Investigation of nerve pathways mediating colorectal dysfunction in a Parkinson’s disease model produced by lesion of nigrostriatal dopaminergic neurons

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/NMO.13893

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Abstract

Background: Gastrointestinal (GI) dysfunction, including constipation, is a common non-motor symptom of Parkinson’s disease (PD). The toxin 6-hydroxydopamine (6OHDA) produces the symptoms of PD, surprisingly including constipation, after it is injected into the medial forebrain bundle (MFB). However, the mechanisms involved in PD-associated constipation caused by central application of 6OHDA remain unknown. We investigated effects of 6OHDA lesioning of the MFB on motor performance and GI function.

Methods: Male Sprague-Dawley rats were unilaterally injected with 6OHDA in the MFB. Colorectal propulsion was assessed by bead expulsion after 4 weeks and by recording colorectal contractions and propulsion after 5 weeks. Enteric nervous system (ENS) neuropathy was examined by immunohistochemistry.

Key Results: When compared to shams, 6OHDA-lesioned rats had significantly increased times of bead expulsion from the colorectum, indicative of colon dysmotility. Administration of the colokinetic, capromorelin, that stimulates defecation centres in the spinal cord, increased the number of contractions and colorectal propulsion in both groups compared to baseline, however, the effectiveness of capromorelin in 6OHDA-lesioned rats was significantly reduced in comparison to shams, indicating that 6OHDA animals have reduced responsiveness of the spinal defecation centres. Enteric neuropathy was observed in the distal colon, revealing that lesion of the MFB has downstream effects at the cellular level, remote from the site of 6OHDA administration.

Conclusions & Inferences: We conclude that there are trans-synaptic effects of the proximal, forebrain, lesion of pathways from the brain that send signals down the spinal cord, at the levels of the defecation centres and the ENS.

Key words: Parkinson’s disease, colonic propulsion, gut dysfunction, constipation, defecation pathway, 6-hydroxydopamine

Abbreviations

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1 INTRODUCTION

Parkinson’s disease (PD) is a multifactorial disorder that affects 1-2% of the senior population, with about 10% of PD patients having symptoms before the age of 50. Chronic and progressive motor impairments, which include resting tremor, bradykinesia, rigidity and postural instability, are the principal characteristics of PD. In addition to the characteristic motor symptoms, PD is accompanied by non-motor symptoms, including gastrointestinal (GI) dysfunction, that further reduce the patient’s health-related quality of life. GI dysfunction includes excessive salivation, difficulty swallowing (dysphagia), nausea, slowed stomach emptying ( gastroparesis), and severe chronic constipation, all of which have been shown to occur more frequently in PD patients compared to aged-matched healthy individuals. Chronic constipation is a common disorder that can affect about 70-80% of patients with PD, suggesting that constipation is an unavoidable comorbidity of PD. Despite dopamine pharmacotherapy to improve motor symptoms caused by central nervous system (CNS) degeneration, PD patients with constipation are often unresponsive to conventional treatments (e.g. changes in diet, enemas, osmotic laxatives), and many of the current treatments cause adverse side-effects, such as nausea and severe abdominal cramps. Hence, an explanation on how Parkinson’s pathology leads to chronic constipation is needed. This knowledge may assist in targeting therapies to treat GI symptoms.

