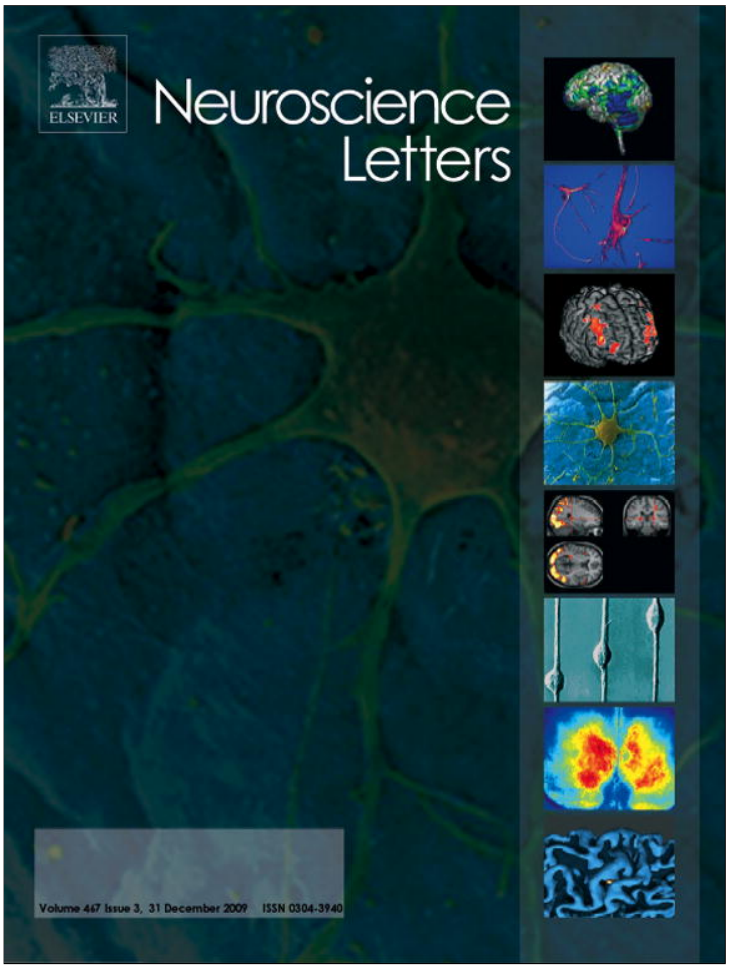

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Functional and neurochemical changes of the gastrointestinal tract in a rodent model of Parkinson's disease

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ABSTRACT

Patients with Parkinson's disease develop motor disturbances often accompanied by peripheral autonomic dysfunctions, including gastrointestinal disorders, such as dysphagia, gastric stasis and constipation. While the mechanisms subserving enteric autonomic dysfunctions are not clearly understood, they may involve the enteric dopaminergic and/or nitrergic systems. In the present study, we demonstrate that rats with unilateral 6-hydroxydopamine lesion of nigrostriatal dopaminergic neurons develop a marked inhibition of propulsive activity compared to sham-operated controls, as indicated by a 60% reduction of daily fecal output at the 4th week of observation. Immunohistochemical data revealed that 6-hydroxydopamine treatment did not affect the total number of HuC/D-positive myenteric neurons in both the proximal and distal segments of ileum and colon. Conversely, in the distal ileum and proximal colon the number of nitrergic neurons was significantly reduced. These results suggest that a disturbed distal gut transit, reminiscent of constipation in the clinical setting, may occur as a consequence of a reduced propulsive motility, likely due to an impairment of a nitric oxide-mediated descending inhibition during peristalsis.

