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Enteric Dysfunctions in Experimental Parkinson's Disease: Alterations of Excitatory Cholinergic Neurotransmission Regulating Colonic Motility in Rats

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ABSTRACT

Parkinson's disease is frequently associated with gastrointestinal symptoms, mostly represented by constipation and defecatory dysfunctions. This study examined the impact of central dopaminergic denervation, induced by injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle, on distal colonic excitatory cholinergic neuromotor activity in rats. Animals were euthanized 4 and 8 weeks after 6-OHDA injection. In vivo colonic transit was evaluated by radiologic assay. Electrically induced and carbachol-induced cholinergic contractions were recorded in vitro from longitudinal and circular muscle colonic preparations, whereas acetylcholine levels were assayed in the incubation media. Choline acetyltransferase (ChAT), HuC/D (pan-neuronal marker), muscarinic M2 and M3 receptors were assessed by immunohistochemistry or western blot assay. As compared with control rats, at week 4, 6-OHDA–treated animals displayed the following changes: decreased in vivo colonic transit rate, impaired electrically evoked neurogenic cholinergic contractions, enhanced carbachol-induced contractions, decreased basal and electrically stimulated acetylcholine release from colonic tissues, decreased ChAT immunopositivity in the neuromuscular layer, unchanged density of HuC/D immunoreactive myenteric neurons, and increased expression of colonic muscarinic M2 and M3 receptors. The majority of such alterations were also detected at week 8 post 6-OHDA injection. These findings indicate that central nigrostriatal dopaminergic denervation is associated with an impaired excitatory neurotransmission characterized by a loss of myenteric neuronal ChAT positivity and decrease in acetylcholine release, resulting in a dysregulated smooth muscle motor activity, which likely contributes to the concomitant decrease in colonic transit rate.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder, characterized by tremor, bradykinesia, and rigidity. PD is also associated with gastrointestinal (GI) dysmotility, including dysphagia, constipation, and defecatory disorder, which contribute to PD morbidity (Braak et al., 2006; Cloud and Greene, 2011; Pellegrini et al., 2015).

Eosinophilic cytoplasmic inclusions (Lewy bodies), containing aggregated α-synuclein, a hallmark of PD, have been detected in the enteric nervous system (ENS) and dorsal motor nucleus of the vagus (DMV) in PD patients, suggesting that the disease could spread from brain to gut (Wakabayashi and Takahashi, 1997; Braak et al., 2006), or rather start from the digestive system and move toward the brain (Hawkes et al., 2007; Pan-Montojo et al., 2010). However, the mechanisms of onset and progression linking PD to enteric dysmotility are poorly understood.

Alterations associated with GI dysmotility have been investigated in PD patients and animal models of PD. In PD models induced by systemic injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, rotenone, or salsolinol, GI motor alterations were ascribed to direct effects of neurotoxins on enteric neurons (Banach et al., 2005; Anderson et al., 2007;

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All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.
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ABBREVIATIONS: PD, Parkinson's disease; ChAT, choline acetyltransferase; DMV, dorsal motor nucleus of the vagus; ENS, enteric nervous system; ES, electrical stimuli; GI, gastrointestinal; GR159897, 5-fluoro-3-[2-[4-methoxy-4-[[(R)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1H-indole; ICSMC, isolated colonic smooth muscle cell; L-732,138, *N*-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzylester; L-NAME, N^{ω}-nitro-L-arginine methylester; MFB, medial forebrain bundle; NK, neurokinin; 6-OHDA, 6-hydroxydopamine; SB218795, (R)-[(2-phenyl-4-quinolinyl)carbonyl) amino]-methyl ester benzeneacetic acid; SNc, substantia nigra pars compacta; TBS, Tris-buffered saline; TH, tyrosine hydroxylase.

Pan-Montojo et al., 2010). However, other authors observed that, under denervation, elicited by 6-hydroxydopamine (6-OHDA) injection into the medial forebrain bundle (MFB), GI dysmotility appears to depend on nigrostriatal dopaminergic degeneration (Blandini et al., 2009). In particular, neurotransmitter alterations (dopamine, nitric oxide, vasoactive intestinal peptide), regulating intestinal motility, have been documented in the 6-OHDA model (Colucci et al., 2012; Zhu et al., 2012). PD-related GI abnormalities have also been explored in transgenic models. Mice overexpressing human wild-type or mutant A53T α-synuclein displayed moderate GI dysfunctions, including reduced gastric emptying, increased transit time, and reduced fecal output (Noorian et al., 2012). Furthermore, changes in colonic myenteric ganglia and propulsive activity were observed in Thy1-αSyn mice, characterized by overexpression of human α-synuclein in colonic myenteric ganglia (Wang et al., 2008, 2012). However, as no consistent nigrostriatal degeneration is associated with synuclein overexpression in these animals, at least at an early age, such models might not be not suitable for studies designed to pinpoint the impact of central dopaminergic denervation on GI motility.