Defecation and fecal continence are under hierarchical control. The voluntary control of defecation involves the cingulate cortex of the frontal lobe, allowing humans to make the decision to defecate or not. The message to defecate is then directed from the cortex to the integrative centres in the lower brain stem (including Barrington’s nucleus), which in turn connect to the lumbo-sacral defecation centre and Onuf’s nucleus in the sacral region through pathways in the spinal cord. The nerve pathways from the lumbo-sacral defecation centre play an important role in activating the enteric nervous system (ENS). The ENS provides intrinsic control of the digestive system and works synergistically with the CNS to control colorectal propulsive activity. The external anal sphincter relaxes in coordination with the propulsive contractions of the colorectum through the voluntary control of neurons of Onuf’s nucleus in the sacral region. Thus, interactions between the brain, spinal cord and ENS are essential in the control of defecation. Constipation in PD could arise from lesions in the CNS, the ENS, or both.
Administration of the toxin 6-hydroxydopamine (6OHDA), which is chemically related to dopamine, is used to produce animal models of PD. 6OHDA has strong oxidative properties and, when injected into the nigrostriatal pathway, it causes rapid degeneration of midbrain dopamine neurons resulting in motor impairments. Previous studies have shown that rats injected with 6OHDA develop GI dysfunction, such as a reduction in daily fecal output and fecal water content, and delayed gastric emptying. This makes 6OHDA-lesioned animals valuable models for studying both the motor and non-motor symptoms of PD, and for investigating the mechanism through which lesions in the brain cause changes in the GI tract.

The purpose of this study was to characterize the GI phenotype in the 6OHDA rat model of PD. GI function was assessed by colonic bead expulsion and by recording colorectal propulsion in vivo, in response to the CNS-penetrant ghrelin receptor agonist, capromorelin. Capromorelin was used because it specifically stimulates the defecation centres in the spinal cord. Evidence for this site of action is discussed in more detail in the Discussion section of this paper. Neuropathy in the ENS was evaluated using immunohistochemistry.

2 MATERIALS AND METHODS
2.1 Animals
All experimental procedures involving rats followed and conformed to the Australian National Health and Medical Research Council (NHMRC) code of practice for the care and use of animals for scientific purposes and were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee (FINMH 18-014). Adult male Sprague-Dawley (SD) rats (200-220g) were obtained from the Animal Resource Centre (ARC, Perth) and kept under controlled illumination (12h light/dark cycle) and temperature (22 ± 2°C). All animals were fed a normal chow diet and water ad libitum. Rats were group-housed and weighed daily post-lesioning.

2.2 6OHDA rat model of PD
Rats (n = 30; 13 sham rats and 17 6OHDA rats) were anesthetized with isoflurane (5% induction in 1L/minute O₂; 2-3% maintenance in 1L/minute O₂), placed on a stereotaxic apparatus and injected with meloxicam (1mg/kg) for post-surgical pain relief. 6OHDA (3µg/µL; Tocris, Cat. no. 2547), dissolved in saline containing 0.02% (w/v) ascorbic acid (Sigma-Aldrich, EC no. 205-126-1), was microinjected unilaterally into two sites of the medial forebrain bundle (MFB), using the following coordinates (mm) from bregma: (i) antero-posterior: -4.0, medio-lateral: -0.8, dorso-ventral: -8.0 (3 µL); (ii) antero-posterior: -4.4, medio-lateral: -1.2, dorso-ventral: -7.8 (2.5µL). All injections were...
performed at a flow rate of 1μL/minute with the needle left in place for 1-2 minutes after the injection to allow for complete diffusion of the toxin and to minimize backflow. Sham rats underwent the same surgical procedure, instead receiving vehicle injections of 0.02% (w/v) ascorbic acid.

2.3 Amphetamine-induced rotation test
An amphetamine-induced rotation test was performed at 2 weeks post 6OHDA lesion, as previously described.31 In brief, animals were placed in cylindrical chambers and recorded for 10 minutes before the injection of amphetamine to observe any baseline rotational bias. The rats then received a single i.p. injection of D-amphetamine (5mg/kg, Tocris, Cat. no. 2813), dissolved in 0.9% saline. Rats were allowed to habituate to their environment for 10 minutes and net ipsilateral rotations were recorded over a 60 minute period.