Parkinson's disease (PD) is a multi-system disorder characterized by the involvement of selected neuronal populations throughout the central and peripheral nervous systems [3,6]. The pathological hallmark of PD is the degeneration of dopaminergic, melanized neurons of the *substantia nigra pars compacta* (SNc) projecting to the striatum, which triggers the motor symptoms of the disease (tremor, rigidity and bradykinesia) [5]. Although PD is considered the prototypical movement disorder, PD patients also experience numerous non-motor symptoms, including cognitive dysfunction, sleep disorders, psychiatric symptoms and, especially, gastrointestinal (GI) dysfunctions [25]. Almost all parkinsonian patients experience GI dysfunctions, such as dysphagia, nausea and other dyspeptic symptoms as well as abdominal distension, and severe constipation [24]. GI dysfunctions are now considered a core component of PD clinical picture [9,22,24,25], since they often precede the onset of motor symptoms by many years and their occurrence in otherwise healthy people has been associated with an increased risk for PD [1,9,22,24,25].

GI motility is controlled by various mechanisms acting both at central and local levels. Local control is exerted by the enteric nervous system (ENS), an intrinsic neural network endowed in the gut wall, that comprises a multitude of neurons mainly organized in the myenteric (Auerbach's) and submucosal (Meissner's) plexuses [16]. Neuronal circuitries of the myenteric plexus include intrinsic primary afferent neurons, ascending excitatory and descending inhibitory pathways involved in the regulation of peristalsis, leading to anal displacement of intraluminal contents [11,16]. Dopamine has been recently claimed to be an enteric neurotransmitter, since dopamine, tyrosine hydroxylase (TH), and the dopamine transporter (DAT) co-localize within a subset of ENS neurons [20].

Lewy bodies, the typical alpha-synuclein positive inclusions found in the parkinsonian brain [7], have been also detected in the myenteric plexus of PD patients, particularly within nitric oxide synthase (NOS) and other enteric neurons [31]. These inclusions

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have been detected in patients with advanced pathology, but also in non-symptomatic subjects with PD-related brain lesions limited to the lower brainstem [8], thereby supporting the hypothesis that the GI tract may be an early target of the disease [18].

So far, the involvement of enteric dopaminergic and nitrergic neurons has been suggested in GI dysmotility. Early studies in rats showed altered myoelectric activity in the duodenum, following systemic administration of parkinsonian neurotoxin 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) [13,27]. However, MPTP does not cause parkinsonism in rats (unlike primates and mice) [19], thus limiting the value of these observations. Anderson et al. [2] reported a 40% reduction of dopamine neurons in the ENS of mice receiving intraperitoneal MPTP administration, without loss of cholinergic or nitrergic neurons. Recently, Tian et al. [28] reported increased expression of TH and DAT in the GI tract of rats bearing bilateral lesions of the SNc obtained by stereotaxic injection of 6-hydroxydopamine (6-OHDA), a neurotoxin with selective toxicity for dopaminergic neurons [30]. Given the inhibitory nature of dopamine in the ENS, an excess of enteric dopamine production caused by 6-OHDA would have been expected to reduce the gastrointestinal propulsive motility, especially gastric emptying [28]. The inhibitory effect of dopamine is exerted through activation of prejunctional D_2 receptors located on cholinergic neurons leading to delayed gastric emptying. Indeed, in patients with PD, peripheral dopamine D_2 antagonist domperidone improves gastric emptying delayed by L-DOPA treatment [29].

This study was designed to investigate whether lesion of the nigrostriatal tract induced by 6-OHDA in rats may be associated with neuroanatomical and neurochemical modifications leading to GI dysfunction. Currently, there is no evidence that a selective nigrostriatal lesion may induce GI dysfunction in PD animal models. Following the lesion, we monitored the daily fecal output of the rats for 4 weeks. Then, we investigated whether changes in the nitrergic population of myenteric neurons occur in segments of proximal and distal ileum and colon.

Male Sprague–Dawley rats (Charles River, Calco, Como, Italy), weighing 250–280 g at the beginning of the experiment, were used. Animals were housed two per cages at 20–22 °C on a 12-h light–dark cycle with food and water *ad libitum*, until moved to the metabolic cages (see below). Animal care and procedures were in accordance with the European Union Directive 86/609 on care and humane use of experimental animals. All experimental procedures were approved by the Animal Research Ethics Committee of the University of Pavia. The number of animals used was kept to the minimum necessary for a meaningful interpretation of data.

