone or motility (figure 3A-C).

In the presence of SCH23,390, the initial increase in tone was reduced from +415±126 to +26±20mg in the antrum (N=10; t=2.723, df=8) and from +352±97 to +115±80 mg in the corpus (N=11; t=2.905, df=10), respectively. Similarly, motility was reduced from +573±150 to +194±48% of baseline in the antrum (N=13; t=2.118, df=8) and from +441±70 to +194±30% of baseline in corpus (N=9; t=4.577, df=8) (p<0.05 vs DA alone for all; Fig 3A-B). Pretreatment with SCH23,390 did not affect any of the parameters of the inhibitory phase (p>0.05 for all).
Figure 3. The excitatory phase of the gastric response to DA microinjection is dependent on the activation of DA1- and DA2-like receptors, while the inhibitory phase is DA2-like dependent only.

(A) Representative trace from a male PQ-treated rat showing the biphasic response to DVC microinjection of DA (top trace). Upon recovery to baseline, the DA1-receptor antagonist SCH23,390 was applied to the floor of the 4th ventricle, a second DA microinjection induced a significantly reduced gastroexcitation. Oblique bars indicate a 5- to 10-min break in the recording. (B) Graphic summary showing the effects of DA microinjection before and after 4th ventricular application of SCH 23,390 in tone (left panel, N=9-14 and 11 for antrum and corpus, respectively) and net percent change in motility (right panel, N=9-14 and 9-13 for antrum and corpus, respectively). * p<0.05 paired t-test. Open black bars: DA in PQ; open red bars: DA after SCH 23,390 in PQ. *p<0.05 (C) Representative trace from a male PQ-treated rat animal showing that application to the 4th ventricle of the D2-like receptor antagonist L741,626 reduces significantly the inhibition of gastric tone. Oblique bars indicate a 5- to 10-min break in the recording. (D) Graphic summary showing the effects of DA microinjection before and after 4th ventricular application of L-741,626 in tone (left panel; N=5-6 and 4-5 for antrum and corpus, respectively) and net percent change in motility (right panel; N=3-6 and 3-4 for antrum and corpus, respectively). Open black bars: DA in PQ; open red bars: DA after L-741,626 in PQ. * p<0.05 paired t-test.

These data indicate that part of gastro-excitative response to DA is mediated, at least in part, by DA1-like receptors.
Application of L741,626 to the floor of the IV ventricle significantly reduced the inhibitory response to microinjection of DA in the DVC. Specifically, the gastroinhibition was reduced from -284±70 to -151±27mg in antrum (N=6; \( t=2.668, df=5 \)) and from -280±57 to -168±64 mg in corpus (N=5, \( t=3.334, df=4; p<0.05 \) for all). The motility decrease was attenuated from +58±9.4 to +83±9.4\% of baseline in antrum (N=6, \( t=1.532, df=3 \)), and from +86±2.7 to +98±30.9 \% of baseline in corpus (N=4, \( t=0.3941, df=3; p>0.05 \) for all).

Interestingly, following pretreatment with L741,626, the DA-dependent increase in gastric tone was significantly reduced in the antrum from +437±110 to +231±66mg (N=5; \( t=2.934, df=4 \)), but not in corpus (from +267±103 to +168±33mg, N=4, \( t=1.225, df=3; p<0.05 \) for antrum only). Gastric motility was significantly reduced in both the antrum (from +437±92 to +169±57\% of baseline; N=3, \( t=4.360, df=2 \)) and the corpus (from +535±19 to +222±3\% of baseline; N=3, \( t=16.68, df=2; p<0.05 \) for all; Fig. 3C-D).

These data indicate that part of gastroinhibitory response to DA is mediated by DA2-like receptors and suggest that the increase in tone in antrum and the increase in motility in both antrum and corpus is also modulated by DA2-like receptors.

Since we demonstrated previously\(^1\) that the nigro-vagal pathway also activates α2-adrenoceptors on neurons of the A2 area to modulate gastric motility, we examined whether the DA-induced biphasic response observed in PQ-treated rats was modulated by pretreatment with the α2-adrenoceptor antagonist, yohimbine.

In a group of rats, following microinjection of DA, yohimbine (500pmoles/2µl) was applied either alone (N=4) of in the presence of SCH 23,390 or L-741 or both (N=6) on the floor of the IV ventricle, and DA was microinjected. In these rats, yohimbine did not affect the inhibitory phase of the response to DA microinjection in the DVC (p>0.05). Data are summarized in table 2.
Table 2. Effect on the inhibitory phase of the gastric response to dopamine microinjection in the dorsal vagal complex upon pretreatment with the α2-adrenoceptor antagonist yohimbine.

<table>
<thead>
<tr>
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<th>DA</th>
<th>DA + Yohimbine</th>
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<tbody>
<tr>
<td>Antrum tone (mg)</td>
<td>-218±45.21 (N=10)</td>
<td>-196±41.76 (N=10)</td>
</tr>
<tr>
<td>Corpus tone (mg)</td>
<td>-163±24.9 (N=7)</td>
<td>-174±33.5 (N=7)</td>
</tr>
<tr>
<td>Antrum motility (% of baseline)</td>
<td>51±11.5 (N=7)</td>
<td>59.3±10.4 (N=7)</td>
</tr>
<tr>
<td>Corpus motility (% of baseline)</td>
<td>80±14.7 (N=6)</td>
<td>56±10.5 (N=6)</td>
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These data suggest that the DA-mediated inhibitory response in PQ-treated rats does not appear to involve catecholaminergic neurons of the A2 area.

CHAPTER 4.6 DISCUSSION

In the present study we report that brainstem dopaminergic modulation of gastric tone and motility is altered in a rodent model of PD.

We confirmed our previous data\textsuperscript{23} that microinjection of DA in the DVC of control rats decreases gastric tone and motility, and weekly injections of paraquat induce misfolding of α-synuclein in both the DMV and the SNpc\textsuperscript{17}. In PQ-treated rats, microinjection of DA in the DVC, however, induced a biphasic gastric response in the majority of the rats. In fact, a combined DA1- and DA2-like receptor mediated gastroexcitatory response preceded the DA-induced gastroinhibition. As shown previously in control rats, the gastroinhibition in PQ-treated rats was mediated by DA2-like receptors, and the catecholaminergic inputs arising from neurons of the A2 area were not involved.

The short time-span between the end of the PQ-administration and the performance of the experiments is unlikely sufficient to induce the degeneration of TH-IR neurons in the SNpc\textsuperscript{26}, thus suggesting that brainstem vagal neurocircuits, rather than nigral degeneration, are involved in the response described herein.

We have shown recently that, in addition to the dopaminergic input onto DMV neurons originating from tyrosine-hydroxylase (TH) positive neurons of the adjacent A2 area\textsuperscript{18,28}, a robust tonic dopaminergic input arising from the SNpc also regulates gastric tone and motility\textsuperscript{17}. The gastric response to central DA, however, differed between the effects obtained upon microinjection in the DVC and the effects obtained upon selective stimulation of dopaminergic projections originating in the SNpc. Indeed, DVC microinjection of DA in naive animals induced
a profound reduction in gastric tone and motility, both of which were prevented by blockade of DA2-like receptors. Indeed, within the DVC, the DA2-like receptor is the predominant and most widely distributed dopaminergic receptor. Conversely, in naive animals, pharmacological and optogenetic manipulation of SNpc neurons and fiber terminals in the DVC caused a consistent increase in gastric tone and motility via nigro-vagal inputs on DA1-like receptors expressed both on neurons of the DMV and the A2 area. Since the DA1-like receptor subtype is not as widely distributed in the DMV as the DA2-like receptor, this response indicated a high level of specificity in brainstem vagal neurocircuits, as suggested previously.

The pharmacological characterization of the monophasic gastroinhibition in response to dopamine microinjection in naïve rats was the focus of our previous manuscript, in which we reported that the dopamine-induced inhibition of gastric tone and motility was mediated by DA2-like receptors only. Conversely, in PQ-treated rats, both DA1- and DA2-like receptors were recruited by dopamine microinjection. Furthermore, the A2 area does not appear to be involved in this biphasic response since pretreatment with the α2 adrenoceptor antagonist, yohimbine, had no effect upon the following dopamine microinjection. The involvement of neurons of the A2 area appears in response to dopamine, thus, appears therefore to be related exclusively to the SNpc-mediated stimulation of gastric tone.

We have also shown recently that the nigro-vagal pathway is compromised both in a paraquat-induced model of PD as well as in a rodent model of environmental PD, such that, in these PD models, stimulation of SNpc results in a reduced vagally-mediated excitation of gastric tone and motility. The expectation was that, in PQ-treated rats, the gastric response to DA microinjection in the DVC would be attenuated. It was then intriguing to observe therefore that the response to DA microinjection in the DVC induced an initial increase in gastric tone and motility, followed by a gastroinhibition similar to that observed in naïve rats. We speculate that this increase in dopaminergic–induced gastric motility may be due to neuroplasticity that induced the expression of otherwise silent or masked receptors (DA1-like receptors). Such a DA1-like mediated excitatory response may indicate the appearance of a compensatory mechanism that attempts, but fails, to overcome the observed gastric hypomotility reported in clinical practice as well as in experimental models of PD. Rather than alleviating gastric dysfunction, however, the unmasking of this receptor mediated an increase in gastric tone and motility observed in PQ-treated animals. This increase in tone and motility might exacerbate the gastrointestinal dysfunctions, by uncoupling the physiological, coordinated activation of antrum and corpus motility, ultimately resulting in delayed gastric emptying and gastroparesis.
Neuroplasticity in brainstem vagal circuits is known to occur as a result of pharmacological or environmental manipulations that affect the levels of cAMP in these circuits\textsuperscript{15,16,32,33}. Indeed, DA1-like receptors are coupled to a Gs subunit that increases cAMP though activation of the adenylyl cyclase\textsuperscript{34}, and their pathophysiological recruitment in this model of parkinsonism is likely to play a role in these neurocircuits.

While in general, neuronal plasticity is a physiological response to environmental changes, maladaptive plasticity as suspected from the experiment described herein, may result in rearrangements that are not necessarily beneficial. In fact, maladaptive plasticity such as that observed in phantom limb pain\textsuperscript{35}, tinnitus\textsuperscript{36}, task specific focal dystonia\textsuperscript{37} and drug induced dyskinesias\textsuperscript{38} may result in behavioral impairments or development of additional disease symptoms, preventing full recovery or even causing undesirable side-effects\textsuperscript{39}.

Focusing on SNpc neuronal degeneration, in aging rats tyrosine hydroxylase immunoreactivity does not decrease, despite the physiological loss of SNpc dopaminergic neurons, suggesting some form of compensatory neuroplasticity\textsuperscript{40}. In PQ-treated rats, however, the significant decrease in both TH-IR and cresyl violet positive neurons of the SNpc, occurs at time points later than that analyzed here\textsuperscript{26}, indicating a delayed neuronal degeneration rather than a decrease in the enzyme levels. Using quantitative morphometry, systematic studies of the human SNpc have shown that despite the neuronal atrophy and degeneration associated with both PD and aging, some SNpc dopaminergic neurons undergo hypertrophy and a neuroplastic increase in size\textsuperscript{41,42}.

Likewise, in an experimental pathological condition, such as following 6-OHDA induced nigral degeneration, the number of dopamine-β-hydroxylase immunoreactive neurons in the A2 area is increased significantly\textsuperscript{2,43}, suggesting a potential upregulation of brainstem catecholaminergic neurotransmission from A2 to vagal motoneurons. This is of particular relevance in the context of PD, because we have shown that activation of the nigro-vagal pathway also mediates the activation of the A2 area neurons, which influences gastric motility via α2-adrenoceptor activation\textsuperscript{17}. Contrary to our expectation, these receptors do not appear to be involved in the biphasic response to DA reported here.

In summary, in the present study we have shown that the response to exogenous administration of dopamine is altered in the vagal neurocircuits that control tone and motility.
This possible maladaptive plasticity may underlie, at least in part, the gastric dysfunctions observed in Parkinson’s disease.

**Acknowledgments, funding, and disclosures:** Supported by a grant from the PA Tobacco settlement fund and a NIH grant NIDDK DK-55530. The authors would also like to thank Cesare M. and Zoraide Travagli for support and encouragement, and Dr. K.N. Browning for critical comments on earlier versions of the manuscript.

**Author Contribution:**
C.B.: acquisition of data, analysis and interpretation of data, drafting of the manuscript, statistical analysis.
L.A.: acquisition of data, analysis.
R.A.T.: study conception and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, obtained funding.
CHAPTER 4.7 REFERENCES


CHAPTER 5

INGESTION OF SUBTHRESHOLD DOSES OF ENVIRONMENTAL TOXINS INDUCES ASCENDING PARKINSONISM IN THE RAT


* Authors contributed equally

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Chapter 5 consists of a previously published research article. Cecilia Bove drafted, edited and revised the completed manuscript. The research article has been reformatted to fit into this thesis. The citation for the research article is:

CHAPTER 5.1 SIGNIFICANCE

The data described in chapters 2 to 4 provided solid evidence for the involvement of the vagus nerve and the associated dorsal motor nucleus of the vagus in the pathophysiology of Parkinson’s Disease (PD). The model utilized in chapters 2 and 4, however, relies on high doses of the herbicide paraquat, which humans are not likely to encounter in real life. The aim of the present study was to show that subthreshold doses of paraquat, that resemble the doses of the herbicide at which humans are more likely to be exposed, in combination with a second exogenous agent, might trigger parkinsonism that starts from the enteric nervous system (ENS) as postulated by Braak and collaborators. The second substance examined herein is lectins, carbohydrate binding proteins that are highly abundant in raw vegetables and grains, and have been linked to higher incidence of PD in previously published epidemiological studies. This model of environmental parkinsonism recapitulated both the classical motor features of PD and the non-motor gastrointestinal dysfunctions observed in humans. Moreover, it allowed the reproduction of the Braak staging of the disease, based on the spatiotemporal distribution of Lewy bodies from the ENS up to the substantia nigra pars compacta (SNpc), and confirmed the involvement of the vagus nerve in the onset of the disease. Indeed, subdiaphragmatic resection of this cranial nerve prevented the spread of the α-synucleinopathy and the onset of the motor deficits. This study is crucial for a more faithful replication of the disease, and has provided an excellent model for the development of disease-preventing approaches that might be potentially more beneficial than the current disease-modifying therapies currently available. Further characterization of this model is provided in chapters 6 and 7, where the electrophysiological and pharmacological properties of the neurons of the dorsal motor nucleus of the vagus (DMV) are described.
CHAPTER 5.2 ABSTRACT

Increasing evidence suggests that environmental neurotoxicants or misfolded α-synuclein generated by such neurotoxicants are transported from the gastrointestinal tract to the central nervous system via the vagus nerve, triggering degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and causing Parkinson’s disease (PD). We tested the hypothesis that gastric co-administration of subthreshold doses of lectins and paraquat can recreate the pathology and behavioral manifestations of PD in rats. A solution containing paraquat + lectin was administered daily for 7 days via gastric gavage, followed by testing for Parkinsonian behavior and gastric dysmotility. At the end of the experiment, brainstem and midbrain tissues were analyzed for the presence of misfolded α-synuclein and neuronal loss in the SNpc and in the dorsal motor nucleus of the vagus (DMV). Misfolded α-synuclein was found in DMV and SNpc neurons. A significant decrease in tyrosine hydroxylase positive dopaminergic neurons was noted in the SNpc, conversely there was no apparent loss of cholinergic neurons of the DMV.

Nigrovagally-evoked gastric motility was impaired in treated rats prior to the onset of parkinsonism, the motor deficits of which were improved by L-dopa treatment. Vagotomy prevented the development of parkinsonian symptoms and constrained the appearance of misfolded α-synuclein to myenteric neurons. These data demonstrate that co-administration of subthreshold doses of paraquat and lectin induces progressive, L-dopa-responsive parkinsonism that is preceded by gastric dysmotility.

This novel preclinical model of environmentally triggered PD provides functional support for Braak’s staging hypothesis of idiopathic PD.

KEYWORDS: Brainstem, vagus, gastric motility, idiopathic parkinsonism
CHAPTER 5.3 INTRODUCTION

While the etiology of Parkinson’s disease (PD) is unknown, both genetic and environmental factors have been theorized to play a role in its pathogenesis. In the search for environmental triggers for the development of idiopathic PD, Braak’s group hypothesized that an ingested “unknown pathogen” enters the gastrointestinal (GI) tract, and is itself either transported retrogradely via the vagus nerve to the dorsal motor nucleus of the vagus (DMV) within the brainstem, or induces retrogradely spreading neural dysfunction. As a consequence of DMV involvement, the finely-tuned vagal modulation of GI motility is disrupted. Autonomic dysfunction, including delayed gastric emptying and reduced gastric motility, can occur long before the onset of the classical motor symptoms of PD. The recent discovery of an anatomical connection between the DMV and the substantia nigra pars compacta (SNpc) (i.e., the nigro–vagal pathway), as well its demonstrated importance in the control of gastric tone and motility, might be the pathway by which the “unknown pathogen” travels or induces the impairment of the neurocircuit from the DMV to SNpc. Disruption of this nigro–vagal circuit may, therefore, explain the prodromal gastric dysmotility observed in PD patients.