2.4 Ledged beam traversal test
Motor coordination and balance were tested using the ledged beam test, as previously described.32 In brief, a beam, 1.3 m in total length, and comprising of eight (10 cm length) sections that decreased in width from 4.5cm to 1.5cm in 0.4cm decrements was used. Each animal was trained to traverse the beam (from widest to narrowest) directly into the animal's home cage (5 trials). During the testing phase, animals were recorded whilst traversing the beam, over 5 trials, with a 15-30 second inter-trial period. Videos were analyzed in slow-motion to quantify the number of footfaults and times taken to cross the beam. The total number of footfaults per section and the times taken to cross the beam were averaged over the 5 trials.

2.5 Colonic bead expulsion test
Colonic propulsion was measured using the bead expulsion test at 4 weeks post-lesion. Rats were lightly anesthetized with isoflurane (3% isoflurane in 1L/minute O₂) to allow insertion of a bead (5 mm in diameter) into the distal colon, 3 cm from the anus, using a flexible plastic rod. Following bead insertion, rats were placed in individual cages to recover from anesthesia. The time taken from insertion of the bead to expulsion was recorded to the nearest second.

2.6 In vivo colorectal propulsion studies
In vivo colorectal propulsion studies were conducted on animals 5 weeks after 6OHDA or vehicle administration. Rats were sedated with ketamine hydrochloride (50-60mg/kg, intramuscular; i.m.) and anesthesia was induced with α-chloralose (60mg/kg, into the lateral tail vein). The femoral artery was cannulated for the infusion anesthetic and blood pressure recording. Anesthesia was
maintained by intra-arterial infusion of α-chloralose (12-20 mg/kg/hour) combined with ketamine hydrochloride (3-5 mg/kg/hour) in phosphate-buffered saline (PBS). The femoral vein was also cannulated for the delivery of the ghrelin receptor agonist capromorelin (4mg/kg). Blood pressure and heart rate were recorded with a Power Lab recording system using Chart 8 software (both from ADInstruments, Sydney, Australia). The distal colon was cannulated at the colonic flexure, which in the rat is at the junction of the proximal and distal colon, where formed fecal pellets are first observed. A second cannula was inserted into the anus. The colon remained in situ, and the muscle and skin were closed around the proximal cannula. The proximal cannula was connected to a Mariotte bottle filled with PBS, and the distal cannula to a pressure transducer via a one-way valve. The baseline intraluminal pressure was maintained at 7-10 mmHg by adjusting the heights of the Mariotte bottle and outlet. Expelled fluid was collected in a cylinder distal to the one-way valve and measured by weighing with a force transducer. At the end of each experiment, the rat was taken for perfusion while under anesthesia.

2.7 Tissue collection and preparation

After the in vivo experiment (at 5 weeks post-lesion), rats were perfused transcardially with PBS followed by paraformaldehyde fixative (PFA, 4% w/v). Brains were removed and postfixed in and PBS at 4°C for 4-5 days. The tissue was frozen with isopentane chilled on dry ice. The entire rostrocaudal extents of the caudate putamen (CPu) and substantia nigra pars compacta (SNpc) were sectioned using a cryostat into 30μm coronal sections. Sections were preserved in cryoprotectant solution (35% (v/v) 0.1M phosphate buffer, 15% (w/v) sucrose, 30 v/v ethylene glycol, 35% (v/v) distilled water) until sections were mounted onto Superfrost slides (ThermoFisher; SF41296SP). Gut tissue (colon and ileum) was flushed of fecal contents and dissected along the mesenteric attachment, pinned flat onto balsa board with the mucosa facing down, and fixed overnight in 2% formaldehyde plus 0.2% picric acid in 0.1M sodium phosphate buffer, pH 7.2, at 4°C. Preparations were cleared of fixative by 3×10 minutes washes in dimethyl sulfoxide followed by 3×10 minutes washes in PBS. Fixed tissue was stored at 4°C in PBS containing sodium azide (0.1% w/v).