Animals were anaesthetized with 50 mg/kg of sodium-thiopental and placed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). 6-OHDA, dissolved in saline solution containing 0.02% of ascorbic acid (Sigma, USA), was unilaterally injected in two sites of the right medial forebrain bundle, at the following coordinates (mm): (i) AP = −4.0, ML = −0.8, DV = −8.0 (9 μg/3 μL); (ii) AP = −4.4, ML = −1.2, DV = −7.8 (7.5 μg/3 μL) [23]. Injections were performed at 1 μL/min, using a Hamilton 10-μL syringe. After injection, the needle was left in place for 5 min before being retracted, to allow complete diffusion of the medium and wounds were clipped. The untreated contralateral area (left nigrostriatal tract) served as internal control. Control rats underwent sham stereotaxic surgery.

After recovering from surgery, rats were individually housed in metabolic cages (Tecniplast, Varese, Italy) with stainless steel grid floor equipped with an apparatus that allowed complete recovery of feces outside the cage. The animals were supplied with water and a standard pelleted diet *ad libitum*. Special feeders allowed accurate measurement of food intake. After an equilibration period of 1 week in the metabolic cage, physiological parameters including food (g) and water (mL) consumption, body weight (g), and fecal excretion (g) were measured daily, beginning from the second week, for 3 weeks. Data were analyzed and plotted as mean ± SEM.

At the end of the 4th week, rats were sacrificed and perfused transcardially with cold saline solution. Brains were removed and frozen at −80 °C. Serial coronal sections (25 μm thick) were cut with a cryostat at the level of the *striatum* and SNc and mounted on polylysine-coated slides. Every fourth section underwent immunohistochemical staining for TH to evaluate dopaminergic terminal damage in the striatum and loss of dopaminergic cell bodies in the SNc. Sections were postfixed in 4% neutral buffered formaldehyde (Carlo Erba, Milan, Italy), rinsed in Tris-buffered saline (TBS), treated with 3% H_2O_2 and incubated in TBS containing 10% normal goat serum/0.6% Triton X-100, for 30 min at room temperature. Sections were then incubated overnight at 4 °C with a mouse anti-TH antibody (diluted 1:2000, Chemicon International Inc., Temecula, CA, USA), rinsed in TBS and re-incubated for 1 h, at room temperature, with a goat biotinylated anti-mouse IgG antibody (diluted 1:1000, Vector Laboratories, Burlingame, CA, USA). Finally, sections were processed with the avidin–biotin technique and reaction products were developed with nickel-intensified 3′,3′-diaminobenzidine tetra-hydrochloride, using commercial kits (Vector Laboratories, Burlingame, CA, USA).

Segments of the GI tract (proximal and distal ileum and colon) were excised and immersed for 15 min in phosphate-buffered saline (PBS) pH 7.2 and type-l calcium-channel blocker nicardipine, as a muscle relaxant. Intestinal segments were then opened along the mesenteric border, flushed out with PBS and pinned on balsa wood, with the mucosa facing down. Specimens were subsequently fixed overnight in 2% paraformaldehyde containing 0.2% picric acid in 0.1 M PBS at 4 °C. Afterwards, specimens were removed from the balsa wood, washed in dimethylsulfoxide, and stored at 4 °C in PBS containing 0.1% Na-azide.

Specimens were then processed as longitudinal muscle-myenteric plexus whole mount preparations (LMMPs) by peeling away the different layers (i.e. mucosa, submucosa and circular muscle). Before immunostaining, LMMPs were incubated in 10% normal goat serum in PBS containing 1% Triton X-100 for 30 min at room temperature to reduce nonspecific binding. For double labeling immunohistochemistry, LMMPs were incubated in a mixture containing a mouse monoclonal anti-HuC/D (pan-neuronal marker) (Invitrogen, USA) and a rabbit monoclonal anti-nNOS (marker of nitrergic neurons) (Swant, Bellinzona, Switzerland) both diluted (1:200 and 1:400, respectively) in a suitable medium (1.8% NaCl in 0.01 M phosphate buffer with 0.1% Na-azide). After a 48-h incubation at 4 °C in a humid chamber, LMMPs were washed in PBS; antigen–antibody complexes were identified with a mixture of secondary antibodies, i.e. donkey anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) and donkey anti-rabbit IgG conjugated to tetramethyl rhodamine iso-thiocyanate (TRITC) (Listarfish, Milan, Italy), both diluted at 1:200 in PBS. LMMPs were then mounted on gelatin-coated slides and cover-slipped with buffered glycerol (pH 8.6).