When considering possible alterations of enteric excitatory pathways in PD, the available evidence is scarce and conflicting. Some authors have observed that the choline acetyltransferase (ChAT) expression in the proximal colon of rats with nigrostriatal denervation by 6-OHDA did not vary (Colucci et al., 2012; Zhu et al., 2012), whereas in the same model, a decreased acetylcholine content was found in the muscular layer of rat stomach (Zheng et al., 2011). Recently, Sharrad et al. (2013) reported that Lewy pathology targets both intrinsic and parasympathetic cholinergic neurons in the large bowel, suggesting the involvement of cholinergic pathways in bowel dysmotility associated with PD.

Despite several investigations supporting the view that GI symptoms in PD are associated with alterations of enteric nerve functions, the role played by cholinergic neurotransmission in the onset and progression of bowel motor abnormalities remains unclear. Therefore, the present study examined the impact of dopaminergic denervation by 6-OHDA injection into the MFB on colonic excitatory cholinergic neuromotility. In particular, functional studies were performed to assess the in vivo colonic transit as well as in vitro cholinergic contractile activity and acetylcholine release, whereas the evaluation of cholinergic neuron density and muscarinic receptor expression in the colonic neuromuscular layer was carried out by means of molecular approaches.

Materials and Methods

Animals. Male Sprague-Dawley rats (body weight 200–250 g) were used throughout the study. The animals were fed standard laboratory chow and tap water ad libitum and were not used for at least 1 week after their delivery to the laboratory. They were housed, three in a cage, in temperature-controlled rooms on a 12-hour light cycle at 22–24°C and 50–60% humidity. Their care and handling were in accordance with the provisions of the European Community Council Directive 86-609, recognized and adopted by the Italian government.

Induction of Nigrostriatal Denervation. Animals were anesthetized with 50 mg/kg sodium thiopental (i.p.) and placed into a stereotaxic frame (Stoelting, Wood Dale, IL). Rats received 6-OHDA (dissolved in saline solution containing 0.02% of ascorbic acid) or saline unilaterally into two sites of the right MFB, at the following coordinates (in millimeters) relative to the bregma and dural surface: 1) antero-posterior = −4.0, medium-lateral = −0.8, dorso-ventral (DV) = −8.0 tooth bar at +3.4 (9 μg /3 μl); and 2) antero-posterior = −4.4, medium-lateral = −1.2, DV = −7.8 tooth bar at −2.4 (7.5 μg /3 μl) (Paxinos and Watson, 1998). The injection rate was 1 μl/min using a Hamilton 10-μl syringe mounting a 26-gauge needle. After injection, the needle was left in place for 5 minutes to avoid leaks. At the end of the process, wounds were clipped, and the animal was allowed to wake up and recover. Animals were euthanized 4 and 8 weeks following 6-OHDA injection. Brains were immediately removed, frozen on dry ice, and stored at −80°C, whereas colonic specimens were dissected and processed for functional experiments and other assays as described later.

Immunohistochemistry of Tyrosine Hydroxylase in Brain Sections. Serial coronal sections (40 μm), including the striatum and substantia nigra pars compacta SNc) from both sham-operated and 6-OHDA animals, were cut on a cryostat and mounted on polylysine-coated slides. Immunohistochemical staining for tyrosine hydroxylase (TH) was carried out to evaluate dopaminergic terminal damage in the striatum and loss of cell bodies in the SNc, as previously described (Blandini et al., 2004). In brief, sections containing the striatum and SNc were postfixed in cold, 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 together with 0.6% Triton X-100 for 30 minutes at room temperature. Sections were incubated overnight at 4°C with a mouse anti-TH antibody (1:2000; Chemicon International, Temecula, CA), then rinsed in TBS and incubated for 60 minutes at room temperature with a goat biotinylated anti-mouse IgG antibody (1:1000; Vector Laboratories, Burlingame, CA). Finally, sections were processed with the avidin-biotin technique using a commercial kit (Vectastain ABC Elite kit; Vector Laboratories), and reaction products were developed using nickel-intensified 3,3′-diaminobenzidine tetrahydrochloride (DAB Substrate Kit for Peroxidase; Vector Laboratories). After rinsing with TBS, sections were dehydrated in ascending alcohol concentrations, cleared in xylene (Carlo Erba), and coverslipped.