2.8 Immunohistochemistry of brain sections

Immunohistochemical staining for tyrosine hydroxylase (TH) was performed to evaluate dopaminergic damage in the CPu and SNpc. CPu and SNpc sections were incubated in rabbit anti-TH primary antibody, diluted in 0.3% (v/v) Triton X-100 and 10% (v/v) normal horse serum (NHS) (1:1000; Millipore AB152) overnight at 4°C. The sections were then washed in PBS (3×5 minutes) before incubation with a goat biotinylated anti-rabbit IgG antibody (Dako E0432, Denmark), diluted to 1:500 with 0.3% (v/v) Triton X-100 and 10% (v/v) NHS, for 1 hour at room temperature.
Brain sections were rinsed in PBS (3×10 minutes) and incubated with streptavidin horseradish peroxidase (1:1000; Dako P0397) for 30 minutes at room temperature. Finally, reaction products were developed using 3,3’-diaminobenzidine tetra-hydrochloride (DAB Substrate Kit, Dako K3468). After rinsing with distilled water (1×5 minutes), the slides were dehydrated in graded alcohol solutions (50%, 70%, 90% and 100% in distilled water), cleared in xylene and coverslipped using DPX mounting medium.

2.9 Immunohistochemistry of intestinal segments
The mucosa and circular muscle were removed from the fixed tissue and wholemounts consisting of the myenteric plexus adhering to the longitudinal muscle were prepared. Wholemount preparations were incubated with human anti-Hu (1:5000; a gift from Dr Vanda Lennon) and sheep anti-neuronal nitric oxide synthase (nNOS, 1:1000; Emson V205) overnight at 4°C. The wholemounts were then washed (3×10 minutes) in PBS before incubation with donkey anti-human Alexa 594 and donkey anti-sheep 488 at 1:500 dilution (Jackson 709-585-149; Invitrogen A11015), for 1 hour at room temperature. Preparations were washed once with PBS, followed by 2×5 minutes washes in distilled water and incubated with Hoeschst 33258 solution (10 μg/mL Bisbenzimide-Blue in distilled water; Sigma-Aldrich, Sydney, NSW, Australia) for 5 minutes. Tissue was washed 3×10 minutes with distilled water before being mounted on glass slides using fluorescence mounting medium (Dako).

2.10 Image Analysis
Images of brain sections were taken using the SteREO Lumar.V12 (Carl Zeiss, Germany) and digitally enhanced (adjusting brightness and contrast) with Image J software (Version 1.51; National Institutes of Health, USA). CPu and SNpc sections corresponded to +0.2 and -6.04 mm from bregma, respectively.
Images for immunofluorescence of the myenteric plexus were captured using the Axio Imager. Z1 microscope (Carl Zeiss, Germany) using 10× and 20× air objectives. For Hu per ganglion studies, approximately 15-20 ganglia were counted per preparation. Approximately 100-200 neurons per preparation were counted for Hu translocation and nNOS quantitative studies. For all quantitative measurements, 8 wholemount preparations were used per cohort.

2.11 Statistical analysis
Data are expressed as the mean ± standard error of the mean (SEM). Comparisons between groups were performed using one- or two-way ANOVA with Sidak’s or Tukey’s multiple comparisons test. Hu and nNOS cells per ganglion, and Hu translocation data were analyzed by unpaired t-test.

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Analyses were performed using GraphPad Prism (GraphPad software Inc.). *P* values of less than 0.05 were considered statistically significant.

3 RESULTS

3.1 Confirmation of effectiveness of 6OHDA in causing motor deficits and SNpc lesions

TH-immunohistochemistry showed that unilateral injection of 6OHDA into the right MFB substantially reduced dopamine immunoreactivity in both the CPu and SNpc, confirming that effective lesioning of the midbrain dopamine system occurred (Figure 1A).

Two weeks after the lesion, injection of amphetamine elicited rotational activity in 6OHDA rats (249.8 ± 60.3 rotations in 1 hour) whereas there were fewer rotations observed in sham rats (56.8 ± 18.2 rotations in 1 hour; *P*<0.05; Figure 1B).