In many studies, different pesticides, e.g., rotenone, herbicides, e.g., paraquat, and toxins, e.g., MPTP and 6-OHDA, have been administered by various routes, including oral administration, in order to model idiopathic PD. Although very useful as animal models, environmentally induced idiopathic PD in humans is unlikely to result from either single or multiple exposures to high doses of an agent over a short period of time. Rather, individuals are exposed to a myriad of environmental toxins over the course of their lifetime. A more likely scenario, therefore, involves repeated exposures to low doses of toxins, or a combination of toxins, whose pathogenicity may be enhanced by external factors, including diet. Epidemiological studies, for example, have demonstrated that the drinking of well water, as well as long-term exposure to pesticides/herbicides and heavy metals, are all associated with an increased incidence of idiopathic PD.

Paraquat is a widely used herbicide and, given the strong positive correlation between its use and the incidence of idiopathic PD (reviewed in ref. 18), paraquat administration (up to 100 mg/kg p.o. or i.p., either alone or in combination with the fungicide, maleb), once or twice a week for three–six consecutive weeks is used commonly to induce experimental idiopathic PD. In this model, parkinsonian symptoms are observed typically after at least four weeks. Other investigators have raised issues, however, with contradictory studies that have not consistently shown loss of dopaminergic neurons in SNpc or replicated reliable parkinsonism following oral administration.
paraquat administration, while other reports have failed to show strong evidence for paraquat contamination of food\textsuperscript{21-24}. Dietary factors such as lectins have been implicated in the pathogenesis of idiopathic PD-like pathology in C. elegans\textsuperscript{20}. Lectins are ubiquitous carbohydrate-binding proteins that are present worldwide in the human diet\textsuperscript{25}. Lectins can penetrate the GI tract, either by endocytosis, via a breakdown in gut barrier function, or via a lectin receptor (saccharide)-mediated mechanism, and can be transported retrogradely within neurons\textsuperscript{26-29}. While lectins are environmentally pervasive, dietary lectins in properly cooked food are harmless and generally thought to pose no health risk\textsuperscript{25}. The consumption of raw uncooked vegetables, grains, and eggs that are rich in lectins, however, can potentially enhance the toxicity of pesticides and herbicides resulting in higher prevalence of idiopathic PD\textsuperscript{16}. By virtue of their membrane permeability, lectins have been developed as a chaperones for drugs, but have also been shown to transport viruses and toxin(s)\textsuperscript{30,31}, including those that may be responsible for α-synuclein inclusions in PD\textsuperscript{32}. As such, a lectin-mediated insult is likely to be gradual, and may be influenced by association with other macro/micronutrients or ingested chemicals. It is possible, therefore, that dietary lectins contribute to the transport from the GI tract to the central nervous system (CNS) of pathogens that induce degeneration of dopaminergic neurons and Lewy body-like protein aggregation, i.e., the histological hallmark of idiopathic PD\textsuperscript{33}. Thus, lectins may represent a key environmental factor in the development of this disease.

In the present study, therefore, we tested the hypothesis that gastric administration of subthreshold doses of paraquat and lectins induces an ascending pattern of α-synuclein aggregation in the vagus nerve and DMV and consequent gastric dysmotility, followed by degeneration of SNpc neurons and motor features of idiopathic PD.

**CHAPTER 5.4 MATHERIALS AND METHODS**

All procedures were conducted in accordance with the National Institutes for Health guidelines, with the approval of the Penn State University-College of Medicine Institutional Animal Care and Use Committee and according to journal policies and regulations on animal experimentation.

**5.4.1 In vitro α-synuclein fibril formation**

To examine the effect of paraquat and lectin on the kinetics of fibril formation, an in vitro fibrillation assay was used, as described previously\textsuperscript{34}. Briefly, solutions containing: (i) purified recombinant α-synuclein alone (35 μM in 50mM Tris-HCl buffer, pH 7.5); (ii) lectin from Pisum
sativum (0.0025%); (iii) paraquat (100 μM); or (iv) a combination of lectin and paraquat, were incubated at 37 °C with constant shaking at 300 rpm for ~40 h. Each sample was plated in triplicate on a 96-well plate, and 20 μM Thioflavine T, a fluorescent dye that binds to fibrillary structures, was added. The fluorescence (excitation at 450 nm and emission at 485 nm) was measured at different time points using a fluorescence plate reader (Spectramax Gemini EM, Molecular Devices, Sunnyvale, CA) interfaced with Softmax® pro 6.3.1 software (Molecular Devices). The relative fluorescence units were averaged and plotted as a function of time; the resulting plot was interpolated, normalized and fitted to a sigmoidal curve using GraphPad Prism® software (GraphPad Software, LaJolla, CA, USA).

5.4.2 Animals and treatment

Male Sprague–Dawley rats were housed in an AAALAC accredited Animal Care Facility maintained at 24 °C on a 12:12 h light/dark cycle. Food and water were provided ad libitum. Rats were gavaged daily, for seven consecutive days, with 1% sucrose (control; n = 12) or (i) 1% sucrose and 0.05% lectin from P. sativum+ paraquat (1 mg/kg, P +L; n = 20), (ii) 1% sucrose and 0.05% lectin (L; n = 5), or (iii) 1% sucrose and paraquat (1 mg/kg, P; n = 5). To promote absorption, gastric emptying was delayed by injection of cholecystokinin (3 μg/kg i.p.) 15 min prior to each gavage. Rats were allowed to recover two (n = 9) or four (n = 11) weeks before experimental procedures were carried out. A group of rats received injections of L-dopa (4 mg/kg) and benserazide (15 mg/kg, i.p. diluted in ascorbate saline; n =11) twice a day for two days, after the third week of recovery. Rats treated with lectins at doses up to 0.2% were observed for up to twelve weeks.

A group of rats was anesthetized with isoflurane (2.5% in 100% O2) and an abdominal laparotomy was performed to expose and sever both posterior and anterior subdiaphragmatic vagal branches, as described previously.6,35 The efficacy of the vagotomy was assessed with i.p. administration of 0.2 mg/kg fluorogold.

5.4.3 Tissue collection

At the conclusion of the behavioral or gastric experiments (see below), rats were euthanized under deep general anesthesia, rapid sternal thoracotomy and transcardiac perfusion with 200 ml of heparinized saline followed by 200 ml of 4% paraformaldehyde (PFA) in PBS. Brains were removed and postfixed in 4% PFA and 20% sucrose for 24–48 h at 4 °C, and then transferred in a solution containing PBS, 0.08% Na azide, and sucrose. The brains were sliced in 50μm-thick
coronal sections using a freezing microtome using either a 1:4 or 1:8 systematic random sampling routine and preserved as floating sections prior to further processing.

5.4.4 Immunohistochemistry

Detailed methodology has been described previously\textsuperscript{36,37}. Primary antibodies were (i) rabbit-α-\textsuperscript{129}Ser α-synuclein (Abcam, Cambridge, UK; 1:1000); (ii) goat-α-ChAT (Chemicon, Temecula, CA; 1:5000); (iii) mouse-α- TH (Immunostar, Hudson, WI; 1:10000) or rabbit α-TH (Pel-Freez biological, Rogers, AR; 1:200). For immunoperoxidase staining, secondary antibodies were biotinylated donkey immunoglobulins (IgGs) for multiple labeling (Jackson ImmunoResearch Laboratories, West Grove PA) diluted 1:1000; the detection complex was ExtrAvidin-horseradish peroxidase (ExtrAvidin- HRP; 1:1500). For immunofluorescence staining, secondary antibodies were donkey immunoglobulins Alexa Fluor 488 or 568 (ThermoScientific, Waltham,MA; 1:1000).

Both primary and secondary antibodies were incubated at room temperature on a shaker for three days or overnight, respectively. Brain slices were rinsed in PBS, mounted on gelatin-coated slides, air-dried overnight, dehydrated in alcohol, cleared in xylene, coverslipped with DePeX (Electron Microscopy Sciences, Hatfield, PA, USA).

5.4.5 Behavioral testing

A well-established rodent behavioral battery of tests\textsuperscript{38,39} was used to identify the parkinsonian phenotype in treated rats, as described previously\textsuperscript{40}. Briefly, these consist of the (1) vibrissae-evoked forelimb placement test (Vibrissae test), a forced reflex test in which the tester restrains three limbs and allows stimulation of the ipsilateral vibrissae to evoke a reflex ipsilateral forelimb placement on a firm surface. This test is repeated 10 × 3 at each testing session. (2) Stepping test\textsuperscript{39,40}, a partial forced reflex test in which the experimenter holds the testing rat, restraining both hind limbs and one forelimb at a time, with the free forelimb touching a flat surface. The rat is moved sideways along the surface at a rate of 90 cm/5 s in the direction of the testing forelimb. The test is repeated 3× separately for both forelimbs. Results are expressed as percentage of baseline. (3) Post L-dopa/benserazide treatments twice daily for 3 days and behavioral assessment using the vibrissae test at 1 and 2 hours post-treatment.

These motor behavioral tests were used to assess the parkinsonian phenotype prior to treatment (baseline), every week thereafter, as well as on the day of the gastric motility test. To avoid any pharmacological interaction, the last L-dopa treatment was conducted at least 1 week prior to the gastric motility studies.
5.4.6 Stereology

In each animal, an entire series of brain sections (1:4 or 1:8), containing the whole SNpc or the whole DVC, were stained using cresyl violet (CV) to identify key anatomical structures and structural integrity. Brain slices in representative groups were stained for TH as described below. TH-positive neurons in the SNpc were quantified using the Stereo Investigator software suite from MBF bioscience with a 100x magnification using a Olympus BX53 microscope (Olympus, Tokyo, Japan) fitted with a digital CCD camera (Hamamatsu, Hamamatsu City, Japan) and a motorized stage (Prior Scientific, Rockland, MA, USA). The total numbers of cells were estimated using the optical fractionator, the coefficient of error was calculated according to Gundersen et al., and values ≤0.05 were accepted as significant. TH stained sections were counterstained with CV to assess neuronal loss, as opposed to TH down-regulation, and estimated independently using design-based stereology as detailed above.

5.4.7 Gastric studies

Gastric tone and motility recordings were performed as described previously. Briefly, animals were fasted overnight (water ad libitum) before being anaesthetized deeply with Na-thiobutabarbital (Inactin® 100–150 mg/kg i.p). After intubation with a tracheal catheter, a midline laparotomy was performed and two custom-made 6 Å–8mm strain gauges (AT Engineering, Hershey, PA) were sutured to the serosal surface of the anterior gastric corpus and antrum in alignment with the circular smooth muscle. Leads were exteriorized, prior to suturing the abdominal laparotomy; the jugular vein was catheterized to permit systemic administration of bethanechol (10 μg/kg), a muscarinic agonist that does not cross the blood-brain barrier and excites the smooth muscle directly supramaximally. Animals were then placed in a stereotaxic frame and were instrumented for measuring the effects of microinjections in SNpc and DVC on gastric tone and motility as described previously.

The ionotropic glutamate receptor agonist, NMDA, (5 nmoles/200 nl) was microinjected into the SNpc (in mm, rostro-caudal (RC): −5.0 to 5.6 from bregma; medio-lateral (ML): 1.6–2.4 from midline; dorso-ventral (DV): −7.6 to 7.8 from the surface of the dura mater). To assess the effects of direct activation of vagal efferent motoneurons, TRH (0.1–3 pmol/60 nl) was microinjected into the left DVC (in mm, RC: 0.0–0.6 from calamus scriptorius; ML: 0.2–0.4 from midline; DV: 0.5–0.65 from the brainstem surface). All drugs were dissolved in isotonic phosphate buffered saline (PBS, in mM: 115 NaCl, 75 Na2HPO4, 7.5 KH2PO4; pH= 7.4).

Strain gauges signals were acquired with a Wheatstone bridge, filtered (low pass filter cutoff= 0.5 Hz; AT Engineering), amplified (EXP CLSG-2; QuantaMetrics, Newton, PA, USA)
and recorded on a computer using Axotape® 10 software (Molecular Devices, San Jose, CA). Gastric tone and motility were recorded for 2–5 min before and 15–20 min after drug application; the drug-induced effects on tone and motility were calculated through average value of the calibration measures as described previously. Since variations in size of the animal and in the strain gauge placement may lead to slight differences in responses between individual animals, each animal served as its own control, and motility data were measured as percentage changes over baseline (=100%).

Gastric motility was calculated using the following formula, as described previously:

\[
\text{Motility index percent} = \frac{[(N1 \times 1) + (N2 \times 2) + (N3 \times 4) + (N4 \times 8)] \times 100}{t}
\]

Where \( N \) = number of peaks in a particular force range and \( t \) = interval time in which the gastric motility is measured. \( N1 = 20–59 \text{ mg}, N2 = 60–100 \text{ mg}, N3 = 101–200 \text{ mg}, N4 \geq 201 \text{ mg}.\)

5.4.8 Materials

Unless indicated otherwise, all chemicals were obtained from Sigma- Aldrich (St. Louis, MO).

5.4.9 Statistical analysis

Data are reported as mean ± SEM and in all instances significance was set at \( p < 0.05 \).

Data were evaluated using one-way ANOVA followed by post-hoc Tukey’s multiple comparison test or one sided, paired t-test using GraphPad® software (Graph Pad Prism).

CHAPTER 5.5 RESULTS

5.5.1 Incubation of α-synuclein with subthreshold concentrations of lectin and paraquat accelerates the rate of fibril formation in vitro

In vitro incubation of α-synuclein alone (n = 6) induced fibril aggregation with a half-time \( (t_{1/2}) \) of + 25 ± 1.9 h. Incubation of α-synuclein in the presence of either paraquat (100 μM; n = 7) or lectin (0.0025% w/v; n = 4) accelerated the rate of fibrillation \( (t_{1/2} = +19 \pm 0.8 \text{ and } +18 \pm 1.1 \text{ h for paraquat and lectin, respectively, } p <0.05 \text{ vs. } \alpha\text{-synuclein alone}) \).

The \( t_{1/2} \) fibrillation was accelerated further upon incubation of α-synuclein with a solution containing both paraquat and lectin \( (t_{1/2} = +16 \pm 1.1 \text{ h, } p < 0.05 \text{ vs. paraquat or lectin alone; } n = 6; \text{ Fig. 1}) \).

These data suggest that, in combination, paraquat and lectin act co-operatively to accelerate the rate of α-synuclein fibrillation in vitro, compared to either paraquat or lectin alone.
Figure 1. Incubation with paraquat + lectin increases the rate of α-synuclein fibrillation.
(a) Time course of α-synuclein fibrillation in the presence of α-synuclein alone (white, α-syn, n = 6), paraquat (light gray, P, n = 7), lectin (dark gray, L, n = 4) or a combination of paraquat + lectin (black, P + L, n = 6). (b) Graphic summary of fibrillation t1/2 for α-synuclein. *p < 0.05 vs. α-synuclein alone; #p < 0.05 vs. paraquat or lectin

5.5.2 Misfolded α-synuclein is present in myenteric neurons of the GI tract, in the DMV, and in SNpc of paraquat + lectin treated animals

In control rats, 129Ser α-synuclein-immunoreactivity (IR) was not detected in myenteric neurons of the stomach (Fig. 2a), small (Fig. 2b) or large intestine (Fig. 2c). Two to four weeks after the end of the treatment with paraquat + lectin, however, expression of 129Ser α-synuclein-IR was observed in myenteric neurons through the whole extent of the GI tract, including rats that received vagotomy prior to the treatment (Fig. 2d-l). At either two–four weeks after the end of the treatment, 129Ser α-synuclein-IR was also observed in choline acetyltransferase (ChAT)- and tyrosine hydroxylase (TH)-positive neurons of the DMV, the A2 area, and the SNpc (Fig. 3d-i) of treated rats, but not in control animals (Fig. 3a-c) or in animals treated with lectin of paraquat alone. In animals (n = 9) that underwent complete subdiaphragmatic vagotomy prior to paraquat + lectin administration, 129Ser α-synuclein-IR was not observed in in vagal neurons of the dorsal vagal complex (DVC) or in SNpc (Fig. 3j-l).

These results provide further support to the observation that a histological hallmark of idiopathic PD is observed in enteric neurons, as well as in key CNS nuclei following treatment with paraquat + lectin, but following subdiaphragmatic vagotomy, 129Ser α-synuclein-immunoreactivity is limited to myenteric neurons.
Figure 2. Paraquat + lectin treatment promotes α-synuclein misfolding in myenteric neurons of the GI tract.
Representative micrographs showing 129Ser α-synuclein in the myenteric plexus isolated from the stomach (a), (d), (g), (j), the small intestine (b), (e), (h), (k), and the large intestine (c), (f), (i), (l) from control animals (top row), animals sacrificed two (second row) or four (third row) weeks after the end of the gavage with paraquat + lectin, or animals that received subdiaphragmatic vagotomy prior to the paraquat + lectin treatment (bottom row). Calibration bars: 50 μm
5.5.3 Paraquat and lectin treated animals have impaired motor functions.