LMMPs were examined with a confocal laser scanning microscope (Leica TCS-SP system mounted on a Leica DMIRBE inverted microscope). An Ar/Vis laser at 500/530 nm and 580/630 nm was used to excite FITC and TRITC fluorescence, respectively. For each sample, the total number of HuC/D-immunoreactive neurons was counted in 10 microscopic fields (0.24 mm^2 each field) previously determined by means of two orthogonal coordinates taken from a table of random numbers and measured on the movable stage of the microscope. Therefore, for each preparation, a total area of 2.4 mm^2 was evaluated. To determine the proportion of nNOS immunolabeled neurons, at least 500 HuC/D-immunoreactive neurons were counted in the LMMPs. Thus, the number of nNOS immunolabeled neurons was expressed as percentage of HuC/D-immunopositive neurons and calculated as mean ± SEM.

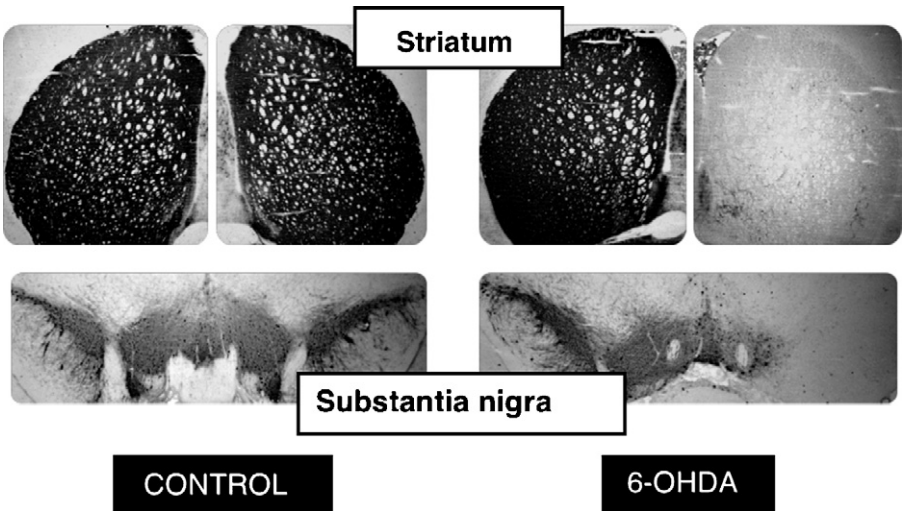

Fig. 1. Representative examples showing TH immunoreactivity (dark staining) in the striatum and substantia nigra of rats treated with 6-OHDA injection into the right medial forebrain bundle. Compared to untreated animals, note the marked decrease (virtual lack) of dopaminergic fibers and neuronal cell bodies in the striatum and substantia nigra (right panel) in rats receiving the central unilateral injection of 6-OHDA.

Statistical analysis was performed using GraphPad Prism 3. Comparisons between groups were made using Student's *t*-test for unpaired data or two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test. *P* values < 0.05 were considered significant.

Rats injected with 6-OHDA in the right hemisphere showed complete, ipsilateral loss of dopaminergic (TH-positive) terminals and cell bodies in the striatum and SNc, respectively (Fig. 1). No changes in TH immunoreactivity were detected in control rats.