Evaluation of Nigrostriatal Degeneration. The striatal dopaminergic terminal damage resulting from 6-OHDA infusion into the MFB was detected by the absence of TH staining within the striatum and expressed as the percentage of striatal volume deprived of TH immunoreactivity, as compared with the overall striatal volume. The striatal expression of TH was also evaluated in the brains of sham-operated animals.

The total number of dopaminergic cells in the SNc of both hemispheres was counted using stereological analysis. Unbiased stereological estimation was performed using the optical fractionator method (West et al., 1991) by the Stereo Investigator software on a Neurolucida computer-controlled microscopy system (Microbrightfield Inc., Williston, VT). The boundaries of SNc at all levels in the rostrocaudal axis were defined with reference to a coronal atlas of the rat brain (Paxinos and Watson, 1998). TH-positive cells in the SNc were counted in every fourth section, on comparable sections for all experimental groups throughout the whole nucleus. Counting frames (75 × 75 μm) were placed at the intersections of a grid (frame size 208,65 × 161,6 μm) that had been randomly placed over the section. Cells were labelled if they were TH-positive and were in focus within the counting area. Results represent the percentage of the number of TH-positive neurons in the injected SNc with respect to the intact side (neuron survival). We set 98% at the cut-off level for the striatal lesion and 95% for the lesion in the SNc.

Radiologic Assessment of Colonic Transit. The radiologic assessment of overall in vivo colonic transit was performed as previously described (Vegezzi et al., 2014). In brief, 4 and 8 weeks after 6-OHDA or saline nigrostriatal injection, overnight fasted rats received a suspension of $BaSO_4$ (2.5 ml, 1.5 g/ml; Prontobario H.D., Bracco Imaging Italia, Milan, Italy) intragastrically, and radiographic exposures were taken 10 and 12 hours later. This time frame was previously shown to allow the detection of contrast medium in radiographs of the cecal and colorectal regions. Focus-film distance

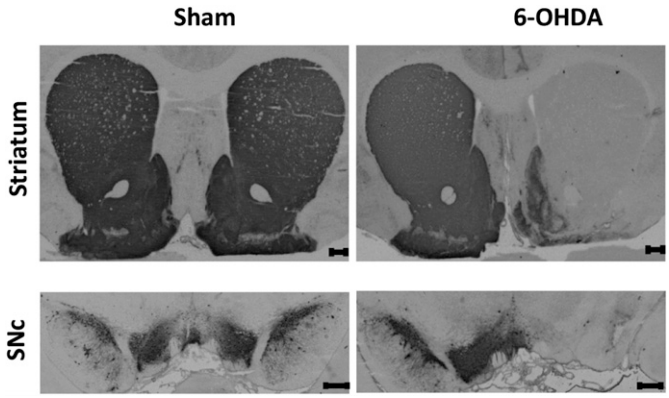

Fig. 1. Representative images of dopaminergic (TH+) striatal terminals and SNc cell bodies of both sham-operated and 6-OHDA–lesioned animals. Scale bar: 500 μm.

was manually fixed at 100 cm, and exposure values were 65 kVp to 4.5 mA (exposure time: 0.01 second). Total body DV radiographic projections were considered. The analysis of radiographic images was carried out according to the scoring proposed by Cabezos et al. (2008) by 6 different observers blinded to the treatment. In detail, the proportion of labeled cecum and colorectum, the intensity of labeling, the organ profile, and the homogeneity of labeling within the organ were all evaluated by each blinded observer and scored for each animal to obtain an overall value ranging from 0 to 12. The score for each rat was the mean of six readings. Final data were the means from eight rats per group.