Prior to treatment with paraquat + lectin, the baseline score for the vibrissae test was $+9.6 \pm 0.1$ successful forelimb placement/10 trials ($n = 12$). Two weeks after the end of the treatment, the score decreased significantly to $+6.1 \pm 0.7$ successful forelimb placement/10 trials ($n = 12$; $p < 0.05$). The impaired motor performance showed no further deterioration, with the score four weeks after the end of the treatment being $+6.2 \pm 0.9$ successful forelimb placement/10 trials ($n = 11$; $p < 0.05$ vs. baseline). A significant amelioration of Parkinsonism was evident after four weeks.
doses of L-dopa, with an increase in vibrissae test scores to +8.3 ± 0.65 successful forelimb placement/10 trials (p < 0.05 vs. paraquat+ lectin; p > 0.05 vs. baseline), supporting the hypothesis that exposure to subthreshold doses of paraquat + lectin induces ongoing nigrostriatal dopaminergic degeneration that is reversibly ameliorated with L-dopa treatment (Fig. 4a).

A similar, but lesser impairment in motor behavior was observed with the stepping test. The baseline stepping test score was decreased significantly to +76.4 ± 4.7% (n = 9; p < 0.05 vs. baseline), two weeks after the end of the treatment with paraquat + lectin. The motor performance continued to deteriorate by four weeks after the end of the treatment to +66.4 ± 4.7% (p < 0.05 vs. own baseline). Administration of L-dopa did not show a significant amelioration of the stepping impairment, likely determined by the minor impairment observed in this partial forced motor test (Fig.4b).

As expected, from the extent of the toxin-induced bilateral lesion of the nigrostriatal pathways (see below), these rats did not exhibit any rotational behavior either spontaneously or following L-dopa administration. These brief treatments did not induce any drug-related dyskinesias.

In contrast, the motor performances remained at baseline levels when rats were gavaged with lectin or paraquat alone (+10 ± 0 vibrissae test, and +92.2 ± 4 and +85 ± 3% stepping test, n = 5 for both groups; p > 0.05 vs. P + L).

Similarly, rats that underwent subdiaphragmatic vagotomy prior to the paraquat + lectin treatment did not show motor impairment two weeks after the end of the gavage in either tests (vibrissae: +9.8 ± 0.1, and stepping: +96 ± 1.7% of baseline; n = 9; p > 0.05 vs. own baseline). At four weeks, their motor score was significantly higher than that of nonvagotomized rats at the same time point (vibrissae: +10 ± 0, and stepping: +86.6 ± 1.6% of baseline; n = 9; p < 0.05 vs. nonvagotomized rats at four weeks; Fig. 4).

These data indicate that paraquat + lectin treatment induces parkinsonism that is relieved by L-dopa treatment and it is prevented by subdiaphragmatic vagotomy prior to the treatment.
Figure 4. Paraquat and lectin treatment impairs motor activity.
Graphic summary showing the motor performance of rats examined with the vibrissae (a) and stepping (b) tests following treatment with paraquat + lectin. Note that the motor activity was significantly reduced two weeks after the end of treatment and persisted thereafter. L-dopa pretreatment induced a significant improvement of motor performance assessed with the vibrissae test (n = 12). Animals that received subdiaphragmatic vagotomy (n = 9) prior to the treatment did not show any motor impairment. *p < 0.05 vs. baseline.

5.5.4 Paraquat and lectin-treated animals have a decreased number of TH-positive neurons in the SNpc

Stereological estimates showed a significant loss of TH-positive neurons in the SNpc four weeks after the end of the treatment (n = 3, 4 for control and P + L, respectively; p < 0.05). Nissl staining showed a comparable decline in SNpc neuronal number, suggesting a loss of neurons, rather than a temporary down regulation of TH expression (Fig. 5). Conversely, the number of ChAT-IR neurons in the DMV was unchanged, i.e., +1900 ± 368 and +2123 ± 126 neurons in control and paraquat + lectin treated rats, respectively (p > 0.05). Rats treated with paraquat or lectin alone did not show any decline in SNpc neuronal number (n = 5 for both groups; p > 0.05 vs. P + L).
These data indicate that animals treated with subthreshold doses of paraquat + lectin induce a significant, bilateral nigrostriatal degeneration of dopaminergic neurons of SNpc, but not of cholinergic DMV neurons.

Figure 5. Treatment with paraquat + lectin induces loss of TH-positive neurons in the SNpc. Representative images of TH-positive neurons in SNpc of control (a) and treated animals (b). Insets are higher magnifications of the boxed areas in the respective panels. Calibration bar: 100 µm. (c) Graphic summary of TH-IR neuronal number in both the left and right SNpc of control (white) and P + L treated (black) rats. Note that a significant loss of neurons was detected in SNpc of animals four weeks after the last gavage of paraquat + lectin (n = 3 for controls and 4 for P + L 4 weeks, respectively; *p < 0.05 vs. control). (d) Graphic summary showing that the mean estimate number of TH-IR neurons is not significantly different from the mean estimate number of CV-positive neurons in the SNpc of P + L treated animals (n = 4).

5.5.5 The Nigro–Vagal pathway that controls gastric motility is impaired following treatment with paraquat and lectin

We confirmed our previous findings showing an increase in gastric tone and motility following microinjection of N-methyl-D-aspartate (NMDA, 5 nmoles/200 nl) into the SNpc of control rats. This NMDA-induced gastroexcitation, observed in both the antrum and corpus, was diminished markedly in rats tested either two or four weeks after treatment with paraquat + lectin. Data for paraquat+ lectin are summarized in Fig. 6 (antrum) and Table 1 (corpus).
Similarly, we confirmed previous reports\textsuperscript{44} showing that DVC microinjection of thyrotropin-releasing hormone (TRH, 0.1–3 pmoles/60 nl) increased gastric tone and motility in control rats in a dose-dependent manner. Following paraquat + lectin treatment, however, the TRH-induced increase in tone and motility was reduced significantly. Data are summarized in Fig. 6 (antrum) and Table 1 (corpus).

<table>
<thead>
<tr>
<th></th>
<th>NMDA (5nmol/200nl) in SNpc</th>
<th>TRH (0.1pmol/60nl) in DMV</th>
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<tbody>
<tr>
<td></td>
<td>Tone (mg)</td>
<td>Motility (% of baseline)</td>
</tr>
<tr>
<td>control</td>
<td>+410 ± 51.9</td>
<td>+553 ± 135</td>
</tr>
<tr>
<td>P+L/2wks</td>
<td>+211 ± 63.8*</td>
<td>++307 ± 49.7</td>
</tr>
<tr>
<td>P+L/4wks</td>
<td>+255 ± 37*</td>
<td>289 ± 38.2*</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>TRH (1pmol/60nl) in DMV</th>
<th>TRH (3pmol/60nl) in DMV</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Tone (mg)</td>
<td>Motility (% of baseline)</td>
</tr>
<tr>
<td>control</td>
<td>+419 ± 81.7</td>
<td>+803 ± 139.7</td>
</tr>
<tr>
<td>P+L/2wks</td>
<td>+776 ± 271.6</td>
<td>+270 ± 20*</td>
</tr>
<tr>
<td>P+L/4wks</td>
<td>+353 ± 75.9</td>
<td>+332 ± 66.7*</td>
</tr>
</tbody>
</table>

Table 1: Tone and motility response in gastric corpus following NMDA and TRH microinjections indicate a compromised nigrovagal innervation.
Values expressed as mean ± S.E.M.; p<0.05

Treatment with either paraquat (n = 5) or lectin (n = 5) alone, did not alter significantly any of the gastric responses to NMDA or TRH microinjection.

To evaluate if the attenuated increase in gastric tone and motility observed in treated animals in response to central microinjection of either NMDA or TRH was due to an impairment of the gastric smooth muscle functionality, bethanecol was administered (dose: 10 μg/kg i.v.). The bethanecol-induced increases in gastric tone and motility were comparable among all groups, indicating that paraquat + lectin treatment did not compromise gastric smooth muscle. Data are summarized in Table 2.
Table 2: The gastric motility response to bethanecol i.v. administration shows no alteration of smooth muscle functionality.
Values expressed as mean ± S.E.M.; p<0.05

Together, these data support the hypothesis that gastric administration of subthreshold doses of paraquat + lectin induces an impairment of the nigro–vagal pathway without compromising gastric smooth muscle.
Figure 6. Treatment with paraquat and lectin induces impairment of the nigro–vagal pathway.

(a) Representative recordings from the gastric antrum showing that the increase of tone and motility following microinjection of NMDA in the left SNpc was reduced progressively at two and four weeks after the last gavage treatment. (b) Bottom panel: graphic summaries showing that two or four weeks after the paraquat and lectin treatment, microinjection of NMDA in the left SNpc increased gastric antrum tone (n = 9, 6, 10 for control, P + L two weeks and P + L four weeks, respectively) and motility (n = 7, 7, 9 for control, P + L two weeks and P + L four weeks, respectively) to a significantly lesser extent than in controls (*p < 0.05 vs. control).

(c) Representative recordings from the gastric antrum showing that the increase of tone and motility following microinjection of TRH (1 pmole/60 nl) in the DMV was reduced progressively at two and four weeks after the last gavage treatment. (d) Graphic summaries showing that, two or four weeks after the paraquat and lectin treatment, microinjection of TRH (0.1–3 pmole/60 nl) in the DMV increased gastric antrum tone and motility to a significantly lesser extent than in controls (*p < 0.05 vs. control)
CHAPTER 5.6 DISCUSSION

In the present study, we demonstrated that co-administration of subthreshold doses of lectin + paraquat produce (i) consistent pathological hallmarks of α-synuclein aggregation in enteric, brainstem, and midbrain neurons, (ii) stable parkinsonism associated with modest, but significant, degeneration of SNpc dopaminergic neurons, and (iii) motor parkinsonism is reversibly treatable with L-dopa. We also demonstrated a sequential progression of α-synuclein aggregation, with inclusions in the DMV preceding those in the SNpc. This temporal pattern of central dysfunction was mirrored functionally, with dysregulated gastric responses to stimulation of either the nigro–vagal pathway or the DVC preceding the development of motor parkinsonism. Finally, animals that received subdiaphragmatic vagotomy prior to paraquat + lectin administration did not show motor parkinsonism, and the accumulation of misfolded α-synuclein was confined to myenteric neurons, further supporting a vagally mediated progression of the synucleopathy.

The temporal pattern and progression of parkinsonism described in our current animal model replicates faithfully the predictions of a temporally distinct pattern for idiopathic PD as outlined by Braak’s staging hypothesis, i.e., a spread of synucleopathy that starts with the ingestion of an unknown pathogen, which enters myenteric neurons of the enteric nervous system, then travels to the CNS via retrograde transport through the vagus nerve, affecting the DMV (without causing neuronal loss) and the fine vagal modulation of GI motility first and, later, higher areas including the dopaminergic neurons of the SNpc, thus impairing motor functions.

Herein, we have shown that subdiaphragmatic vagotomy restricted misfolded α-synuclein to gastric myenteric neurons following paraquat + lectin administration, with no progressive synucleinopathy being observed in either DMV or SNpc, or Parkinsonism, thus supporting a ENS–vagal route of the pathology. Support for the involvement of the vagus nerve in different phases of PD is found in studies showing that patients who received truncal vagotomy, thereby severing the myenteric neuron–vagal–DMV connection, showed a clear reduction in the incidence of PD. Moreover, a potential vagal pathology in PD is reinforced by findings that the electrogastromyography patterns of PD patients are similar to those of vagotomized patients.

Despite multiple studies demonstrating that misfolded α-synuclein can spread and propagate in a prion-like fashion, it is important to note that it is likely that other factors such as unique histological features, mitochondrial stress, and cytosolic calcium levels are responsible for the regional distribution of idiopathic PD pathology. Therefore, it is possible that a combination of the spread of pathogenic α-synuclein together with endogenous factors renders neurons susceptible to damage.
As with most paradigms, the experimental models of PD used currently have many advantages and disadvantages\textsuperscript{51,52}. A plethora of studies have shown that exposure to paraquat is correlated positively with parkinsonism in humans\textsuperscript{13,53,54}. Indeed, several studies have reported that systemic chronic administration of high doses of paraquat in experimental animals induce some of the hallmark parkinsonian disturbances\textsuperscript{12,13,22,24,53,55}. The ability of paraquat itself to cause idiopathic PD has, however, been called into question due to the lack of reproducibility of the pathology using oral dosing models, the high doses used in most animal studies, the lack of paraquat residues in food, and scientific fraud in some reported studies (reviewed in recent letter by Cook et al.\textsuperscript{217-10,19,23,56}). Other toxin models that have stable behavioral outcomes, such as the 6-OHDA induced model in rats, require intracranial administration of the toxin, while systemically administered toxins like rotenone, MPTP, and paraquat do not replicate the route of entry of environmental toxins\textsuperscript{19,57}. Moreover, these models induce severe Parkinsonism over a relatively short time period, which does not reflect the temporal pattern and progressive nature of idiopathic PD. Additionally, models that overexpress α-synuclein, either via genetic manipulation or via recombinant vector administration\textsuperscript{58}, either do not replicate the natural course of the disease or fail to replicate the full repertoire of motor deficits\textsuperscript{51}.

In contrast, our model replicates the likely route of environmental agents that instigate idiopathic PD pathology, namely ingestion and enteric entry, follows a temporally defined pattern inducing brainstem disruption that precedes the nigral pathology that results in bradykinesia, i.e., the behavioral hallmark of idiopathic PD. The extent of the behavioral deficits observed in our model are milder and more variable, which also simulates the natural course of early idiopathic PD. Our data also provide a putative reason for the variability noted in previous oral paraquat toxicity models in that co-administration of lectin is required to induce a more consistent pathology. Furthermore, this raises the possibility that consumption of raw lectin-rich food in rural communities where chronic environmental exposure to toxins such as paraquat is endemic, provides an underlying mechanistic basis for the pathogenesis of idiopathic PD\textsuperscript{12-16,55,59}. Ingestion of such a diet, therefore, by virtue of the chemical properties of lectins\textsuperscript{26-29} may facilitate the absorption and/or transport of toxins in susceptible individuals\textsuperscript{30,31}. At the doses and administration route, i.e., oral gavage, used in the present study, however, lectins or paraquat, when administered alone, did not induce any significant effect on the parameters analyzed, i.e., motor performance, gastric emptying, tone and motility, and immunohistochemical properties.

Our data suggest that impairment of both the recently described nigro-vagal\textsuperscript{6}, as well as the TRH-activated vagal efferent pathway\textsuperscript{44} occur in the absence of functional disruption of the gastric smooth muscle itself. Such gastric dysfunctions were observed prior to impairment of
motor control and in advance of SNpc neuronal degeneration, mimicking the prodromal GI issues observed in many parkinsonian patients.

There has also been considerable debate on the mechanism(s) through which paraquat may enter the CNS across the blood–brain barrier\textsuperscript{60}. Results from our study suggest that the presence of lectins is also required to induce α-synuclein aggregation in the gut as well as its spread into the CNS via the vagus nerve, and subsequently into the SNpc via the newly discovered nigro–vagal pathway.\textsuperscript{6} Further support for this novel mechanism of action is provided by the recent description of α-synuclein accumulation in a subpopulation of enteroendocrine cells that exhibit neuron-like properties and have direct connections to enteric and/or extrinsic nerves\textsuperscript{61}. Indeed, the suggestion has been raised that α-synuclein itself could act as a lectin.

In conclusion, our study shows that the ability of orally administered subthreshold doses of paraquat in the presence of lectins to trigger parkinsonian pathology. Although we did not characterize the mechanistic features of this model, the range of neurodegenerative and pathophysiological changes induced by co-administration of paraquat + lectin reproduces many of the cardinal features of the human disease, including, parkinsonism that is responsive to L-dopa therapy, neurocircuit dysfunction, induction of neuronal α-synucleinopathy, neurodegeneration leading to the loss of SNpc dopaminergic neurons while sparing the DMV, as well as prodromal gastric motility disturbances that were observed in absence of smooth muscle impairment. The appearance of gastric dysfunction and Parkinsonism, its prevention by subdiaphragmatic vagotomy, and the distinct sequence of pathological and degenerative changes described herein, make for an attractive experimental model that will help identify triggering factors essential to idiopathic PD etiology, as well as being of use in the discovery of biomarkers and testing of new therapeutics at a stage where interventions would be disease modifying rather than symptom-alleviating.

Acknowledgments, funding, and disclosures: We thank Cesare M. and Zoraide Travagli for support and encouragement. We acknowledge Elizabeth Neely and Brian Chiou from Dr. James Connor’s lab for their help with the in vitro fibrillation assay. We also gratefully acknowledge the discussion, suggestions and editing of Dr. Kirsteen N. Browning.

This work was supported by NIH grant DK55530, a grant from Michael J. Fox Foundation for Parkinson’s Disease and a grant from the Tobacco Settlement Fund of PA to R.A. Travagli. Additional support for this work was provided via NIH R01NS42402, R21AT001607, and DIBTH0632, Grace Woodward Fund and the Pennsylvania Tobacco Settlement Funds.
Biomedical Research Grant, Penn State University Brain Repair Research Fund, Anne M. and Phillip H. Glatfelter III Foundation and Ron and Pratima Gatehouse Trust Fund to T. Subramanian and K. Venkiteswaran.