Compared to controls ($n = 12$), animals with nigrostriatal lesions ($n = 13$) showed a significant ($P < 0.001$) reduction in daily fecal output throughout the 3rd and 4th post-lesional weeks (Fig. 2A), reaching a nadir at the end of the observational period (−60%). Conversely, food and weight gain throughout the 3 weeks of observation were equivalent in the two groups (Fig. 2B and D). Concerning water consumption, the difference upon time between treated rats and controls was not significant; however, direct comparison of daily data revealed a significant difference in water consumption during days 7-14 and 17 and 21 (values ranging from $P < 0.05$ to $P < 0.001$) (Fig. 2C).

The total number of HuC/D immunoreactive myenteric neurons did not change between 6-OHDA treated rats and controls in any of the selected GI regions (Fig. 3). However, we observed differences when the percentage of HuC/D immunolabeled neurons expressing nNOS was assessed. In particular, rats with nigrostriatal lesions showed a significant reduction of nNOS-immunoreactive neurons in the distal ileum (−15%) ($P < 0.005$) and in the proximal colon (−14%) ($P < 0.05$), whereas no difference was observed in the proximal ileum and distal colon (Fig. 3).

The neurochemical substrate of PD-related GI dysfunction is still unclear, mostly because of the paucity of experimental studies addressing this issue. In this study, we investigated the effects of a complete, unilateral lesion of the nigrostriatal pathway on a specific parameter of GI function, such as the daily fecal output. Furthermore, we tested whether the central dopaminergic denervation was able to affect the nitrergic component of myenteric innervation, one of the preferential targets of PD within the ENS [8,18]. We found that lesioned rats showed both functional and neurochemical alterations of the GI tract, including a reduction of daily fecal output and percentage of myenteric nitrergic neurons.

The reduction of daily fecal output was present throughout the 3rd and 4th week of observation following 6-OHDA injection, the lowest value (−60%) being recorded at the end of the observation period. Previous evidence in the same model showed that 6-OHDA injection causes a fast lesion that reaches full expression within the first week after surgery. In fact, cell loss in the SNc occurs in 12–24 h followed, within 3–4 days, by marked lesions of striatal

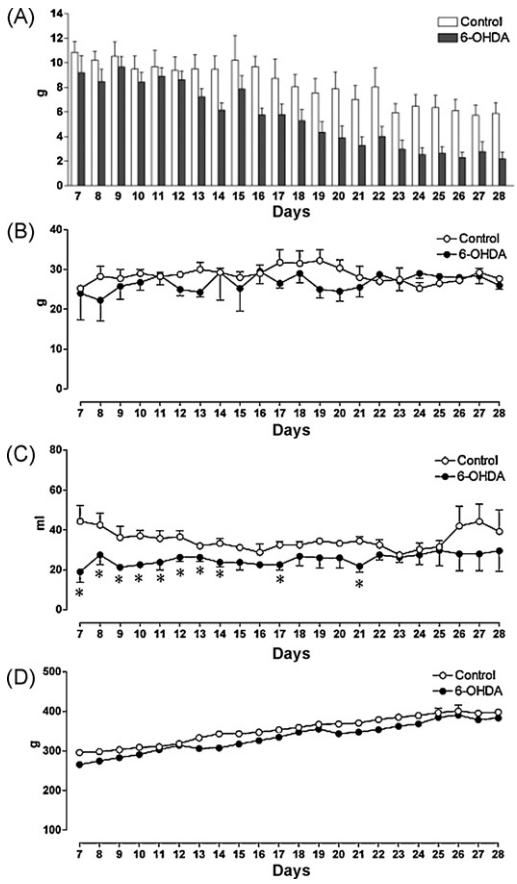

Fig. 2. Fecal daily output (A), food (B) and water consumption (C) and weight gain (D) in control and 6-OHDA treated rats. Compared to controls, treated rats showed a significant ($P < 0.001$) reduction in daily fecal output throughout the 3rd and 4th post-lesional weeks (A). A direct comparison of the daily data revealed a significant difference in water consumption during the days 7–14 and 17 and 21 (values ranging from $P < 0.05$ to $P < 0.001$) (C).