Recording of Colonic Contractile Activity In Vitro. Contractile activity of colonic longitudinal and circular smooth muscle was recorded as previously described (Antonioli et al., 2014). After euthanization, the colon was removed and placed in cold Krebs' solution. Longitudinal and circular muscle strips from the distal colon, approximately 3 mm wide and 20 mm long, were set up in organ baths containing Krebs' solution at 37°C, bubbled with 95% O_2 + 5% CO_2. The strips were connected to isometric force transducers (2Biological Instruments, Besozzo, VA, Italy). A tension of 0.5 g for circular muscles and 1.0 g for longitudinal muscles was slowly applied to the preparations. Mechanical activity was recorded by BIOPAC MP150 (2Biological Instruments). Krebs' solution had the following composition (in mM): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, and glucose 11.5 (pH 7.4±0.1). Each preparation was allowed to equilibrate for at least 30 minutes, with intervening washings at 10-minute intervals. A pair of coaxial platinum electrodes were positioned at a distance of 10 mm from the longitudinal axis of each preparation to deliver transmural electrical stimulation by a BM-ST6 stimulator (Biomedica Mangoni, Pisa, Italy). Electrical stimuli (ES) were applied as follows: 10-second single trains consisting of square wave pulses (0.5 ms, 30 mA). At the end of the equilibration period, each preparation was repeatedly challenged with electrical stimuli (10 Hz), and experiments were started when reproducible responses were obtained (usually after two or three stimulations). Frequency-response curves (from 1 to 20 Hz) were constructed under the different in vitro experimental conditions reported later. The tension developed by each preparation was normalized by the wet tissue weight and expressed as grams per gram of wet tissue (g/g tissue).

In the first set of experiments, electrically evoked motor responses were recorded from colonic preparations maintained in standard Krebs' solution.

In the second series of experiments, contractions were assessed in colonic preparations maintained in Krebs' solution containing N^{ω}-nitro-L-arginine methylester (L-NAME; 100 μM), guanethidine (10 μM), *N*-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzylester (L-732,138, neurokinin NK_1 receptor antagonist; 10 μM), 5-fluoro-3-[2-[4-methoxy-4-[[(R)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1H-indole (GR159897, NK_2 receptor antagonist; 1 μM), and (R)-[[(2-phenyl-4-quinolinyl)carbonyl]amino]-methyl ester benzeneacetic acid (SB218795, NK_3 receptor antagonist; 1 μM) to examine the patterns of colonic contractions driven by excitatory nerve cholinergic pathways.

In the third series, colonic cholinergic contractions where evoked by direct pharmacological activation of muscarinic receptors located on smooth muscle cells. For this purpose, colonic preparations were maintained in Krebs' solution containing tetrodotoxin (1 μM) and stimulated with carbachol (0.01–100 μM).

Measurement of Acetylcholine Release from Colonic Longitudinal Muscle Preparations. Longitudinal muscle strips of colon, containing the myenteric plexus, were prepared and incubated in Krebs' solution containing L-NAME, guanethidine, L-732,138, GR159897, and SB218795 as reported earlier. After equilibration, aliquots of Krebs' solution (200 μl) were collected at −300, −180, −60, +60, +180, and +300 seconds with respect to the onset of ES (0.5 ms, 30 mA, 10 Hz). At the end of the 10-second period of ES application, one additional aliquot was collected to evaluate the amount of electrically induced acetylcholine release, as previously described by Yajima et al. (2011). To better appreciate the alterations of electrically