Author Contribution:
### 5.7 REFERENCES


CHAPTER 6

CHARACTERIZATION OF THE BASIC MEMBRANE PROPERTIES OF NEURONS OF THE RAT DORSAL MOTOR NUCLEUS OF THE VAGUS IN PARAQUAT-INDUCED MODELS OF PARKINSONISM

Cecilia Bove, Florence H. Coleman, and R Alberto Travagli¹

¹ Chapter 7 consists of a submitted research manuscript. Cecilia Bove was the first author, drafted, edited and revised the completed manuscript. The review article has been reformatted to fit into this thesis.
CHAPTER 6.1 SIGNIFICANCE

In chapters 2, 4 and 5 we have demonstrated that in two different rodent models of parkinsonism, we were able to replicate the prodromal gastric dysfunctions observed in Parkinson’s disease (PD) patients, as well as the staging of disease progression proposed by Braak and collaborators. Although the role of the vagus nerve in the etiology of PD was confirmed in chapter 6, in addition to corroborating the role in the DMV as the first brain region affected by the presence of α-synuclein aggregates in both models, no description of the effects of paraquat on the intrinsic activity of DMV neurons was provided. In the present study, a combination of current and voltage clamp recordings of DMV neurons was used to examine the intrinsic membrane properties of these neurons in both the classical paraquat model of PD induction, as well as in the novel model of PD described in chapter 6. Our data support a study published in a model of α-synuclein overexpression in which the DMV has been described as a resilient area capable of initiating a stressless pacemaking activity to resist the oxidative stress resulting from accumulation of Lewy bodies. Indeed, we report that despite DMV neurons responding to administration of paraquat, either alone or in combination with lectins, by accelerating the kinetics of the afterhyperpolarization phase, no increase in excitability was detected, suggesting the maintenance of a normal pacemaking activity. Interestingly, voltage-clamp analysis of miniature excitatory postsynaptic currents (mEPSCs) shows that gastric administration of paraquat and lectins primes the postsynaptic terminal, i.e. DMV neurons, to be more responsive to the release of excitatory neurotransmitters from the presynaptic terminal. This might suggest the initiation of a mechanism by which DMV neurons try to counteract this neuronal loss and diminished gastric function, which will be examined further in chapter 7.
CHAPTER 6.2 ABSTRACT

Most of Parkinson’s disease (PD) patients experience gastrointestinal dysfunctions, including gastric hypomotility. The dorsal motor nucleus of the vagus (DMV) modulates the motility of the upper gastrointestinal (GI) tract. Paraquat (P) administration induces Parkinsonism in experimental models, and we have developed recently an environmental model of Parkinsonism in which rats are treated with subthreshold doses of P and lectins (P+L), in both models rats develop reduced gastric motility. The aim of the present study was to examine whether the membrane properties of DMV neurons in these two experimental models of Parkinsonism were altered.

Whole cell recordings in slices containing DMV neurons were conducted in male Sprague Dawley rats which received either injections of paraquat (10mg/kg i.p.; 10P), or oral administration of paraquat (1mg/kg) and lectin (0.05% w/v; P+L). Morphological reconstructions of DMV neurons were conducted at the end of the recordings.

The decay kinetics of the afterhyperpolarization phase of the action potential was reduced in 10P neurons vs control, while the phase plot revealed a slower depolarizing slope. At baseline, the amplitude of miniature excitatory postsynaptic currents was increased in P+L neurons. No differences in the morphology of DMV neurons were observed.

These data indicate that the membrane and synaptic properties of DMV neurons are altered in rodent models of Parkinsonism, in which neurons of 10P and P+L rats demonstrate an increased excitatory transmission, perhaps in an attempt to counteract the paraquat-induced gastric hypomotility.
CHAPTER 6.3 INTRODUCTION

Parkinson’s disease (PD), the second most frequent neurodegenerative disorder after Alzheimer’s disease, is characterized by the chronic and progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). The lack of dopaminergic inputs causes the striatum to fire irregularly, resulting in an overall dysregulation of basal ganglia activity underlying the cardinal motor symptoms of the disease, i.e. tremor at rest, rigidity and bradikynesia. In addition to the motor dysfunctions, a variety of non-motor symptoms are associated with PD, including gastrointestinal (GI) complications such as esophageal dysfunctions, delayed gastric emptying, and constipation. These GI dysfunctions are prodromal to the emergence of the motor symptoms and the clinical diagnosis of possible PD appearing up to 20 years prior to diagnosis. Indeed GI symptoms in healthy individuals have been positively correlated with an increased risk of developing PD later in life.

The fine modulation of the functions of the upper GI tract is under the control of preganglionic parasympathetic neurons of the dorsal motor nucleus of the vagus (DMV). DMV neurons are spontaneously active with a firing rate of about 1 pulse per second (p.p.s.). Their spontaneous activity is modulated mainly by GABA, glutamate, and catecholamines inputs arising from the adjacent nucleus tractus solitarius (NTS). In addition to NTS inputs, the DMV receives inputs from other higher centers, including a tonic dopaminergic input which originates from the SNpc via the newly described nigro-vagal pathway, which activation increases gastric tone and motility.

Evidence supports a strong correlation between the use of the herbicide paraquat (P) and the incidence of PD. Indeed, in both the classical model of PD, i.e. intraperitoneal injections of P, as well as in the newly described model of environmental PD, i.e. oral administration of subthreshold doses of P with lectins from Pisum sativum, we showed an impairment of the nigro-vagal pathway resulting in GI symptoms, the presence of misfolded α-synuclein, a histological hallmark of PD, in enteric, DMV, and SNpc neurons, as well as motor symptoms of Parkinsonism which were relieved by L-Dopa treatment.

Despite the clear correlation between PD and GI disorders, little is known about the consequences of the disease on DMV neuronal properties. A recent paper by Lasser-Katz and collaborators has shown that, in a model of α-synuclein over-expression, DMV neurons initiate a “stressless-pacemaking” that prevents the increase in excitability observed in SNpc neurons in the
same model\textsuperscript{18}. A comprehensive analysis of the biophysical properties of DMV neurons in rats treated with P alone or P+L, however, has not been conducted.

The aim of the present study was to examine whether the membrane properties of DMV neurons in these two experimental models of Parkinsonism were altered.
CHAPTER 6.4 MATERIALS AND METHODS

All procedures were conducted in accordance with the National Institutes for Health guidelines, with the approval of the Penn State University College of Medicine Institutional Animal Care and Use Committee and according to journal policies and regulations on animal experimentation.

6.4.1 Animals

Male Sprague-Dawley rats (Charles River, Kingston, NY, USA) were housed under a standard 12-hour light/dark cycle at 24°C and had ad libitum access to food and water. After weaning (post-natal day 21) rats were separated into 4 groups: i) naïve animals that received no treatment (control group; CTL), ii) 10P group, which received i.p. injections of 10mg/kg of paraquat (Sigma-Aldrich, St. Louis, MO), weekly for three consecutive weeks \(^\text{15}\), and iii) P+L group, which received a daily oral administration of a solution containing 1mg/kg of P and 0.05% (w/v) of lectin from pisum sativum (Sigma-Aldrich)\(^\text{16}\). Rats from group ii were tested 2 days after the final injection of P and rats from group iii were tested 2 weeks after the end of the treatment. These are the time points at which the gastrointestinal dysfunction associated with Parkinsonism were observed in previous publications \(^\text{15,16}\).

6.4.2 Electrophysiology

Rats were anesthetized with Isoflurane (VEDCO, St. Joseph, MO) in a custom-made anesthetic chamber and a bilateral pneumothorax was performed after abolition of the foot-pinch withdrawal reflex. The brainstem was then removed and placed immediately in chilled (4°C), oxygenated Krebs’ solution (see Solution composition section). Four-six coronal slices (250-300μm thick) spanning the entire rostro-caudal extent of the DVC were cut using a vibratome. The slices were then incubated in oxygenated Krebs’ solution at 25±1°C for at least 90 min prior to recording. Electrophysiological recordings were performed from a single slice held in place by a nylon mesh in a custom-made 500μl perfusion chamber with flowing Krebs’ solution at 32±1°C. A Nikon E600FN was used to locate the DVC in the slice.

Whole cell recordings of DMV neurons were made with patch pipettes (3-5 MΩ resistance) filled with a potassium gluconate solution or a potassium chloride solution (see Solution composition section). Data were sampled every 100μs, filtered at 2 kHz, digitized via a Digidata 1322A interface (Molecular Devices, San Jose, CA), acquired, stored and analyzed on a PC utilizing pClamp 10 software (Molecular Devices) or with MiniAnalysis 60.
(Synaptosoft, Fort Lee, NJ). The junction potential was corrected manually and recordings were accepted only if the series resistance was <20 MΩ.

Electrophysiological properties measured included, in current clamp configuration: (1) duration of the action potential at threshold, (2) amplitude and decay kinetics of the afterhyperpolarization (AHP) phase of the action potential, and (3) frequency of action potential firing, expressed as pulses s⁻¹, in response to 400 ms-long DC pulses (20 to 270 pA in step increments). In voltage clamp configuration: (1) membrane input resistance (measured from the current deflection obtained by stepping the membrane from -70 to -80 mV for 400 ms), (2) amplitude and decay time of the current underlying the AHP evoked by stepping the membrane from -50 to +10 mV for 24 ms, and (3) frequency, amplitude, rise and decay time, and charge transferred of miniature excitatory and inhibitory postsynaptic currents in the presence of tetrodotoxin (TTX; 0.3μM) and bicuculline (10μM) or kynurenic acid (1mM), respectively. To analyze the decay time of the current underlying the evoked AHP, the current was fitted with the following formula to obtain the decay values for τ1 and τ2:

$$f(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i} + C$$

To characterize and compare action potentials across groups, the dynamic changes of the events were evaluated by performing a phase plot analysis. Briefly, changes of membrane potential as differential of the time (expressed as mV/ms) were plotted against the instantaneous voltage value (mV). The resulting loop graph was utilized to compare the threshold value (Vthresh), peak voltage value (Vpeak), repolarizing voltage value (Vrepol) as well as the rates of depolarization and repolarization calculated from the slopes of the loop graph. The differential equation dV/dt was calculated in pClamp 10, and the data was plotted and analyzed in SigmaPlot 11.0 (Systat Software, San Jose, CA).

At the end of the experiment, neurons were filled with neurobiotin (2.5% w/v; 0.3nA depolarizing pulse, 600ms duration, every 2 s) for 20 min to permit postfixation reconstruction. Slices were then immersed overnight in Zamboni’s fixative (see Solutions composition), transferred in PBS+0.05% Na Azide, and stored at 4°C until analyzed.
6.4.4 Morphological reconstruction of neurons

The morphological reconstruction of patched neurons was performed as described previously. Briefly, slices were cleared of fixative in PBS-TX and kept at 4°C until the injected neurobiotin (Vector Labs) was visualized using a cobalt–nickel enhancement of the Avidin D–horseradish peroxidase (Avidin D–HRP). Slices were incubated in Avidin D–HRP solution (see Solution composition section) for 2 h. After rinsing in PBS, and incubation for 15–20 min in Avidin D–HRP and DAB solutions (see Solution composition section), the slice was incubated for an additional 15 min in the presence of 3% H2O2. The slice was then rinsed in PBS, placed on a coverslip, air dried, cleared in alcohol and xylene, and mounted in Cytoseal™60 (Thermo Scientific, Cheshire, WA).

Three-dimensional reconstructions of individual neurobiotin-labeled neurons, digitized at a final magnification of 600X were made using Neurolucida® software (Microbrightfield, Williston, VT). The morphological features that were assessed include: soma area and diameter, form factor, whether the cell has bipolar or multipolar somata, number of segments (i.e., branching of dendrites), branch order, and extension in the x- and y-axes. Data analysis was performed as described previously.

6.4.5 Solutions composition

Krebs’ solution (in mM): 126 NaCl, 25 NaHCO3, 2.5 KCl, 1.2 MgCl2, 2.4 CaCl2, 1.2 NaH2PO4, and 10 D-Glucose maintained at 295-300 mOsm, and at pH 7.4 by bubbling with 95% O2 and 5% CO2.

Intracellular potassium gluconate solution (in mM): 128 potassium gluconate, 10 KCl, 0.3 CaCl2, 1 MgCl2, 10 HEPES, 1 EGTA, 2 ATP-Na, 0.25 GTP-Na, adjusted to pH 7.35-7.4 with KOH, with osmolarity 270-285 mOsm.

Intracellular potassium chloride solution (in mM): 140 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 1 EGTA, 2 ATP-Na, 0.25 GTP-Na, adjusted to pH 7.35-7.4 with KOH, with osmolarity 270-285 mOsm.

Zamboni’s fixative was made with 1.6% (w/v) paraformaldehyde, 19 mM KH2PO4, and 100 mM Na2HPO4•7H2O in 240 ml saturated picric acid-1,600 ml H2O, adjusted to pH 7.4 with HCl. PBS-TX was composed of (in mM): NaCl 115, Na2HPO4 75, KH2PO4 7.5, and 0.3% Triton X-100. Avidin D–HRP solution was made with 0.002% Avidin D–HRP in PBS containing 1% Triton X-100; 0.05% DAB in PBS containing 0.5% gelatin supplemented with
0.025% CoCl$_2$ and 0.02% NiNH$_4$SO$_4$. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich.

### 6.4.6 Statistical analysis

Data were analyzed by using one-way ANOVA followed by post-hoc Tukey’s multiple comparison test, and $\chi^2$ square test (Graph Pad Prism, Graph Pad Software Inc., La Jolla, CA) and are reported as mean±SEM. Data points with a value over two standard deviations from the mean were excluded. In all instances, significance was set at p<0.05.

### CHAPTER 6.5 RESULTS

#### 6.5.1 Paraquat treatment alters the basic membrane properties and action potential characteristics of DMV neurons

Systemic administration of paraquat (10P group) accelerated significantly the decay kinetics of the action potential afterhyperpolarization phase from 131±8ms in control animals (N=71) to 99±6ms in 10P treated animals (N=80; p<0.05). Gastric administration of paraquat and lectins (P+L group) did not alter the decay kinetics of the action potential afterhyperpolarization (146±11ms for P+L; N=64; p>0.05).

The amplitude of the action potential AHP, the input resistance (Rinp), and the duration of the action potential measured at firing threshold were similar among the groups (p>0.05 for all). Data are summarized in figure 1.
Figure 1. Paraquat treatment alters the basic membrane properties and action potential characteristics of DMV neurons.

(A) Representative traces of evoked action potentials from control (CTL, black), 10P- (blue), and P+L- (red) treated rats. Note that the action potentials have similar amplitude among the groups, but the AHP of DMV neuron from the 10P treated rat has faster kinetics of decay. Holding potential = -60 mV. (B) Whisker box plot showing the amplitude (left panel) and decay kinetics (right panel) of the afterhyperpolarization phase of the action potential. A significant reduction in the decay kinetics of the afterhyperpolarization phase of the action potential was observed in DMV neurons from 10P treated rats. Dots above the whisker box plot are outliers. Black bar: CTL, N=71; N=41 blue bar: 10P, N=80; red bar: P+L, N=64. *p < 0.05 vs control. (C) Whisker box plot showing that the input resistance is unaffected by the different treatments. Dots above the whisker box plot are outliers. Black bar: CTL, N=30; blue bar: 10P, N=26; red bar: P+L, N=30. (D) Whisker box plot showing that the duration of the action potential at threshold value is similar across all groups. Dots above the whisker box plot are outliers. Black bar: CTL, N=71; blue bar: 10P, N=80; red bar: P+L, N=64. p > 0.05.

The contribution of calcium-dependent potassium currents that underlies the action potential AHP \(^{24}\) was also examined in the voltage-clamp configuration. Similarly to that observed in current-clamp configuration, the amplitude of the resulting current was comparable across groups (p>0.05). Conversely, the fast component of the kinetic of decay was reduced significantly in all treated groups compared to control (44±3.8ms for controls, N=19; 11±0.9ms for 10P, N=41; 13±0.9ms for P+L, N=23; p<0.05; fig. 2).

Data are summarized in figure 2 and Table 1.
Figure 2. Paraquat treatment alters the kinetics of decay of the Ca^{2+}-K^+ currents. 

(A) Representative voltage clamp traces of $I_{K(Ca)}$ recorded from CTL (black), 10P (blue), and P+L (red) neurons after application of a depolarization step from -50 to +10 mV. (B) Whisker box plot showing the $I_{K(Ca)}$ amplitude. Dots above the whisker box plot are outliers. Black bar: CTL, N=57; blue bar: 10P, N=53; red bar: P+L, N=35. p > 0.05 for all. (C) Whisker box plot showing the two components of the $I_{K(Ca)}$ decay kinetics expressed as $\tau_1$ (left panel) and $\tau_2$ (right panel). A significant reduction in the decay kinetics of the fast component of the $I_{K(Ca)}$ was observed in all treatment groups vs control. Black bar: CTL, N=33-19; blue bar: 10P, N=41; red bar: P+L, N=23. *p < 0.05 vs control.