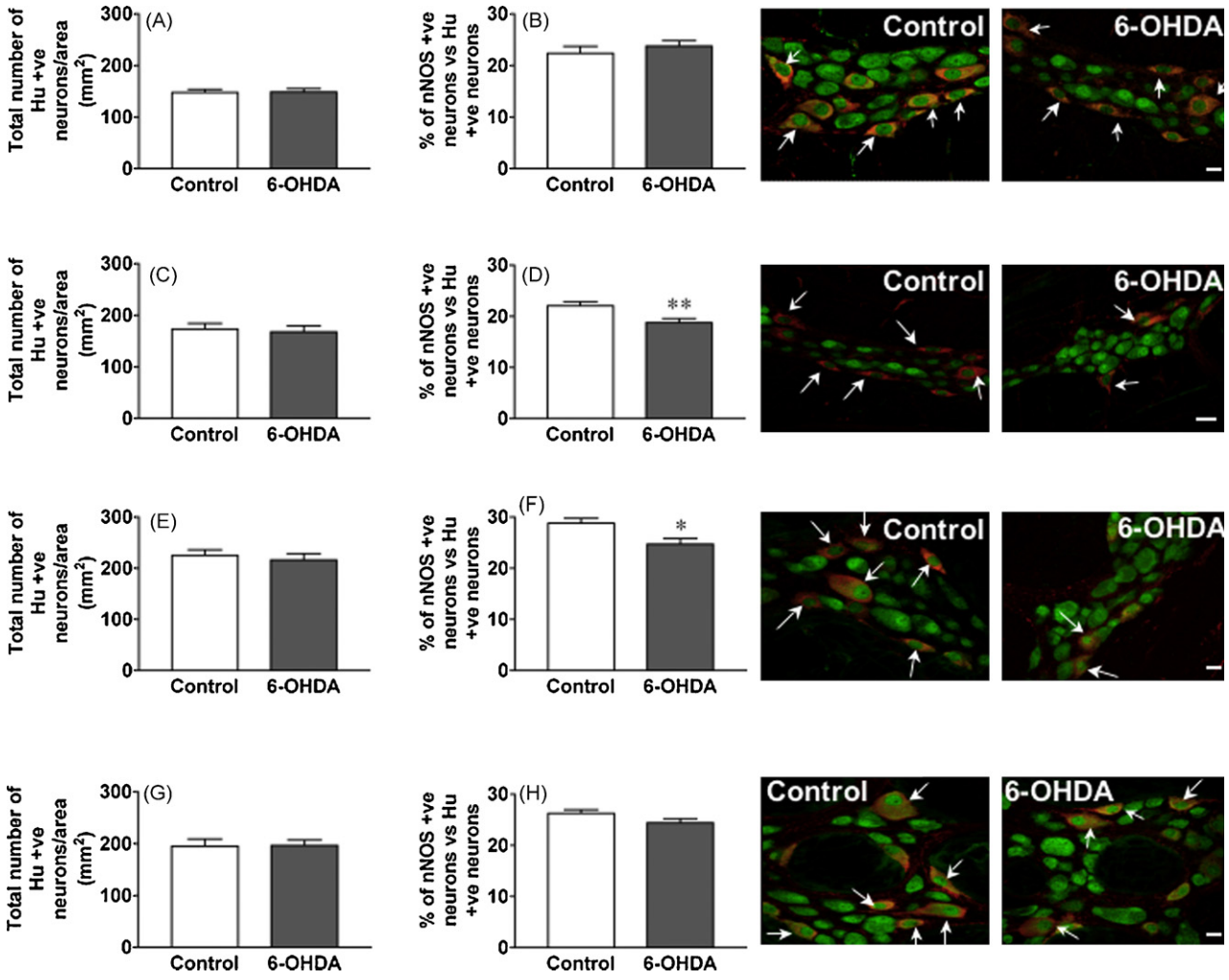

Fig. 3. Quantitative analysis of Hu and nNOS immunoreactive myenteric neurons. The number of Hu positive myenteric neurons is unchanged in the proximal and distal ileum, as well as in the proximal and distal colon (A, C, E and G, respectively) of controls and 6-OHDA treated rats. Conversely, compared to control rats, the percentage of nNOS expressing neurons is significantly reduced in the distal ileum (D: $P < 0.005$) and proximal colon (F: $P < 0.05$), whereas unchanged in the proximal ileum (B) and distal colon (H), of 6-OHDA treated rats. Calibration bars: 10 μm.

dopaminergic terminals [12]. Thus, the nigrostriatal damage is not immediately associated with a reduced fecal output, implying that enteric dysfunction occurs later with respect to the early central lesions.