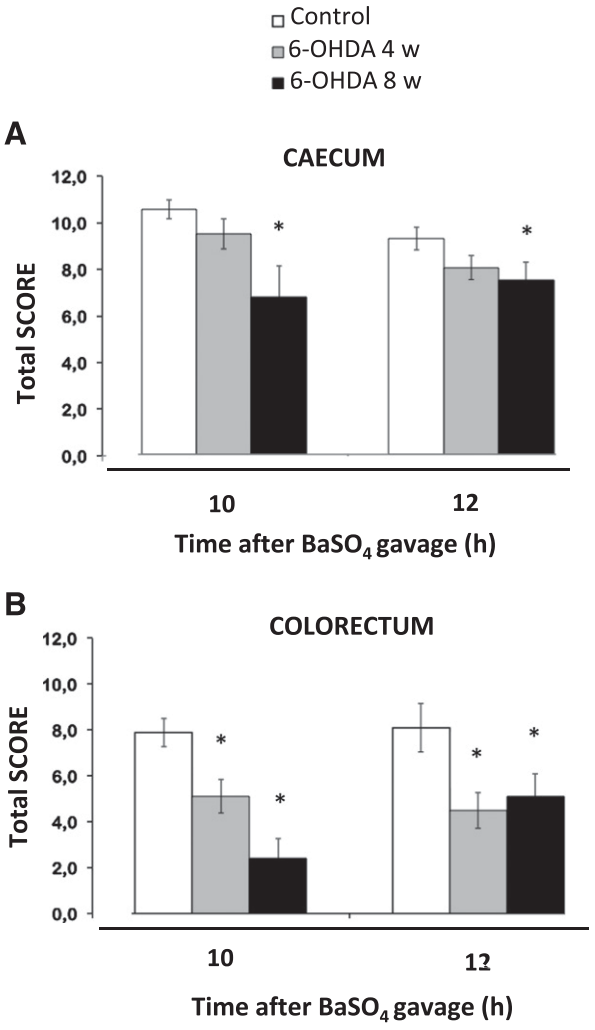

Fig. 2. Radiographic contrast study of large bowel motor function in control rats and animals 4 and 8 weeks after nigrostriatal denervation with 6-OHDA. Total score was assessed 10 and 12 hours after barium sulfate gavage in the cecum (A) and colorectum (B). Each column represents the mean ± S.E.M. score obtained from eight animals. Statistics: Kruskal-Wallis test followed by Dunn's post-test. *$P < 0.05$, significant difference versus control.

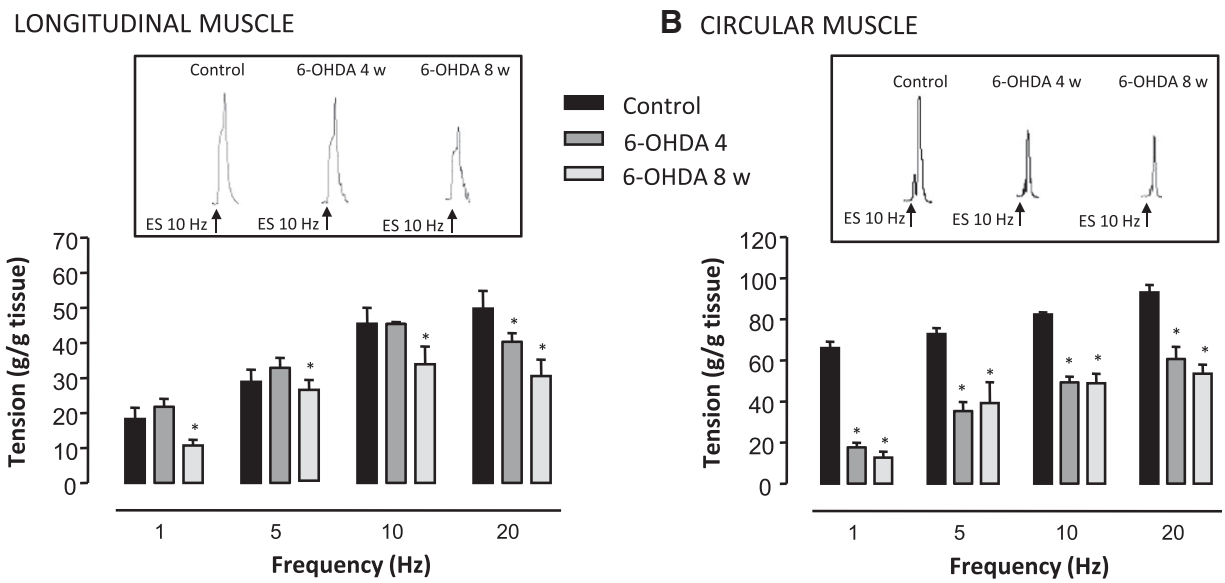

Fig. 3. Effects of ES (1–20 Hz) on the mechanical activity of colonic longitudinal (A) and circular (B) smooth muscle preparations isolated from control animals or rats 4 and 8 weeks after nigrostriatal denervation with 6-OHDA. Colonic preparations were maintained in standard Krebs' solution. Tracings in the inset on the top of each panel display contractile responses to ES recorded at a frequency of 10 Hz. Each column represents the mean ± S.E.M. obtained from eight animals. Statistics: one-way analysis of variance followed by post-hoc analysis with Student-Newman-Keuls test. *$P < 0.05$, significant difference versus control.

evoked acetylcholine release, the variations of acetylcholine concentrations in the bathing fluid upon application of electrical stimulation were calculated for each group as a percentage of the values at the end of the 10-second stimulation period over the baseline values assessed at −60 seconds. Aliquots were stored at −80°C to determine acetylcholine content (Choline/Acetylcholine Assay Kit; Abcam, Cambridge, UK). Acetylcholine release was expressed as choline concentration normalized to the weight of the colonic preparation.