<table>
<thead>
<tr>
<th>Groups</th>
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<th>10P</th>
<th>P+L</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\tau_1$ (ms)</td>
<td>58 ± 3.5</td>
<td>57 ± 2.9</td>
<td>52 ± 2.5</td>
</tr>
<tr>
<td>N</td>
<td>33</td>
<td>41</td>
<td>23</td>
</tr>
</tbody>
</table>

Table 1. Slow decay time constant of the Ca^{2+}-dependent K^+ current measured in voltage-clamp configuration.

To better understand the characteristics of the action potential, a phase plot analysis of the action potential was performed on a subset of neurons from each group.

Neurons from control animals exhibit a much steeper depolarization slope compared to the treated groups (5.6±0.39 ms$^{-1}$ for controls; 3.7±0.4 ms$^{-1}$ for 10P; 3.6±0.4 ms$^{-1}$ for P+L; N=6 for all; p<0.05). No differences were observed in the other phase plot parameters analyzed. Data are summarized in figure 3 and Table 2.
Figure 3. Phase plot analysis of the action potential reveals changes in the depolarizing slope following paraquat treatment

(A) Schematic representation of the phase plot analysis. Top: sample action potential showing the voltage at: rest (Vrest), threshold (Vthresh), peak (Vmax), and repolarization (Vrepol), and the slope for the measurement of depolarizing and repolarizing phases. Bottom: sample phase plot calculated from the above action potential, with the corresponding aforementioned parameters.

(B) Representative phase plots from control (black), 10P (blue), and P+L (red). (C) Whisker box plot showing that the slope of repolarization is reduced significantly after paraquat treatment. Black bar: CTL; blue bar: 10P; red bar: P+L; N=6 for all. *p < 0.05 vs 10P and P+L.

Table 2. Action potential parameters as described by the phase plot analysis.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>10P</th>
<th>P+L</th>
</tr>
</thead>
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<tr>
<td>Slope of repolarization (ms⁻¹)</td>
<td>-1.4 ± 0.11</td>
<td>-1.4 ± 0.09</td>
<td>-1.3 ± 0.07</td>
</tr>
<tr>
<td>Vmax (mV)</td>
<td>29.5 ± 3.4</td>
<td>28.3 ± 2.5</td>
<td>29.4 ± 2.5</td>
</tr>
<tr>
<td>Vrepol (mV)</td>
<td>-87.7 ± 1.1</td>
<td>-81.1 ± 1.7</td>
<td>-82.5 ± 1.4</td>
</tr>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
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</table>

These data suggest that paraquat treatment acts directly on DMV neurons to alter their basic membrane properties by accelerating the rate of depolarization and the kinetics of decay of the action potential AHP phase.
6.5.2 Paraquat treatment does not affect the response of DMV neurons to current injection

Since we observed an acceleration in the kinetics of the AHP phase of the action potential following P treatment, the number of action potentials fired in response to injection of direct current pulses of increasing amplitude were examined (400 ms duration; 20–270 pA). When averaging the number of action potentials fired at each current step, no significant differences were observed across groups (N=75 for control; N= 87 10P; and N= 52 for P+L p< 0.05). Similarly, when analyzing the frequency of distribution of action potentials in discrete bins, we observed that, when stimulated with 270 pA of direct current, all groups have similar distribution of the number of action potentials fired, although the 10P-treated group shows a trend towards a rightward shift in the frequency (Fig. 4; $\chi^2>0.05$).
Figure 4. **Paraquat treatment does not affect the response to current injection of DMV neurons**

(A) Representative traces showing the response after injection of 20 (top trace) and 270 pA (bottom trace) direct current (DC, 400 ms long). (B) Frequency-response curves for DMV neurons from control (black), 10P (blue), and P+L (red). CTL (black; N=75), 10P (blue N=87), and P+L (red N=52). p>0.05 (C) Frequency distribution plot of neurons in the different bins (x axis) showing the maximum number of action potentials fired by DMV neurons at 270 pA. Black open bars: CTL; blue open bars: 10P; red open bars: P+L. χ² > 0.05.

Collectively, these data suggest that the changes in the basic properties of the action potential of neurons from animals treated with 10P do not appear to impact the response of DMV neurons to current injection significantly.

### 6.5.3 Paraquat and lectins increase the amplitude of miniature excitatory postsynaptic currents at baseline

Neurons from P+L rats showed a significant increase in the amplitude of miniature excitatory postsynaptic currents (mEPSCs) from 26±1.0 to 33±1.6 pA at baseline in control (N=37) and P+L (N=23), respectively (p<0.05). Conversely, the increase in mEPSCs amplitude was not observed
in neurons from 10P-treated rats. Likewise, no differences among the groups were observed when frequency, rise and decay time, and area of the mEPSCs were analysed (p>0.05).

Data are summarized in figure 5 and Table 3.

No differences in the amplitude, frequency, rise and decay time, or area under the curve were observed in miniature inhibitory postsynaptic currents. Data are summarized in Table 3.

Figure 5. The amplitude of mEPSCs is larger in DMV neurons from rats treated with paraquat and lectins.
(A) Representative traces showing miniature excitatory post-synaptic current events (mEPSC) recorded from control (CTL, black), 10P- (blue), and P+L (red) treated rats in the presence of 0.3 μM TTX and 10 μM bicuculline. Holding voltage= -50mV. Whisker box plot showing the amplitude (B), frequency (C), rise (D) and decay (E) times, and area (F) of mEPSCs. Dots outside the whisker box plot are outliers. Black bars: CTL (N=37); Blue bars: 10P (N=40); Red bars: P+L (N=23). *p<0.05
These data suggest that following oral administration of a combination of paraquat and lectin there is an increase in the baseline amplitude of mEPSCs, suggesting post-synaptic alterations in glutamatergic transmission.

### 6.5.5 The morphological properties of DMV neurons are unaltered by paraquat treatment

To evaluate whether paraquat exposure alters the morphology of DMV neurons, neuronal reconstructions were performed after the electrophysiological recordings by filling neurons with neurobiotin. The morphological reconstruction of DMV neurons revealed that there were no differences in neuronal size, number of segments, segments length, and branch order across groups. Data are summarized in table 4.
<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>10P</th>
<th>P+L</th>
</tr>
</thead>
<tbody>
<tr>
<td>X axis</td>
<td>313 ± 21.4</td>
<td>274 ± 18.2</td>
<td>301 ± 21.4</td>
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<tr>
<td>Y axis</td>
<td>170 ± 12.1</td>
<td>187 ± 11.5</td>
<td>195 ± 20.5</td>
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<tr>
<td>Soma area</td>
<td>267 ± 14.6</td>
<td>282 ± 13.6</td>
<td>241 ± 16.3</td>
</tr>
<tr>
<td>Number of segments</td>
<td>8.5 ± 0.6</td>
<td>8.4 ± 0.4</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td>Average segment length</td>
<td>220 ± 15.9</td>
<td>260 ± 42</td>
<td>222 ± 12</td>
</tr>
<tr>
<td>Branch order</td>
<td>3.7 ± 0.2</td>
<td>3.7 ± 0.2</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>N</td>
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<td>21</td>
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</table>

Table 4. Morphological characteristics of DMV neurons.

Altogether, these data suggest that induction of parkinsonism does not affect the DMV neuronal morphology in rats.

CHAPTER 6.6 DISCUSSION

In the present study we report that some of the membrane and synaptic properties of DMV neurons are altered in rodent models of Parkinsonism. Specifically, we have shown that (i) following an established model of PD induction, i.e. three weekly i.p. injections of 10mg/kg of paraquat, DMV neurons have faster kinetics of decay of the AHP phase; (ii) voltage clamp recording of $I_{KCa}$ show faster kinetics of the fast component in the PD models analyzed herein; (iii) the phase plot analysis of the action potential revealed that DMV neurons from both models of PD showed a slower depolarization slope compared to control; and, (iv) DMV neurons from P+L treated rats have a larger amplitude of mEPSCs at baseline.

Our previous studies reported that, at the time points utilized in the present work, rats from both the 10P and the P+L groups display gastric dysfunction\textsuperscript{15,16} despite the fact that this relatively short time-span does not allow for a widespread degeneration of SNpc neurons\textsuperscript{17}, supporting the idea that damage may be restricted to the vagal complex rather than also involving the nigro-vagal pathway\textsuperscript{15}.

The involvement of the DMV in the pathogenesis of environmental PD has been confirmed by several studies. Immunohistochemical analysis of tissues harvested from PD patients revealed the presence of Lewy Bodies (LB), pathologic aggregates of α-synuclein, in isolated myenteric
plexus neurons as well as motor neurons of the DMV. This distinct spatial pattern of distribution of LB, which correlates with the appearance of GI symptoms prior to the development of the motor deficits, was interpreted by Braak and collaborators as a possible triggering factor of PD pathology. Indeed, under this hypothesis, an environmental “unknown pathogen” is absorbed in the ENS and then transported retrogradely via the vagus nerve to the DMV. Our recent description of a monosynaptic nigro-vagal pathway supports the involvement of the DMV, and provides a direct point of connection between the vagus nerve and the SNpc, the area ultimately affected by neurodegeneration and responsible for the classic motor symptoms associated with PD. Impairment of this pathway has been proven to be responsible in part for the gastric dysfunction observed in a paraquat model of PD.

The importance of the vagus nerve and the DMV in the progression of PD was further confirmed in other animal models, as well in a novel model of environmental PD which utilizes oral administration of subthreshold doses of paraquat and lectin, and it is able to recapitulate the histological, motor, and GI features of PD. Although the involvement of the nigro-vagal pathway and the vagus nerve is supported by the aforementioned studies, what is less understood are the direct effects of environmental toxins on DMV neurons.

The data presented herein suggest that systemic administration of paraquat decreased the kinetics of decay of the AHP phase of the action potential of DMV neurons. Increasing the rate at which DMV neurons repolarize, however, did not increase their excitatory response to current injection. This evidence supports the “stressless pacemaking theory” observed in an α-synuclein over-expressing mouse model in which DMV neurons are able to engage an antioxidative response to α-synuclein over-expression to avoid disruption of their function. The authors of this study suggested that, unlike SNpc neurons, DMV neurons maintain their physiological autonomous firing rate by reducing the number of functional CaV 1.2 and 2.3 channel complexes, resulting in a reduction of the mean CaV currents. The CaV 1.2 subunit seems to have a role in slowing down pacemaking, so the reduction in CaV 1.2 levels observed in this α-synuclein over-expressing model of PD might underlie the results described in the present manuscript.

The importance of Ca²⁺ influx in DMV neurons is also highlighted by the variations in I_{K(Ca)} observed in this manuscript. Indeed, in all the experimental models used in the present study the decay kinetics of this current were faster. I_{K(Ca)} specifically the apamin-sensitive small
conductance (SK), is an important contributor of the afterhyperpolarization phase of DMV neurons. The faster kinetics of the afterhyperpolarization phase observed in the 10P treated rats, and the reduction in the kinetics of the \( I_{K(Ca)} \) observed in all PD models might represent a mechanism by which DMV neurons attempt, although unsuccessfully, to overcome the gastric dysfunctions observed in these two models.

The phase plot analysis of action potential, first described by Jenerick, was used to uncover subtle differences in the action potential characteristics following PD induction. While the slope of repolarization was not different between the various groups, we observed a reduction in the slope of depolarization following paraquat treatment. This alteration, which indicates that the paraquat-treated neurons require more time to reach the peak of the action potential (Vmax), has been reported in other neuronal types in neurological conditions such as epilepsy. Prior studies on hippocampal and neocortical principal neurons suggested that Na+-dependent dendritic-generated action potentials also exhibit slowing of the depolarization slope, implying that seizure-related action potentials might be generated in the dendrites, rather than the soma. Changes in action potential features are also correlated to an increase in extracellular K\(^+\), which is related to the generation of non-somatic (i.e. dendritic) events. Indeed, \([K^+]_o\) levels increase during a seizure, and local \([K^+]_o\) increase has been shown to cause extrasomatic firing in hippocampal in vitro slices. Changes in either Na\(^+\) or K\(^+\) levels might potentially happen at the DMV synapses, and would consequently increase the excitability of a neuronal population with only subtle changes in the membrane properties of the postsynaptic neurons. This evidence might further support the idea that DMV neurons are resilient to environmental stressors by initiating an antioxidative response that is not observed in SNpc neurons of animals exposed to PD-related stressors, i.e. overexpression of \(\alpha\)-synuclein.

The idea that, in these models, there is a disruption of the delicate microenvironment of the DVC, rather than the intrinsic membrane properties of DMV neurons, would explain some of the changes observed in the present manuscript and, by consequence, have a major role in the gastric dysfunctions reported previously, and supports the theory that PD is a circuit-based disorder rather than a neuronal-deficit disease. Indeed, we have shown that, at baseline conditions, neurons of animals treated with P+L have larger mEPSCs. This postsynaptic effect might be explained as a form of activity-dependent homeostasis, a phenomenon related to synaptic plasticity. Generally speaking, homeostatic changes require a long time course to become

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stable, and strengthen steadily. Increase in mEPSCs amplitude has been observed to be triggered following neuronal silencing, without a concurrent increase in mEPSCs event frequency\textsuperscript{44,46,47}. These changes depend on the neuronal population studied however, on the time course of neuronal silencing, and the state of maturation of the neurons\textsuperscript{43}. Neuroplasticity in brainstem neurocircuits has been described extensively\textsuperscript{11,12,48-50}, and we have recently hypothesized that maladaptive neuroplasticity could underlie the unusual gastric biphasic response to DA microinjection in the DVC observed in 10P-treated animals\textsuperscript{51}. Given that the increase in the amplitude of mEPSCs reported in this manuscript has only been observed in our P+L model of environmental PD\textsuperscript{16}, the involvement of the sensory component of the vagus nerve cannot be excluded. Further studies focusing on the NTS to DMV synapse are hence necessary. This postsynaptic adaptation could also be part of the attempt of DMV neurons to avoid the gastrointestinal dysfunction observed in PD.

Axotomized and/or damaged nerves show morphological changes and retraction of the dendritic tree\textsuperscript{52,53}, and damaged motoneurons show a progressive reduction in postsynaptic EPSP\textsuperscript{54}. Although the vagal neurocircuitry plays a role in PD pathogenesis, our data appear to indicate that, at the time points analysed in the present study, major morphological changes have not occurred yet.

In summary, in the present study we have shown that the membrane and synaptic properties of DMV neurons are altered in rodent models of parkinsonism. The faster AHP phase observed in neurons of 10P rats, the changes in I\textsubscript{K(Ca)} observed in all models, and the increased excitatory transmission in P+L rats suggest a futile attempt to counteract the paraquat-induced gastric hypomotility.

Acknowledgements

Funding: This work was supported by NIH grant DK-55530, and a grant from Michael J. Fox Foundation for Parkinson’s Disease to R. A. Travagli.

The authors would like to thank Dr. K.N. Browning for proof-reading and providing critical comments to earlier versions of the manuscript, to Cesare M. and Zoraide Travagli for support and encouragement, and to V. Gentile for his continued support. A special thanks to J. Dylan Weissenkampen for providing crucial help with some of the statistical analysis.
CHAPTER 6.7 REFERENCES


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CHAPTER 7

PARKINSONISM ALTERS SYNAPTIC INPUTS TO DORSAL MOTOR NUCLEUS OF THE VAGUS NEURONS IN RODENTS

Cecilia Bove, and R Alberto Travagli
CHAPTER 7.1 SIGNIFICANCE

In the present chapter, I used a combination of current and voltage clamp electrophysiological recordings to determine if the alterations in dorsal motor nucleus of the vagus (DMV) neuronal activity described in chapter 6 are accompanied by changes in the responsiveness to dopamine (DA) following Parkinson’s Disease (PD) induction in the rat. Previous studies in our laboratory showed that DMV neurons are responsive to DA acting on either DA1-like or DA2-like receptors. Here we report that this response is altered and may possibly be due to a presynaptic mechanism of action involving neurons of the nucleus of tractus solitarius (NTS). The results presented herein suggest that the gastrointestinal dysfunctions presented in chapters 2, 4 and 5 might be due to dysregulation of the synaptic inputs impinging onto DMV neurons, and confirm the idea that the basic membrane properties presented in chapter 6 might be altered in an attempt to counteract the alterations to the dopaminergic receptors described herein.
CHAPTER 7.2 ABSTRACT

Individuals affected by Parkinson’s Disease (PD) experience gastrointestinal dysfunctions, including gastric hypomotility, that appear prodromal to the appearance of the motor symptoms. The dorsal motor nucleus of the vagus (DMV) modulates the motility of the upper gastrointestinal (GI) tract, and it is regulated, among others, by the nucleus of tractus solitarius (NTS) receiving peripheral signals from the GI tract. Administration of paraquat (P) induces Parkinsonism in experimental models; treated rats show gastric dysfunction, and both high doses of paraquat as well as low doses of paraquat in combination with lectins alters some of the membrane properties of DMV neurons. The aim of the present study was to examine whether the synaptic inputs to DMV neurons were altered in two experimental models of Parkinsonism.