Following 6OHDA injection into the MFB, rats showed significantly poorer motor coordination, when tested with ledged beam traversal four weeks after lesioning, with a greater number of footfaults at each section of the beam, compared to sham animals (Figure 1C, *P*<0.05). Moreover, there was a significant 2-fold increase in times taken to cross the beam for lesioned animals (6OHDA: 10.8 ± 1.2 seconds; sham: 5.6 ± 0.3 seconds; *P*<0.05; Figure 1D).

3.2 Changes in colonic bead expulsion time and in colorectal responses to a ghrelin receptor agonist (capromorelin)

The bead expulsion test was performed in conscious rats to evaluate the ability of the distal colon to propel solid contents. The bead expulsion time was significantly greater for 6OHDA rats (366.3 ± 44.2 seconds) compared to sham rats (246.8 ± 36.4 seconds; Figure 2A; *P*<0.05) four weeks post-lesioning.

Colorectal contractile activity was measured under anesthesia in sham and 6OHDA rats. The rats were injected intravenously with the ghrelin receptor agonist capromorelin (4mg/kg), which acts at the spinal defecation centre (see Discussion). Capromorelin substantially increased the number of propulsive contractions at baseline for both sham rats (from 5.0 ± 1.1 to 51.4 ± 10.4 contractions per 60 minutes) and 6OHDA rats (from 3.1 ± 1.2 to 26.7 ± 5.5 contractions per 60 minutes; *P*<0.05; Figure 2B). This increase in contractile activity in the colorectum elicited a significant increase in the amount of fluid expelled for sham rats (from 0.3 ± 0.1 to 3.5 ± 0.7mL per 60 minutes) and 6OHDA rats (from 0.0 ± 0.05 to 1.0 ± 0.3mL per 60 minutes; *P*<0.05; Figure 2C). However, in
response to capromorelin, 6OHDA rats produced fewer contractions and lower fluid output, when compared to sham rats (P<0.05).

3.3 Unilateral injection of 6OHDA induces ENS neuropathy

In order to investigate whether the GI dysfunction was related to effects on the ENS, alterations at the cellular level were assessed using immunohistochemistry. ENS neuropathy was examined by staining for the RNA binding protein Hu that is expressed in all enteric neurons. There was a statistically significant decrease in the number of Hu-positive neurons per ganglion in the myenteric plexus in the distal ileum of 6OHDA rats (46.4 ± 1.4; P<0.05; Figure 3A) compared to sham (54.9 ± 3.2). No change in the number of Hu-positive cells per ganglion was observed in the distal colon (Figure 3C).

An index of damage to enteric neurons is Hu translocation from the cytoplasm to the nucleus.33 There was a significant increase in the number of neurons with nuclear translocation of Hu in both the distal ileum (Figure 3B) and distal colon (Figure 3D) for 6OHDA rats (ileum: 32.0 ± 2.8; colon: 32.0 ± 2.3; Figure 3F) when compared to sham rats (ileum: 23.0 ± 0.8; colon: 22.3 ± 2.6; Figure 3E; P<0.05).

Previous studies indicate that the type of enteric neurons that is most sensitive to stress is nNOS neurons.34 There was a trend for a decrease in the proportion of nNOS neurons in the myenteric plexus in the distal ileum of 6OHDA rats (22.6 ± 1.7) compared to sham rats (26.1 ± 0.8; Figure 4A). Moreover, there was a statistically significant decrease in the proportion of nNOS-positive neurons in the distal colon of 6OHDA rats (23.2 ± 2.2; Figure 4D) in comparison to sham rats (30.9 ± 1.8; P<0.05; Figure 4B, C). Interestingly, the proportion of nNOS-positive neurons that had nuclear Hu translocation was significantly increased in both the distal ileum and distal colon of rats that had 6OHDA injected into the MFB (ileum: 52.8 ± 4.5; colon: 54.4 ± 2.3) when compared to rats with sham MFB injection (ileum: 40.0 ± 1.7; colon: 32.5 ± 2.7; P<0.05).