We considered the possibility that changes in food and water intake may have influenced these results. Indeed, Greene et al. [17] reported that rats chronically treated with systemic rotenone, a mitochondrial toxin that replicates PD features in rodents [4], showed a transient decrease in stool frequency and weight loss. Aphagia and weight loss have been described in rotenone-treated animals [4,14] and may play a major role in the decreased stool frequency observed in this model. Marked aphagia also represents a complication in animals with 6-OHDA bilateral lesion of the nigrostriatal pathway, although it is not usually reported in animals with a unilateral lesion [12]. For this reason, we monitored food consumption and weight gain in lesioned and unlesioned animals. Both parameters were similar in the two groups, thereby ruling out the possibility that a reduced fecal output in lesioned rats was due to a reduced food intake. Concerning water intake, a significant difference was observed at days 7–14 and occasionally at other time points (days 17 and 21). This implies that there was no difference in water intake during the 3rd and 4th week, when the reduction in daily fecal output was observed in lesioned rats. Thus, we believe the early difference in water intake is unlikely to have influenced the late changes in defecatory behaviour. Nevertheless, the present data cannot rule out an influence of modified water intake on daily fecal output in treated rats and further experiments are necessary to address this issue. Our findings are reminiscent of the constipation that affects the lower GI tract in PD patients, which can precede the onset of motor symptoms by many years [1]. In fact, infrequent bowel movements assessed in mid-life have been associated with an increased risk of developing PD [1]. An association between constipation in PD patients and a dopaminergic defect in the ENS has been previously demonstrated [26]. Apparently, this is a potential mechanism leading to gut transit abnormalities observed in patients with PD.

Based on our results, however, the enteric nitrergic system may also play a role. Overall, available data indicate either unchanged [2] or increased numbers of myenteric nitrergic neurons in the MPTP model of PD [10]. In our hands, rats lesioned with 6-OHDA showed a significant reduction in the percentage of myenteric neurons expressing nNOS in the distal ileum and proximal colon, while the total number of neurons was unchanged. These findings hold a scientific rationale to explain the reduced daily fecal output, as observed in our functional experiments. Indeed, enteric nitrergic neurons, which are strategically placed on descending pathways of peristalsis, exert a major contribution in the regulation of gut propulsion [15,16]. An impaired descending inhibition related to a

nitrergic defect in the distal gut may very well explain the reduction of fecal output in lesioned rats.

An important question arising from this study is how the nigrostriatal dopaminergic denervation may affect the nitrergic system in the ENS. We discard the hypothesis that centrally administered 6-OHDA may have systemic effects by diffusing from the injection site into the blood stream. In recent experiments, we found no detectable levels of 6-OHDA in the bloodstream during the first 24 h following stereotaxic injection (Blandini, unpublished data). Furthermore, the reduced expression of enteric nNOS in a region-specific manner argues against the possibility that a "humoral circulating factor", triggered by 6-OHDA treatment, might be responsible for the nitrergic system alterations. An involvement of extrinsic pathways (parasympathetic and/or sympathetic), linking the CNS to the ENS, is probably the most appropriate explanation of the neurochemical plasticity in our model. In line with this, there is evidence that sympathetic denervation is associated with increased nNOS expression in the ENS, suggesting that extrinsic nerve pathways may have an impact on the enteric nitrergic expression [21,32].

In conclusion, in our rat model of PD, we demonstrated a marked reduction of daily fecal output, reminiscent of constipation in the clinical setting, along with a reduced percentage of enteric nitrergic neurons. Taken together, these data provide a basis to understand the pathophysiology of GI tract abnormalities in PD patients.

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