Whole cell recordings in slices containing DMV neurons were conducted in male Sprague Dawley rats which received either injections of paraquat (10mg/kg i.p.; 10P), or oral administration of paraquat (1mg/kg) and lectin (0.05% w/v; P+L). Miniature excitatory and inhibitory postsynaptic currents, as well as the number of action potentials fired in response to DC current injection were measured before and after application of dopamine (DA), the selective DA1- or DA2-like receptor antagonists SCH 23,390 and L-741,626 respectively, and the α2 adrenoreceptor antagonist yohimbine in combination with SCH 23,390.

DA decreases the frequency of both mEPSCs and mIPSCs in all animal groups, independently of the treatment. Blockade of DA2 like receptors decreases mEPSC and mIPSC frequency in all groups, but has an additional action to increase mIPSC frequency in a small population of neurons from control and 10P rats. Blockade of DA2 like receptors also increases the frequency of action potential firing in DMV neurons in 10P rats.

Blockade of DA1-like receptors reduces the frequency if mEPSCs and mIPSCs in all groups, but has a bimodal action (increase and decrease) in mEPSC and mIPSC frequency in 10P rats, as well as in mIPSCs in control rats. When combined with DA1-like receptors, addition of α2 adrenoreceptor antagonists enhances the reduction in mEPSC frequency in P+L rats.

These data indicate that the synaptic inputs from NTS to DMV neurons are altered in rodent models of Parkinsonism, and might be the cause of the gastric dysfunction described in the same models.
CHAPTER 7.3 INTRODUCTION

Parkinson’s disease (PD) is a movement disorder characterized by loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and by the presence of intracellular aggregates of α-synuclein in the form of Lewy bodies and neurites. Non-motor symptoms have also been recognized as part of the clinical presentation of the disease, and of particular interest are gastrointestinal (GI) dysfunctions, which appear even 20 years prior to the clinical manifestation and diagnosis of PD.

The neural control of the GI tract is under regulation of the dorsal vagal complex (DVC), which includes the dorsal motor nucleus of the vagus (DMV), the nucleus tractus solitarius (NTS), both of which include neurons of the catecholaminergic A2 area, and the area postrema. Peripheral sensory information from the GI tract is received by NTS neurons, where it is integrated with other inputs, modulated and transferred to the DMV, which ultimately regulate the function of the GI tract. NTS neurons release GABA, glutamate, and catecholamines, which are also released by A2 area neurons acting directly on DMV neurons. Recently, a nigro-vagal pathway originating in the SNpc has been described, which, upon its activation, releases DA on DMV neurons to increase gastric tone and motility via activation of DA1-like receptors. DA2-like receptors are also expressed on the membrane of DMV neurons, and constitute the majority of the DA receptor population in this area. Indeed, previous studies from our laboratory have demonstrated that DA application on DMV brainstem slices results in changes, in either directions, of the membrane potential.

Interestingly, in two different paraquat (P) models of PD, i.e. the classic 10 mg/kg P i.p. administration model and the recently described environmental model of PD that utilizes subthreshold gastric administration of P in combination with the carbohydrate-binding protein lectin (L), the nigro-vagal control of DMV neuronal activity is compromised, resulting in a reduction in the nigro-vagal inputs. We also demonstrated recently that DA microinjection into the DVC of anesthetized, naïve animals results in a profound gastroinhibition dependent on activation of DA2-like receptors, while in animals treated with 10 mg/kg P i.p. DA microinjection results in a biphasic gastric response, possibly indicative of a maladaptive neuroplasticity that fails to overcome the alteration in synaptic control of DMV neurons due, at least in part, to reduced DA input.

Our recent data on the DMV membrane properties following either 10P or P+L administration demonstrated that, while the excitability of DMV neurons in response to current...
injection is not affected by PD induction, there is an increase in the baseline amplitude of miniature excitatory postsynaptic currents (mEPSCs) following P+L treatment, possibly indicating alterations at the level of the NTS to DMV synapse.

The aims of the present study were to evaluate the modulation of miniature excitatory and inhibitory postsynaptic currents by dopamine in rodent models of environmental parkinsonism.

CHAPTER 7.4 MATERIALS AND METHODS

All the experimental procedures described below were conducted according to the National Institutes for Health guidelines, with the approval of the Penn State University College of Medicine Institutional Animal Care and Use Committee and according to journal policies and regulations on animal experimentation.

7.4.1 Animals

Male Sprague-Dawley rats (Charles River, Kingston, NY, USA) were housed under a standard 12-hour light/dark cycle at 24°C, with ad libitum access to food and water. At post-natal day 21 rats were separated into 3 groups: i) naïve animals that received no treatment (control group; CTL), ii) rats which received i.p. injections of 10mg/kg of paraquat (Sigma-Aldrich, St. Louis, MO) weekly for three consecutive weeks (10P group) 7, and iii) rats which received a daily oral administration of a solution containing 1mg/kg of P and 0.05% (w/v) of lectin from pisum sativum (P+L) 14.

Experiments were conducted at time points at which the gastrointestinal dysfunction associated with PD were observed previously 7,14. Specifically, rats from group ii were tested 2 days after the final injection of P and rats from group iii were tested 2 weeks after the end of the treatment.

7.4.2 Electrophysiology

Rats were anesthetized with Isoflurane (VEDCO, St. Joseph, MO) in a custom-made anesthetic chamber and, after abolition of the foot-pinחלת withdrawal reflex, a bilateral pneumothorax was performed. The brainstem was removed and immediately submerged in chilled (4°C), oxygenated Krebs’ solution (see below for composition). Four-six coronal slices (250-300μm thick) of the DVC were cut with a vibratome and incubated in oxygenated Krebs’ solution at 25±1°C for at least 90 minutes prior to recording.
Each recording was performed from a single slice held in place by a nylon mesh in a custom-made 500µl perfusion chamber with flowing Krebs’ solution at 32±1°C. DMV neurons were identified with a Nikon E600FN microscope equipped with DIC filters.

Whole-cell voltage-clamp recordings of DMV neurons were made with patch pipettes of 3-5 MΩ resistance when filled with K gluconate or KCl solutions (see below for composition). Data were sampled every 100µs, filtered at 2 kHz, digitized via a Digidata 1322A interface (Molecular Devices, San Jose, CA), acquired, stored on a PC utilizing pClamp 10 software (Molecular Devices) and analyzed with MiniAnalysis 60 (Synaptosoft, Fort Lee, NJ). The junction potential was corrected manually and recordings were accepted only if the series resistance was <20 MΩ.

Neurons were held at -50mV while recording miniature excitatory (mEPSCs) and inhibitory postsynaptic (mIPSCs) currents in the presence of tetrodotoxin (TTX; 0.3μM) and bicuculline (10μM) or kynurenic acid (1mM), respectively. The properties of the miniature postsynaptic currents measured included frequency, amplitude, rise and decay time, and area of the charge transferred. Neurons were held at the potential that allowed action potential firing of approximately 1 pulse per second (p.p.s.) while recording in the current clamp configuration, and the number of action potentials per second were measured before and after application drugs (see below). Drugs were applied for a period of time sufficient for the response to reach its maximum effect, or a minimum of 3 minutes, if no effect was observed. Drugs were applied at concentrations demonstrated previously to be effective 11.

Miniature postsynaptic current properties and variations in the number of action potentials per second were measured in response to the following drugs:

i) 30 µM dopamine (DA); ii) 10 µM of the DA1-like receptor antagonist, SCH 23,390; iii) 10 µM of the DA2-like receptor antagonist, L-741,626; and, iv) 10 µM of the α2-adrenoreceptor antagonist yohimbine (Research Biochemicals International, Natick, MA) alone or in combination with SCH 23,390. Cells were considered responsive to the drugs if a change in ±20% in the frequency of the miniature postsynaptic currents, and if a change in ±30% in action potential firing rate was observed. Variations in the miniature postsynaptic currents properties after drug applications were normalized to baseline and expressed as percentage.

7.4.3 Solutions composition
i) Krebs’ solution (in mM): 126 NaCl, 25 NaHCO3, 2.5 KCl, 1.2 MgCl2, 2.4 CaCl2, 1.2 NaH2PO4, and 10 D-Glucose maintained at 295-300 mOsm, and at pH 7.4 by bubbling with 95% O2 and 5% CO2;

ii) intracellular K-gluconate solution (in mM): 128 K-gluconate, 10 KCl, 0.3 CaCl2, 1 MgCl2, 10 HEPES, 1 EGTA, 2 ATP-Na, 0.25 GTP-Na, adjusted to pH 7.35-7.4 with KOH, with osmolarity 270-285 mOsm;

iii) Intracellular KCl solution (in mM): 140 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 1 EGTA, 2 ATP-Na, 0.25 GTP-Na, adjusted to pH 7.35-7.4 with KOH, with osmolarity 270-285 mOsm.

Unless otherwise stated, all chemicals and drugs were purchased from Sigma-Aldrich.

7.4.4. Statistical analysis

Changes in the miniature postsynaptic currents properties were compared across groups using one-way ANOVA followed by post-hoc Tukey’s multiple comparison test, and one-way paired t-test when two drugs were applied consecutively (Graph Pad Prism, Graph Pad Software Inc., La Jolla, CA) and are reported as mean±SEM. The numbers of responsive versus non responsive neurons were compared across groups with the \( \chi^2 \) test. Data points with a value over two standard deviations from the mean were excluded. In all instances, significance was set at \( p<0.05 \).

CHAPTER 7.5 RESULTS

7.5.1 DA decreased the frequency of the miniature excitatory postsynaptic currents.

We showed previously that exogenous administration of DA in vivo inhibits gastric tone and motility, and induces a biphasic response, i.e. excitation followed by inhibition, in 10P treated rats\textsuperscript{15,16}.

In all groups, bath application of 30 \( \mu \)M DA decreased the mEPSCs frequency to a similar degree. Specifically, mEPSCs frequency was reduced to 65±8.8, 50±10.3 and 66±6.2% of baseline in CTL (\( N=4 \) of 7 neurons), 10P (\( N=4 \) of 8 neurons), and P+L (\( N=5 \) of 8 neurons), respectively (\( p>0.05 \) for all; \( p>0.05 \) for proportion of responding neurons; fig.1). The remaining neurons did not respond with any variation in mEPSCs frequency to DA perfusion.
Figure 1. DA acts presynaptically to decrease the frequency of mEPSCs, but not amplitude.

(A) Representative traces from DMV neurons voltage clamped at -50 mV showing miniature EPSCs before (top) and after (bottom) application of 30μM DA in control, 10P- (blue; (B)), and P+L-treated animals (red; (C)).

(D) Computer-generated graphics from the same neurons as in (A), (B), and (C) showing the DA-induced decrease in mEPSC frequency (dashed lines) from baseline (solid lines).

(E) Summary of the DA-induced decrease in mEPSC frequency, but not amplitude (F). Open bars: control; blue bars: 10P; red bars: P+L. p>0.05

DA perfusion did not induce any variation in amplitude, rise and decay time, or area of the mEPSCs. Data are summarized in Table 1.

These data indicate that the predominant response of exogenous DA application is a presynaptic inhibition of glutamatergic transmission.
<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>10P</th>
<th>P+L</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEPSCs frequency %</td>
<td>64.9 ± 8.8</td>
<td>49.6 ± 10.3</td>
<td>65.5 ± 6.1</td>
</tr>
<tr>
<td>mEPSCs amplitude %</td>
<td>103.5 ± 1.1</td>
<td>98.1 ± 2.6</td>
<td>95.6 ± 5.4</td>
</tr>
<tr>
<td>mEPSCs rise time %</td>
<td>109.7 ± 8.6</td>
<td>106.0 ± 4.5</td>
<td>106.6 ± 8.0</td>
</tr>
<tr>
<td>mEPSCs decay time %</td>
<td>109.4 ± 10.3</td>
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</tr>
<tr>
<td>mEPSCs area %</td>
<td>113.4 ± 8.4</td>
<td>110.4 ± 10.1</td>
<td>112.7 ± 12.5</td>
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</tbody>
</table>

Table 1. DA decreases mEPSCs frequency without affecting any other parameter measured.

As expected, in 2 of 14 neurons from control rats, perfusion with the DA2-like receptor antagonist L-741,626 increased the frequency of mEPSCs to 257 and 125% of baseline in control, however, perfusion with L-741,626 did not increase mEPSCs frequency in neurons from 10P (N=10) or P+L (N=5) treated rats. Indeed, perfusion with L-741,626 decreased the frequency of mEPSCs to 57±6, 63±4.3 and 73±2.4% of baseline in CTL (N=7 of 14 neurons), 10P (N=7 of 10 neurons), and P+L (N=4 of 5 neurons), respectively (p >0.05 for all; fig.2) without affecting the amplitude of the events (p>0.05). Data is summarized in figure 2 and table 2. In any of the other neurons, i.e. 5 control, 3 10P and 1 P+L, perfusion with L-741,626 did not alter mEPSCs frequency.
Figure 2. L-741,626 acts presynaptically to decrease the frequency of mEPSCs, but not amplitude.

(A) Representative traces from DMV neurons showing mEPSCs before (top) and after (bottom) application of 10μM of the DA2-like receptor antagonist L-741,626 in control, 10P- (blue), and P+L-treated animals (red). Holding voltage: -50mV. (B) Graphic summary of the L-741,626-induced decrease in mEPSC frequency, but not amplitude (C). Open bars: control; blue bars: 10P; red bars: P+L. p>0.05

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>10P</th>
<th>P+L</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEPSCs frequency %</td>
<td>57±6</td>
<td>63±4.3</td>
<td>73±2.4</td>
</tr>
<tr>
<td>mEPSCs amplitude %</td>
<td>105.4 ± 4.8</td>
<td>98.4 ± 3.0</td>
<td>102.2 ± 3.9</td>
</tr>
<tr>
<td>mEPSCs rise time %</td>
<td>118.2 ± 4.2</td>
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<td>108.2 ± 8.6</td>
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<td>mEPSCs decay time %</td>
<td>107.5 ± 7.7</td>
<td>102.1 ± 5.4</td>
<td>110.4 ± 5.7</td>
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<tr>
<td>mEPSCs area %</td>
<td>113.7 ± 6.6</td>
<td>105.5 ± 7.5</td>
<td>110.2 ± 6.4</td>
</tr>
<tr>
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<td>4</td>
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Table 2. L-741,626-mediated decrease of mEPSCs frequency.
We demonstrated previously that the nigro-vagal pathway activates DMV neurons via tonic activation of DA1-like receptors.

Bath perfusion with 10μM of the DA1-like receptor antagonist, SCH 23,390 decreased the frequency of mEPSCs to 51±7.3, 59±12.6, and 56±5.6 % of baseline in CTL (N=7 of 11), 10P (N=4 of 13), and P+L (N=9 of 10), respectively (p>0.05 between 10P and CTL and P+L; p>0.05 for χ² test; fig. 3). In 5 of the 13 neurons from 10P treated rats, however, perfusion with SCH 23,390 increased the frequency of mEPSCs to 139±6% of baseline (p<0.05; fig. 3).

Figure 3. SCH 23,390 mediates both increase and decrease in mEPSCs frequency following 10P treatment.
(A) Representative traces from DMV neurons voltage clamped at -50 mV showing miniature EPSCs before (top) and after (bottom) application of 10μM of the DA1-like receptor antagonist SCH 23,390 in control, 10P- (blue), and P+L-treated animals (red). (B) Graphic summary of the SCH 23,390-induced modulation of mEPSC frequency, but not amplitude (C). Open bars: control; blue bars: 10P; red bars: P+L, p>0.05.
SCH 23,390 perfusion did not induce any differences in the amplitude, the rise and decay time, or the area of the mEPSCs. Data are summarized in Table 3.

<table>
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<tr>
<th>Groups</th>
<th>Control</th>
<th>10P</th>
<th>P+L</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEPSCs frequency %</td>
<td>51±7.3</td>
<td>59±12.6 / 139±6</td>
<td>56±5.6</td>
</tr>
<tr>
<td>mEPSCs amplitude %</td>
<td>93.1 ± 4.2</td>
<td>101.4 ± 2.5</td>
<td>98.5 ± 3.7</td>
</tr>
<tr>
<td>mEPSCs rise time %</td>
<td>116.9 ± 3.8</td>
<td>116.9 ± 7.9</td>
<td>110.1 ± 9.3</td>
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<tr>
<td>mEPSCs decay time %</td>
<td>115.8 ± 8.2</td>
<td>112.5 ± 6.3</td>
<td>121.2 ± 4.6</td>
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<tr>
<td>mEPSCs area %</td>
<td>105.1 ± 4.4</td>
<td>114.1 ± 7.8</td>
<td>114.3 ± 5.3</td>
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<tr>
<td>N</td>
<td>7</td>
<td>9</td>
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</table>

Table 3. SCH 23,390 effect on mEPSCs.

These data indicate that there is a tonic DA1-like input impinging on DMV neurons and that in rats treated with 10P the dopaminergic neurocircuitry is altered.