4 DISCUSSION

The use of 6OHDA injected into the MFB to provide a model of PD is established and widely used in rodents.22 In the present study, we have confirmed that 6OHDA injected into the MFB of rats causes motor symptoms, that resemble aspects of the motor symptoms of PD, associated with loss
of dopaminergic neurons of the nigrostriatal pathway. We also observed changes in colorectal function and in the neurons of the ENS. It was previously found that injection of 6OHDA into the MFB of rats caused up to a 60% reduction in daily fecal output at 4-8 weeks and a reduced fecal water content. The defecation control pathways pass through the forebrain and the deficiency in fecal output caused by 6OHDA injected into the MFB might be expected to be a consequence of damage at this level. The neural pathway has a series of synapses between the cortex and the muscle of the colon (Figure 5) and we find evidence of changes in neurons that are several synapses removed from the injection site. In fact, we found that the expulsion of a bead from the colorectum, which depends on propulsive reflexes within the ENS, was approximately 50% slower after 6OHDA was injected into the MFB. This is consistent with a previously reported reduced propulsive efficiency, including a reduction in peak pressures of propulsive contractions, in the colon that was removed and investigated *in vitro*. Evidence for the dependence of colorectal propulsion on the ENS includes that if the ENS is disrupted, as in Hirschsprung disease or Chagas’ disease, propulsion does not occur, leading to constipation in both cases.

The effectiveness of stimulation of the defecation centres in the spinal cord with a ghrelin receptor agonist (capromorelin) was reduced, the number of propulsive contractions being halved. Ghrelin receptor agonists activate neurons of the defecation centres at spinal levels (L6 to S2 in the rat). Moreover, ghrelin agonists do not elicit defecation when applied at other levels of the neuraxis, and their effects are not reduced by severing the spinal cord rostral to the defecation centres. However, responses to ghrelin agonists are lost if the connections between the spinal cord defecation centres and the ENS are severed. Thus, the action of capromorelin is in the lumbo-sacral spinal cord. The reduced effectiveness of capromorelin after 6OHDA was injected into the MFB must therefore be due to a deficiency at or downstream from the lumbo-sacral defecation centre. A downstream site is suggested by the slowed bead propulsion (current study) and also by the previously reported reduction in propulsive efficiency of the colon isolated from the 6OHDA-lesioned rats. These functional changes in the colon can be related to the structural changes observed in enteric neurons after 6OHDA injection into the forebrain in previous studies and in the current work. A reduction in the number of enteric neurons that were immunoreactive for nNOS and an increase in vasoactive intestinal peptide (VIP) in the ENS, have been reported. We also found a reduction in the proportion of neurons immunoreactive for nNOS. Deficiencies in nNOS neurons is one of the most common changes that occurs when there is challenge to enteric neurons, probably because of the propensity of the free radical, NO, to form damaging peroxynitrites under conditions of oxidative stress. In addition, acetylcholine release was lower after electrical stimulation of isolated colon from 6OHDA-lesioned rats, indicating altered excitatory control. We measured the extent of Hu translocation from the cytoplasm to the nucleus,
which is an indicator of enteric neuron stress.\textsuperscript{33,44} There was a significant increase in the occurrence of nuclear Hu in both the distal ileum and distal colon in rats injected with 6OHDA into the MFB, compared to sham injected rats. The increase in Hu translocation indicates that enteric neurons were stressed by 6OHDA. Therefore, the central dopaminergic denervation leads to neurochemical changes and enteric neuron damage in the ENS, that is hypothesized to result in colonic dysmotility.