We showed previously that the SNpc-mediated modulation of DMV neurons occurs via release of DA which activates both DA1-like receptors on DMV neurons, as well as DA1-like receptors on neurons of the A2 area resulting in the activation of α2-adrenoreceptors on vagal neurons. Hence, we tested the effect of α2 adrenoreceptors antagonist yohimbine on the DA1-like receptor-mediated modulation of mEPSCs.

In the presence of SCH 23,390, which per se decreased mEPSCs frequency, application of yohimbine reduced the frequency further from 54±9.3 to 30±5.4% of SCH 23,390 in neurons from P+L treated rats (N=4 of 4; p<0.05 for yohimbine + SCH 23,390 vs SCH 23,390 alone), while not affecting neurons from other groups, i.e. 50±12.0 to 40±13.0% in CTL (N=3 of 4), and 82 to 61 and 60 to 55% in 10P respectively (fig.4). In one neuron from the control and one from the 10P groups, perfusion with yohimbine increased the mEPSC frequency. The amplitude of the mEPSCs was reduced in the P+L group only from 93±6.7 to 79±5.2% of baseline (fig. 4).
Figure 4. Yohimbine further decreases mEPSCs frequency and reduces the amplitude in P+L-treated animals only.

(A) Representative traces from DMV neurons showing mEPSCs before (top) and after (bottom) application of 10\(\mu\)M of the SCH 23,390 and SCH 23,390+10\(\mu\)M yohimbine in control, 10P- (blue), and P+L-treated animals (red). Holding voltage: -50mV. (B) Summary graphic showing that concomitant application of SCH 23,390 and yohimbine further reduces the frequency of mEPSCs. (C) Summary graphic showing that in P+L treated animals yohimbine and SCH 23,390 significantly reduce the amplitude of the mEPSCs compared to SCH 23,390 only. Open bars: control; blue bars: 10P; red bars: P+L. p>0.05

Collectively, these data suggest that DA reduces the excitatory inputs onto the DMV by decreasing the frequency of the mEPSCs, both DA1- and DA2-like receptors activate tonically mEPSCs, and that in P+L treated animals catecholamines enhance the DA-mediated modulation by reducing further the frequency and amplitude of the events.
7.5.2 DMV inhibitory miniature postsynaptic currents are decreased in the presence of DA

Similar to that described earlier for mEPSCs, bath perfusion with 30 μM DA reduced mIPSCs frequency to 63±5.8, 45±12.0 and 59±7.0% of baseline in CTL (N=3 of 11 neurons), 10P (N=4 of 7 neurons), and P+L (N=5 of 6 neurons), respectively (p>0.05 for all; fig.5) without affecting the mIPSCs amplitude.

Data are summarized in figure 5 and table 4.

Figure 5. DA decreases the frequency of mIPSCs, but not amplitude.
(A) Representative traces from DMV neurons showing miniature IPSCs before (top) and after (bottom) application of 30μM DA in control, 10P- (blue; (B)), and P+L-treated animals (red; (C)). Holding: -50 mV. (D) Computer-generated graphics from the same neurons as in (A), (B), and (C) showing the DA-induced decrease in mIPSC frequency (dashed lines) from baseline (solid lines). (E) Summary of the DA-induced decrease in mIPSC frequency, but not amplitude (F). Open bars: control; blue bars: 10P; red bars: P+L. p>0.05
<table>
<thead>
<tr>
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<th>P+L</th>
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<tr>
<td>mIPSCs frequency %</td>
<td>63 ± 5.8</td>
<td>45.2 ± 12.0</td>
<td>59.3 ± 7.0</td>
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<tr>
<td>mIPSCs amplitude %</td>
<td>93 ± 9.5</td>
<td>91.1 ± 5.0</td>
<td>93.3 ± 5.1</td>
</tr>
<tr>
<td>mIPSCs rise time %</td>
<td>87 ± 7.9</td>
<td>140.4 ± 13.5</td>
<td>113.9 ± 14.2</td>
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<tr>
<td>mIPSCs decay time %</td>
<td>106 ± 2.5</td>
<td>119.8 ± 6.5</td>
<td>109.8 ± 9.5</td>
</tr>
<tr>
<td>mIPSCs area %</td>
<td>97 ± 6.3</td>
<td>111.4 ± 6.4</td>
<td>104.9 ± 7.8</td>
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<td>N</td>
<td>3 of 11</td>
<td>4 of 7</td>
<td>5 of 6</td>
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Table 4. Paraquat does not affect how DA modulates mIPSCs recorded from DMV neurons.

As expected, blockade of DA2-like receptors with L-741,626 increased mIPSCs frequency to 190 and 172% of baseline in control (N=2 of 14 neurons) and to 162±18.5% in 10P (N=5 of 14 neurons), respectively. In the remaining neurons, however, perfusion with L-741,626 reduced the mIPSCs frequency to 47±7.9, 46±6.9, and 53±9.0% of baseline in control (N=7 of 14 neurons), 10P (N=6 of 14 neurons), and P+L (N=5 of 6 neurons), respectively (p>0.05). Similar to that was observed with blockade of DA1-like receptors, this bimodal response, i.e. either increased or decreased mIPSCs frequency was observed in neurons from control and 10P, but not P+L groups.

Data are summarized in figure 6 and table 5.
Figure 6. Blockade of DA2-like receptors results in both increase and decrease in mIPSCs frequency in all groups, except in P+L treated animals.

(A) Representative traces from DMV neurons voltage clamped at -50 mV showing miniature IPSCs before (top) and after (bottom) application of 10μM of the DA2-like receptor antagonist L-741,626 in control, 10P- (blue), and P+L-treated animals (red).

(B) Graphic summary of the L-741,626-induced bimodal modulation of mIPSCs frequency, but not amplitude (C). Open bars: control; blue bars: 10P; red bars: P+L. p>0.05
<table>
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<th>P+L</th>
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<tr>
<td>mIPSCs frequency %</td>
<td>47±7.9 / 181±9.1</td>
<td>46±6.9 / 162±18.5</td>
<td>53±9.0</td>
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<tr>
<td>mIPSCs amplitude %</td>
<td>88.2 ± 6.2</td>
<td>94.4 ± 2.9</td>
<td>89.6 ± 13.6</td>
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<td>mIPSCs rise time %</td>
<td>120.2 ± 7.2</td>
<td>106.7 ± 5.3</td>
<td>123.3 ± 21.1</td>
</tr>
<tr>
<td>mIPSCs decay time %</td>
<td>126.6 ± 9.9</td>
<td>103.4 ± 3.1</td>
<td>120.1 ± 12.43</td>
</tr>
<tr>
<td>mIPSCs area %</td>
<td>108.1 ± 18.1</td>
<td>101.4 ± 4.9</td>
<td>105.4 ± 10.5</td>
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<td>N</td>
<td>9</td>
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Table 5. Effects of blockade of DA2-like receptors on mIPSCs.

When DA1-like receptors were blocked with SCH 23,390 most of the cells responded with the anticipated decrease in the frequency of the mIPSCs that was similar across groups. Specifically, the frequency of the events was reduced to 65±6.3, 63±8.4, and 52±10.7% of baseline in control (N=8 of 18), 10P (N=7 of 15), and P+L (N=4 of 7). In a small proportion of neurons from control or 10P-treated animals, however, perfusion with SCH 23,390 increased mIPSCs frequency to 176±17.7 and 173±28.9% of baseline in control (N=4 of 18) and 10P (N=3 of 15), respectively (p>0.05). This bimodal response was not observed in the P+L group. No differences in the other parameters analyzed were observed.

Data are summarized in figure 7 and table 6.
Figure 7. SCH 23,390 results in both increase and decrease in mIPSCs frequency in all groups, but not in P+L treated-animals.  

(A) Representative mIPSCs from DMV neurons voltage clamped at -50 mV before (top) and after (bottom) application of 10μM of the DA2-like receptor antagonist SCH 23,390 in control, 10P- (blue), and P+L-treated animals (red).  

(B) Graphic summary of the SCH 23,390-induced bimodal modulation of mIPSCs frequency, but not amplitude (C). Open bars: control; blue bars: 10P; red bars: P+L. p>0.05
Table 6. Effects of blockade of DA1-like receptors on mIPSCs.

<table>
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<th>10P</th>
<th>P+L</th>
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<tbody>
<tr>
<td></td>
<td>mIPSCs frequency%</td>
<td>65±6.3 / 176±17.7</td>
<td>63±8.4 / 173±28.9</td>
</tr>
<tr>
<td></td>
<td>mIPSCs amplitude%</td>
<td>86.9 ± 4.4</td>
<td>115.4 ± 16.9</td>
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<td></td>
<td>mIPSCs rise time%</td>
<td>103.2 ± 5.9</td>
<td>96.9 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>mIPSCs decay time%</td>
<td>110.3 ± 5.6</td>
<td>110.0 ± 6.7</td>
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<td></td>
<td>mIPSCs area%</td>
<td>103.2 ± 9.8</td>
<td>152.4 ± 24.8</td>
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Concomitant perfusion with yohimbine and SCH 23,390 did not increase the inhibition of mIPSCs frequency in any of the groups (control N=5), 10P (N=7). In control neurons, however, addition of yohimbine significantly increased the rise time from 105±10.8 to 133±11.8%, this effect was not observed in the 10P group (data not shown).

These data suggest that DA reduces the inhibitory inputs onto DMV neurons via both DA1- and/or DA2-like receptor mediated mechanisms. While blockade of these receptors mediates a bimodal response in control, as well as 10P-treated animals, a unimodal reduction in the frequency of the events only was observed in P+L-treated animals.

7.5.3. Paraquat treatment increases the magnitude of the response to DA2-like antagonist

Bath application of 30µM DA resulted in a bimodal response in all groups tested. Specifically, in a subset of neurons DA increased the number of action potentials fired from baseline to 196±25.3, 174±13.0, and 238±61.5% in control (N=7/53), 10P (N=12/53), and P+L (N=3/10) respectively (p>0.05 across groups; p>0.05 for χ² test). Conversely, DA decreased the number of action potentials fired from baseline to 29±7.6, 21±7.1, and 2 and 45% in control (N=13/53), 10P (N=12/53), and P+L (N=2/10) respectively (p>0.05 for all).

Data are summarized in Figure 8.
Figure 8. DA induces a bimodal response in DMV neurons from all groups tested. (A) Representative traces from DMV neurons before (top) and after (bottom) application of 30μM of DA in control (black), 10P- (blue), and P+L-treated animals (red). (B) Graphic summary of the DA-induced bimodal modulation of firing frequency. Open bars: control; blue bars: 10P; red bars: P+L. p>0.05

By reducing the activation of DA2-like receptors, bath perfusion with L-741,626 also mediated a bimodal response in all treatment groups tested. Interestingly, DMV neurons from the 10P group showed a bigger increase in number of action potentials fired in response to L-741,626 compared to the other groups. Specifically, the frequency of firing increased from baseline to 156±9.8, 281±37.3, and 134 and 156% in control (N=6/26), 10P (4/20), and P+L (2/7) respectively (p<0.05). No differences in the magnitude of the response to the DA2-like receptor antagonist were observed in neurons that responded with a decrease in the number of action
potentials fired (from baseline to 29±8.0, 20±6.7, and 22 and 36% in control, 10P, and P+L respectively; N=6/26, 5/20, and 2/7 in control, 10P, and P+L respectively; p>0.05).

Data are summarized in Figure 9.

Figure 9. 10P-treated rats respond more potently to blockade of DA2-like receptors. (A) Representative traces from DMV neurons before (top) and after (bottom) application of 10μM of L-741,626 in control (black), 10P- (blue), and P+L-treated animals (red). (B) Graphic summary of the DA-induced bimodal modulation of firing frequency. Open bars: control; blue bars: 10P; red bars: P+L. p<0.05

Reduction of the DA1-like receptor activation with bath perfusion of the selective antagonist SCH 23,390 also mediated a bimodal response, with no differences across the groups. Specifically, the number of action potentials fired was increased from baseline to 161±10.5,
152±10.0, and 152±14.5 in control (N=10/25), 10P (11/28), and P+L (3/15) respectively, and was reduced from baseline to 49±6.6, 45±10.9, and 24±11.2 in control (N=6/25), 10P (4/28), and P+L (3/15) respectively.

Data are summarized in Figure 10.

Figure 10. Blockade of DA1-like receptors produces a bimodal response in DMV neurons. (A) Representative traces from DMV neurons before (top) and after (bottom) application of 10µM of SCH 23,390 in control (black), 10P- (blue), and P+L-treated animals (red). (B) Graphic summary of the DA-induced bimodal modulation of firing frequency. Open bars: control; blue bars: 10P; red bars: P+L. p<0.05
Perfusion with a combination of SCH 23,390 and the α2-adrenoreceptor antagonist yohimbine showed a trend, towards a reduction, although not significant, in the SCH 23,390-mediated increase in the frequency of firing in all neurons from control and 10P, but not P+L, groups.

Data is summarized in Figure 11.

Figure 11. Concomitant blockade of DA1-like receptors and α2-adrenoreceptors produces a more profound reduction of action potentials fired by DMV neurons.
(A) Representative traces from DMV neurons before (top) and after (bottom) application of 10μM of SCH 23,390 alone, or in combination with 10μM yohimbine in control (black), 10P-(blue), and P+L-treated animals (red). (B) Graphic summary of the yohimbine-induced potentiation of SCH 23,390 modulation of firing frequency. Open bars: control; blue bars: 10P; red bars: P+L. p<0.05
These data suggest that the variations observed in the miniature postsynaptic currents translate in changes observed in miniature postsynaptic currents in response to DA1-like receptor blockade translate to changes in DMV neuronal excitability only following 10P treatment.

CHAPTER 7.6 DISCUSSION

In the present study we report that the responsiveness of DMV neurons to exogenous DA perfusion is altered in rodent models of Parkinsonism. Specifically, we report that (i) DA decreases the frequency of both mEPSCs and mIPSCs in all animal groups, independently of the treatment; (ii) blockade of the excitatory DA1-like receptors reduces the frequency of both mEPSCs and mIPSCs in all animal groups, however it elicits a bimodal response, i.e. both increased or decreased frequency of mEPSCs in 10P-treated animals only, and a bimodal response in the frequency of mIPSCs in both control and 10P-treated rats; (iii) blockade of the inhibitory DA2-like receptors increases the mIPSCs frequency of the minority of neurons from control and 10P-treated rats, but decreases mEPSCs and mIPSCs in all groups; (iv) blockade of α2 adrenoreceptors enhances the DA1-like mediated reduction of mEPSCs frequency in P+L treated rats only, and (v) following 10P treatment blockade of DA2-like receptors has a much greater increase in the frequency of firing of DMV neurons. Taken together these results indicate that the response of DMV neurons, hence, the vagal output to the gastrointestinal tract, to DA is altered in rodent models of Parkinsonism.

The time course of the current experiments were similar to those in which 10P and P+L treated rats displayed gastric dysfunction, as shown previously. Indeed, while stimulation of the SNpc with the glutamate agonist NMDA produces an increase in both gastric tone and motility in naïve rats, this response is blunted in 10P- and P+L-treated rats. We have also shown that direct stimulation of the DVC with DA results in a profound gastroinhibition in control rats, while, following 10P treatment, the response is initially excitatory, and then inhibitory, i.e. biphasic. As such, in the present study we were able to observe and investigate alterations at the level of the dorsal vagal complex only, independently of SNpc degeneration, which is observed only at later time points.
At this stage of PD development in the rat, we were also able to show that DMV neurons already display alterations in their membrane properties. Indeed, while their excitability in response to current injection does not seem affected by the paraquat treatments described herein, DMV neurons from treated animals display an acceleration of the afterhyperpolarization decay time, and neurons from P+L-treated rats also showed a baseline increase in the amplitude of mEPSCs (Bove et al. 2019, under revision). The increase in baseline amplitude of the mEPSCs following P+L treatment might be indicative of a possible postsynaptic compensatory mechanism to overcome the changes in receptor activation described.

Vagal control of the digestive tract includes two different, but related processes. Peripheral signals are acquired by vagal afferent fibers, elaborated by the NTS and relayed to the DMV via selective synaptic inputs; this modulation of the pacemaking activity of DMV neurons, which innervate the GI tract directly via excitation of cholinergic or NANC myenteric neurons, determines the resulting output towards the GI tract. Since DMV neurons preganglionic parasympathetic motorneurons, they release acetylcholine to their target enteric neurons. These postganglionic neurons belong to two distinct pathways that ultimately modulate GI motility. The excitatory pathway also releases acetylcholine to activate the smooth muscle fibers or the interstitial cells of Cajal; the inhibitory pathway utilizes non-adrenergic non-cholinergic (NANC) neurotransmitters that induce muscle relaxation through release of nitric oxide, vasoactive intestinal polypeptide, or adenosine triphosphate. While the postganglionic release of acetylcholine is tonic, NANC transmission is phasic and it is activated by specific DMV neurons/pathways. Hence, the response of the GI tract to neuronal stimuli depends on the type of neurotransmitter released by the enteric neurons, and not on the activity of DMV neurons per se. The existence of these two distinct neurocircuits implies that the synaptic alterations described herein might affect GI output by either inhibiting the population of DMV neurons connected to the cholinergic pathway, or by exciting the second population of DMV neurons activating the NANC pathway. However, no molecular markers exist to distinguish these two populations of DMV neurons, hence these considerations cannot yet be confirmed.

The importance of the vagus nerve in PD has emerged from, amongst other studies, retrospective analyses of patients who received a truncal vagotomy. In this cohort a significant and clear reduction in the incidence of PD was observed. Moreover, electromyogastrography recordings from Parkinsonian patients closely resemble those recordings obtained from patients
in the immediate, acute phase after a vagotomy\textsuperscript{18,19}, indicating a disrupted pattern in vagal activity. The prodromal GI pathologies in Parkinsonian patients, and the presence of misfolded $\alpha$-synuclein aggregates in both ENS and vagal motoneurons\textsuperscript{20} suggests that the etiology of idiopathic PD may start in the GI tract and involve the vagus nerve, as postulated by Braak\textsuperscript{21,22}. Indeed, according to this theory of PD development, $\alpha$-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\textsuperscript{20,22}. Our recently developed model of parkinsonism allowed us to replicate these stages of PD development in the rodent, and provided further demonstration of vagal involvement in the etiology of PD\textsuperscript{14}.

The data presented herein confirm previous findings from our laboratory showing that DMV neurons are responsive to DA, and express both DA1- and DA2-like receptors \textsuperscript{11}. While the current clamp recordings presented in this manuscript suggest that DA and DA1-like receptors are not affected by paraquat treatment, analysis of the miniature excitatory and inhibitory postsynaptic current showed more profound alterations, where there may be the involvement of neurocircuits normally not engaged in the modulation of DMV activity and that mainly compromise the DA1-expressing DMV neurons. Indeed, analysis of mEPSCs revealed that following 10P treatment engagement of DA1-like receptor produces an unexpected inhibition that could suggest the involvement of a dormant neurocircuit. Moreover, following P+L treatment the neurons of the A2 area become predominant in modulating DMV activity. Inhibitory GABAergic transmission does not appear to be as affected, although P+L treatment appear to mask the bimodal response observed in control and 10P-treated animals. These preliminary data suggest, however, that in the two models used in this study there is an alternation in the DA1-like receptors dependent response, while the synaptic, inhibitory inputs mediated by activation of DA2-like receptors seem to be unaffected, confirming our hypothesis that two neurocircuits may exist in the DMV. Indeed, only DA1-like expressing DMV neurons seem to be innervated by dopaminergic inputs coming from the SNpc\textsuperscript{7}. More experiments are necessary to better characterize the results described here.

Given the pivotal role of the NTS in regulating DMV function and, hence, gastrointestinal output, the possibility that NTS neurons are affected by PD induction becomes of crucial importance in the understanding of non-motor dysfunctions associated with PD. In addition to the gastrointestinal symptoms discussed in the present manuscript, in fact, PD is associated with
other autonomic dysfunctions that are strictly correlated to NTS activity, including orthostatic hypotension. Dysregulation of NTS activity has been described in other diseases, including Rett syndrome, Infant Sudden Death syndrome, and it has been shown to be the cause of neuroanatomical and functional respiratory changes in the 6-hydroxydopamine model of PD. Understanding the triggering factors that lead to NTS alterations may be beneficial in preventing the onset of these critical symptoms that accompany PD, and may also be important in understanding the etiology of PD in general.

In conclusion, our study suggests that the synaptic inputs from the NTS on DMV neuron are altered in two different models of Parkinsonism. These findings shed the light on the possibility that PD is not a neuronal dysfunction disorder, but rather a circuit-based disorder, and might challenge the way this disease is described and treated.

Acknowledgements

Funding: This work was supported by NIH grant DK-55530, and a grant from Michael J. Fox Foundation for Parkinson’s Disease to R. A. Travagli.

The authors would like to thank to Cesare M. and Zoraide Travagli for support and encouragement, and Dr. K.N. Browning for proof-reading and providing critical comments to earlier versions of the manuscript to be submitted.

CHAPTER 7.7 REFERENCES


CHAPTER 8

DISCUSSION

Cecilia Bove
CHAPTER 8.1 SUMMARY OF SIGNIFICANT FINDINGS

In the present proposal, we investigated the overarching hypothesis that environmental toxins disrupt the brain-gut axis through a vagally-dependent ENS-DMV-SNpc pathway prior to the development of parkinsonism. The present studies are the first to investigate in detail the alterations in brainstem vagal neurocircuits following induction of parkinsonism, and the pathophysiological effects of these alterations on gastric functions and contributed to the development of a novel rodent model of parkinsonism that replicates the disease progression in human cohorts.

The experiments detailed in Chapter 2 investigated the hypothesis that a nigrovagal pathway connecting the substantia nigra pars compacta and the dorsal motor nucleus of the vagus exists in the rat, that this pathway regulates GI function, and it is compromised in a well-recognized rodent model of PD. Results from this study demonstrated that (i) dopaminergic neurons of the substantia nigra pars compacta send a monosynaptic projection in the DVC impinging on DMV neurons directly or indirectly through the neurons of the A2 area, (ii) the nigrovagal pathway tonically modulates gastric tone and motility, and, when excited, it increases gastric tone and motility, and that (iii) following administration of 10 mg/kg of the herbicide paraquat, known to cause PD in humans and laboratory animals, the nigrovagal pathway control of gastrointestinal function was diminished. These results were crucial in determining that the DMV is indeed involved in the pathogenesis of PD, and led us to investigate the functional consequences of dopaminergic inputs on this area both in vitro and in vivo. Confirming the presence of an anatomical and functional connection between the DMV and the SNpc was necessary to develop the toxins gastric administration regimen which the novel model of environmental PD described in chapter 5 is based on.

Before producing this novel model, we deemed necessary to assess the physiological and pathophysiologic gastric response to the pharmacological stimulation of the DVC with DA. The results of chapter 3 show that DA microinjection in the DVC mainly produce a profound gastroinhibition through activation of the inhibitory DA2-like receptors. However, as observed in chapter 4, following paraquat administration this response becomes biphasic, and engages the excitatory DA1-like receptors that, as shown in chapter 2, were activated by DA release from the SNpc. These results suggest that induction of parkinsonism might have a role in determining a form of maladaptive neuroplasticity. We hypothesize that engagement of DA1-like receptors and dopaminergic synaptic connections is a futile attempt to rescue the normal gastric function, which might actually worsen the condition by impairing the normal vagal activity.
An essential confirmation of the central hypothesis of this study came from the data presented in chapter 5. Here, we described our novel model of environmental parkinsonism which employs gastric administration of a combination of subthreshold doses of paraquat and the carbohydrate-binding lectins. This model allowed us to follow the progression of α-synuclein misfolding starting in the enteric nervous system, progressing in the DMV, and reaching the SNpc to cause the motor dysfunctions and the loss of DA neurons that characterizes the parkinsonian pathology. Moreover, we were able to replicate the gastric dysfunctions observed in the classic model of PD induction. All these hallmarks of PD were absent following vagus nerve resection, confirming the central role of this cranial nerve in the etiology of PD.

Once we were able to confirm that two different models of PD produce similar degree of impairment of gastric function, we went back to the in vitro level to investigate the involvement of the DMV through whole-cell patch clamp recordings. In chapter 6 we described the membrane properties of DMV neurons in the two models used in the previous chapter, and showed that although DMV neurons are affected by paraquat systemic and gastric administration, there is no consequence in their firing properties. Moreover, in the paraquat + lectin model of PD we observed an increase in the amplitude of the miniature excitatory postsynaptic events at baseline. This, together with the alterations in dopaminergic modulation of synaptic inputs on DMV neurons following PD induction presented in chapter 7, suggest that perhaps also the NTS, and not solely the DMV, is involved in the pathogenesis of PD, and might be involved in the disruption of the ENS-DMV-SNpc axis. These results are the first to show that gastrointestinal symptoms in PD might be due to dysfunction of a neuronal circuit, rather than neuronal dysfunction per se.

CHAPTER 8.2 THE NIGRO-VAGAL PATHWAY IN SUPPORT OF BRAAK’S HYPOTHESIS

Before investigating the role of the DMV in the gastrointestinal dysfunctions associated with PD, we first needed to closely examine the relationship between the DMV and the SNpc. Indeed, while Braak’s hypothesis of PD development was supported by the clinical GI manifestations, as well as by the post-mortem histochemical proof of Lewy bodies aggregation in the enteric nervous system, DMV and higher CNS centers, no direct connection between the SNpc and the DMV has ever been shown. The results presented in chapter 2 bridge that gap, by demonstrating the existence of an anatomical pathway connecting the SNpc to DMV neurons. Specifically, by combining tract tracing with pharmacological and optogenetical stimulation of the pathway, we demonstrated the physiological relevance of this monosynaptic connection.
Many previous studies have associated the vagus nerve with Parkinson’s Disease in both animals and humans. α-synucleinopathy was shown to spread from the ENS to the CNS through the DMV in the rotenone model of PD \(^8,9\) and in a genetic mouse model expressing a mutated form of α-synuclein\(^{10}\). Injection of human post-mortem brain lysate from PD-affected individuals, or administration of recombinant α-synuclein in the ENS\(^{11}\), directly in the vagus nerve\(^{12}\), or in peripheral neurons associated with vagal neurocircuits\(^{13}\) recapitulated the spread of Lewy bodies in the rodent. In humans, truncal vagotomy showed a clear preventative effect towards PD onset \(^14\), and electrogastrographic recordings of vagal nerve activity of PD patients resemble those of vagotomized patients in the acute phase of recovery following the surgery\(^{15,16}\). Our results are the first to show that the DMV and the vagus nerve are directly connected to the SNpc in the rodent. Moreover, the results presented in chapter 5 show that in our model of environmental parkinsonism α-synuclein is unable to spread to the CNS when rats undergo subdiaphragmatic vagotomy. This is particularly relevant considering that these rats do not develop motor dysfunctions\(^2\), highlighting that an intact vagus nerve is necessary for disease onset.

Preliminary unpublished data have also confirmed the existence of this pathway in humans. This discovery may lead to the development of a potential non-invasive biomarker for early PD detection, that could also be utilized to monitor disease progression from the earlier stages to allow better disease management.
MRI scan and diffusion tensor imaging (DTI), has been used extensively for white matter odology in the brain. Here, we show the presence of an ipsilateral connection between the SNpc and the DMV in a non-PD individual. Courtesy of Dr. J. Wang.

CHAPTER 8.3 A NEW MODEL TO UNDERSTAND PARKINSON’S DISEASE

In chapter 5 we presented a novel model of environmental parkinsonism. This model reproduced the stages of PD development described by Braak, and allowed us to study the progression of nigro-vagal pathway dysfunction, as well as the motor dysfunction that characterize the disease.

The peculiar feature of this model is that it replicates the likely route of environmental agents that are correlated with idiopathic PD pathology. Indeed, neurotoxins such as the herbicide paraquat are likely to be ingested through treated crops, and once entered in the enteric nervous system cause the molecular events that follow the spatiotemporal defined pattern of Lewy body formation starting in the brainstem. While other models that do not involve paraquat use have been deemed useful to replicate parkinsonian symptoms more reliably and with a higher degree of reproducibility, they do not replicate the stages of PD development proposed by Braak, and induce severe Parkinsonism over a relatively short time period, which does not reflect the natural progression of the disease.
Our experimental model has the potential of being a powerful tool to understand the triggering factors that start \( \alpha \)-synuclein aggregation in the gut and, hence, might potentially identify a novel therapeutic target to either prevent disease progression, if caught in early stages, or to block disease worsening at later stages. The in vitro fibrillation experiments presented in chapter 5 suggest that an exogenous agent in addition to the herbicide paraquat, i.e. dietary lectins, accelerate the rate of \( \alpha \)-synuclein fibrillation, and hence Lewy body formation. However, the molecular mechanism that hastens the kinetics of Lewy bodies formation is still unknown. While this experiment suggest that lectins play a direct role perhaps acting as a molecular chaperone\(^{21,22}\), to either allow a small molecule such as paraquat to alter the biophysical structure of \( \alpha \)-synuclein or to directly promote misfolding, a definite confirmation of this direct interaction between these three elements is yet to be described.

**CHAPTER 8.4 PARKINSON’S DISEASE: A NEURONAL DEFICIT DISORDER OR A CIRCUIT BASED DISORDER?**

Once we were able to establish that the DMV and the SNpc are actually connected, and that the vagus nerve is crucial in PD etiology, we took a closer look at the consequences of nigro-vagal dysfunctions on gastric function.

Administration of 10 mg/kg of paraquat i.p. mediated a reduction in the SNpc-induced increase in gastric tone and motility that suggested an impairment of the nigro-vagal pathway that we surmised was determined by a diminished release of DA from the SNpc (chapter 3). Given this premise, we were surprised to observe that in the same model, direct pharmacological stimulation of DVC neurons with DA mediated a change in the response to this neurotransmitter, rather than a decrease in the magnitude of the response. Specifically, while in chapter 4 we report a gastroinhibition in response to DA microinjection in the DVC, due most likely to the fact that DA2-like receptors are the most abundant receptor type in this area\(^{23-25}\), direct microinjection of DA in the DVC of animals treated with paraquat mediated an initial increase in gastric tone and motility followed by a gastroinhibition comparable to that measured in naïve rats. Interestingly, the initial increase in tone and motility was dependent on the activation of excitatory DA1-like receptors. These receptors are found expressed on the membrane of DMV neurons innervated by the nigro-vagal pathway, and are virtually never engaged by direct stimulation of the DVC in naïve rats. This intriguing result prompted us to examine the DMV neurons more closely.

The results shown in chapters 6 and 7 revealed that the alterations observed in vivo do not seem to be due to DMV neuronal alteration, but rather to dysfunction at the level of the NTS to DMV synapse. In a recently published study, overexpression of \( \alpha \)-synuclein, which causes
evident SNpc neuronal alterations, does not alter the membrane properties of DMV neurons significantly\textsuperscript{26}. Indeed, in chapter 6 we report similar results in the 10 mg/kg i.p. paraquat model, as well as in the paraquat + lectin model. While some changes in the afterhyperpolarization phase of the action potential were reported, no physiological consequences in DMV neuronal activity were described, suggesting that DMV neurons might be indeed resilient to stressors compared to SNpc neurons\textsuperscript{26,27}; however, an interesting increase in the amplitude of the miniature excitatory postsynaptic currents was reported in the P+L model, suggesting the occurrence of plasticity at the NTS to DMV synapse. This plasticity, however, does not appear to benefit DMV neurons. The preliminary results presented in chapter 7 reveal that DA possibly modulates the NTS rather than the DMV, and that induction of parkinsonism alters the DA-mediated modulation of glutamate and GABA from this brain area. Indeed, while in the 10P model there seems to be the unmasking of a normally dormant excitatory circuit that is disinhibited by blockade of the excitatory DA1-like receptors, we observe the silencing of a GABA-related circuit in the P+L model only. Moreover, the contribution of A2 area neurons observed in chapter 3 in the modulation of the nigro-vagal pathway, seems to be relative to glutamatergic NTS transmission only. While more experiments are required to completely characterize this challenging circuit, these results suggest that within the DVC multiple microcircuits exist with different purposes, and with different susceptibility to the plastic changes associated with PD induction.

Loss of dopaminergic neurons in SNpc and dopaminergic inputs into the striatum results in dysregulation of basal ganglia, with emergence of pathological oscillatory patterns of burst firing in output neurons, increased synchrony of the discharge of the neighboring basal ganglia, and an overall increase in basal ganglia output\textsuperscript{28}, which result in alterations on the motor thalamus activity, and diminished motor cortical activity. This circuit dysregulation is also associated with glutamate neurotoxicity in basal ganglia\textsuperscript{29-32}, and altogether lead to the clinical motor symptoms of PD\textsuperscript{33}. When taken together, these evidences may support the idea that even the motor symptoms of PD should be considered as the result of a circuit-based disorder rather than the result of the degeneration of a single neuronal population.

**CHAPTER 8.5 CONCLUDING REMARKS AND ADDITIONAL FUTURE DIRECTIONS**

Further studies should be conducted to better characterize the alterations of the NTS to DMV synapse in the PD models presented in this thesis. Confirmation of the involvement of the NTS in disease progression might create a new venue for investigation of new interventions to ameliorate the autonomic dysfunctions associated with PD.
Preliminary accounts from our laboratory begun to investigate potential preventative interventions to block Lewy bodies formation in the ENS and, hence, PD onset. Indeed, the amino acid serine has been shown to reduce the formation of intracellular aggregates similar to Lewy bodies in other diseases\textsuperscript{34,35}. Our data shows that a dietary intervention as simple as serine supplementation might slow down disease progression, putatively by reducing the spread of \( \alpha \)-synucleinopathy through the vagus nerve. Understanding the molecular mechanisms behind these results might lead to a better understanding of the triggering factors behind ENS invasion by Braak’s “unknown pathogen”, and possibly lead to the development of a disease-preventing therapeutic strategy.

CHAPTER 8.6 REFERENCES


EDUCATION

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