A possibility to be considered is that 6OHDA injected into the MFB leaks into the periphery and reaches the large intestine. This has been investigated directly and no 6OHDA was found in the peripheral circulation after injections of 4-8 µg of 6OHDA into the MFB.\textsuperscript{25} Even if there was leakage from the MFB into the periphery, the small dose of 6OHDA would not have affected colorectal motility in the animal, as it has been shown that repeated peripheral treatments with high doses of 6OHDA (50-100 mg/kg) are necessary to significantly influence GI motility.\textsuperscript{45} Toti and Travagli (2014), who found changes in gastric function after administration of 6OHDA to the MFB, also deduced that escape of 6OHDA into the periphery was an unlikely explanation of the downstream effects. In their study, gastric emptying was slowed and effects on the stomach of tyramine injection into the dorsal vagal complex were reduced after 6OHDA administration.\textsuperscript{46} The numbers of cholinergic neurons in the myenteric plexus of the esophagus, stomach and duodenum were decreased, whereas the percentages of myenteric neurons with nNOS immunoreactivity were increased. It should be noted that the dorsal vagal complex is a central control centre for the stomach that is rather analogous to the defecation centre for the colorectum. It is thus interesting that effects of tyramine in the dorsal vagal complex and capromorelin in the defecation centres on their respective end organs were both reduced.

It seems likely in the case of current experiments and of the previous experiments on gastric control\textsuperscript{46} that there are down-stream, trans-synaptic consequences of lesion of the MFB. This implies that there are on-going trophic influences on the ENS across multiple synapses in the case of defecation control and across several synapses in the case of the stomach. The present work demonstrates a brain to gut influence, but it is also clear that Parkinsonian disorders may originate from the gut. Recent studies have shown that alpha-synuclein administered into the gut is capable of propagating through vagal fibers to the midbrain to case progressive PD-like pathology.\textsuperscript{47-50} After alpha-synuclein fibril inoculation into the gut there was a loss of dopamine neurons in the SNpc and motor impairment in mice.\textsuperscript{49,50} Thus, there is a complex two-way gut-brain-gut interaction, where symptoms of PD can be initiated either from the gut or after lesions in the CNS that lead to enteric neuropathies.
5 CONCLUSION

Our study shows that the unilateral injection of 6OHDA into the MFB induces both motor deficits and GI dysfunction. Deficits in colonic bead expulsion and colorectal propulsion in response to stimulation of the lumbo-sacral defecation centre with capromorelin provide a clear indication of compromised ENS function, which is supported by evidence of enteric neuron pathology. The results imply that there are trans-synaptic effects that compromise down-stream neuronal functions in pathways from the brain to the defecation centres and thence to the ENS of the colorectum.

ACKNOWLEDGEMENTS

This work was supported by funding from the National Health and Medical Research Council (grant APP1145686). CLP is supported by an NHMRC Senior Research Fellowship.

DISCLOSURES

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

SD, JBF and XC designed the research study. XC, SD, RVP, RMM, MDN, CME, CLP and DIF conducted the experiments. XC and SD analyzed the data. JBF and XC wrote the manuscript. All authors contributed discussion and approved the manuscript.

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**Figure descriptions**

**FIGURE 1** Unilateral 6OHDA injections into the MFB ablated the nigrostriatal dopaminergic system and worsened motor coordination. A: TH immunohistochemical staining of the rat CPu and SNpc. There were fewer TH-positive nerve cells and fibres on the side of the 6OHDA injections compared to sham. B: Amphetamine injections increased the number of rotations made by rats.
receiving intracerebral injections of 6OHDA, compared to sham animals. C and D: Hind limb motor function is impaired in 6OHDA rats, as revealed by an increase in the number of footfaults made, compared to sham rats. The time taken to cross the beam was also significantly increased for 6OHDA rats when compared to sham rats. C: Data were analyzed by two-way ANOVA followed by Sidak’s multiple comparisons test. Data represent the mean±SEM; *P<0.05; sham n = 13, 6OHDA n = 17.

FIGURE 2 Impaired colon motility was observed in animals injected with 6OHDA into the MFB compared to sham animals. A: Colonic bead expulsion time was significantly slower in 6OHDA rats when compared to sham-injected rats four weeks post-lesioning. B and C: The number of contractions and amount of fluid expelled in response to capromorelin were greater for sham-injected compared to 6OHDA-injected rats. D: Representative traces of colorectal contractions (blue) and fluid expulsion (red) in sham (Di) and 6OHDA (Dii) rats, highlighting their sensitivity to the ghrelin receptor agonist capromorelin, that was less in the rats in which the MFB was lesioned. Data expressed as mean±SEM; *P<0.05; sham, n = 13; 6OHDA, n = 17.

FIGURE 3 The effects of 6OHDA on the myenteric plexus of the distal ileum and distal colon. A, B: In the myenteric plexus of the distal ileum of 6OHDA rats, when compared to sham rats, there was a significant decrease in the number of Hu-positive neurons per ganglion and an increase in the translocation of Hu to the nucleus. C, D: In the distal colon there was no significant change in the number of Hu-positive neurons per ganglion although there was a significant increase in the number of neurons exhibiting Hu translocation to the nucleus. E and F: Representative photomicrographs of Hu staining in distal colon tissue from rats with sham and 6OHDA injection into the MFB. Asterisks: In E, neurons with low levels of nuclear Hu immunoreactivity; in F, neurons with Hu translocation to the nucleus. Data are mean±SEM; *P<0.05; n = 8 per group.

FIGURE 4 Changes in the proportion of nNOS neurons in the distal ileum and distal colon of 6OHDA rats. A: No significant change was seen in the proportion of nNOS-positive neurons in the distal ileum. B: The proportion of nNOS-positive neurons in the distal colon was significantly decreased in 6OHDA rats compared to sham rats. C, D: Images of Hu and nNOS immunoreactivity in the myenteric plexus of distal colon of 6OHDA and sham rats. Hu is in red and nNOS in green. Data represent the mean±SEM; *P<0.05; n = 8 per group.

FIGURE 5 Sites of 6OHDA administration, ghrelin receptor agonist action and enteric neuronal damage in the neural pathways for colorectal control. 6OHDA was administered into the MFB, rostral to the brain stem centres, including rostral to Barrington’s nucleus, through which cortical control of defecation is relayed. Responses to ghrelin agonists, whose effects on defecation were reduced, are exerted in the defecation centres of the lumbo-sacral spinal cord.
Immunohistochemical evidence of neuropathy was found in the ENS, including loss of inhibitory motor neurons (nNOS neurons). The defecation centres and the ENS are several synapses removed from the site of administration of 6OHDA.

**Graphical Abstract** Lesion of the medial forebrain bundle caused reductions in the responses to activation of the defecation centres in the lumbo-sacral spinal cord, reduced ENS-mediated propulsion and enteric neuropathy 4-5 weeks later, indicating trans-synaptic changes in neuronal properties.
A TH localization

Sham

6OHDA

Caudate

Substantia nigra

5mm

B Amphetamine rotation test

Rotations in 1 hr

MFB

Sham

6OHDA

C Ledged beam - footfaults

No. of footfaults

Sham

6OHDA

D Ledged beam - time to cross

Time (sec)

Sham

6OHDA

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A Bead expulsion test

B Contraction in response to capromorelin

C Fluid propelled in response to capromorelin

Di Sham

Dii 6OHDA lesioned

Capromorelin 4 mg/kg

10 mmHg

4 mL

10 min

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Title:
Investigation of nerve pathways mediating colorectal dysfunction in Parkinson’s disease model produced by lesion of nigrostriatal dopaminergic neurons

Date:
2020-06-08

Citation:

Persistent Link:
http://hdl.handle.net/11343/275873