Immunohistochemistry of HuC/D and ChAT. Sections (8 μm) from formalin-fixed full-thickness distal colonic samples were processed for immunostaining, as described by Ippolito et al. (2015). In brief, sections were incubated overnight at 4°C with primary antibodies against the pan-neuronal HuC/D (A-21271; Molecular Probes, Eugene, OR) and ChAT (Mab AP144P; Chemicon, Temecula, CA), and then exposed to biotinylated immunoglobulins, peroxidase-labeled streptavidin complex, and 3.3′-diaminobenzidine tetrahydrochloride (DakoCytomation, Glostrup, Denmark). Five sections for each colonic sample ($n = 6$ animals per group) were examined by a Leica DMRB light microscope, and representative photomicrographs were taken by a DFC480 digital camera (Leica Microsystems, Cambridge, UK) for quantitative evaluation. Neuronal density was estimated as the number of HuC/D-immunostained cells within the ganglionic area. To measure the myenteric ganglionic area, a morphometric analysis was carried out on 10 microscopic fields per section, randomly selected along the myenteric ridge and captured with a 40× objective using the Image Analysis System L.A.S. software version 4.5 (Leica Microsystems); the ganglia were then highlighted and their area was expressed in mm^2. ChAT expression was evaluated as positive pixels on the total ganglionic tissue area examined and expressed as a percentage of positive pixels. Quantitative variations were expressed as fold changes, which were calculated as the ratio of the final value over the initial value.

Isolated Colonic Smooth Muscle Cells. Rat colonic smooth muscle cells were explanted from tunica muscularis, as described by Ippolito et al. (2015). In brief, colonic specimens from controls and 6-OHDA rats at week 4 were washed repeatedly with cold, sterile phosphate-buffered saline, and the muscular layers were separated from the mucosa and submucosa. The specimens of colonic muscular tissue were then minced and incubated in complete Dulbecco's modified Eagle's medium (Gibco, Life Technology Italia, Monza, Italy) under 5% CO_2 at 37°C. Upon confluence, the explants were dissociated by trypsin. Isolated colonic smooth muscle cells (ICSMCs) were then maintained in Dulbecco's modified Eagle's medium added with 10% fetal bovine serum and used until the fifth passage. Care was taken to verify that ICSMCs displayed and maintained a smooth muscle cell phenotype by immunostaining for standard markers (Nair et al., 2011) (data not shown).

Western Blot Analysis of Muscarinic M2 and M3 Receptors in Colonic Tissues and ICSMCs. Colonic specimens were dissected to separate the mucosal/submucosal layer from underlying neuromuscular tissues. Samples of colonic muscular tissue or ICSMCs were homogenized in radioimmunoprecipitation assay lysis buffer (Cole Parmer homogenizer, Milan, Italy). Homogenates were spun by centrifugation at 20,000 rpm for 15 minutes at 4°C. Supernatants were then separated from pellets and stored at −80°C. Protein concentration was determined by the Bradford method (Protein Assay Kit; Bio-Rad, Hercules, CA). Equivalent amounts of protein lysates (50 μg for both tissues and ICSMCs) were separated by 8% SDS-PAGE for immunoblotting. After transfer onto a polyvinylidene fluoride membrane, the blots were blocked and incubated overnight with a rabbit anti-M2 antibody (MR002; Alomone Laboratories, Jerusalem, Israel) or a rabbit anti-M3 antibody (87199; Abcam). After repeated washings with Tris-Buffered saline and Tween 20 buffer, appropriate secondary peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added for 1 hour at room temperature. Immunoreactive bands were then visualized by incubation with chemiluminescent reagents (Immobilon reagent; Millipore, Billerica, MA) and examined by Kodak Image Station 440 (Celbio, Milan, Italy) for signal detection. To ensure equal sample loading, blots were stripped and reprobed for determination of β-actin by a specific antibody (P5747; Sigma-Aldrich, Milan, Italy).

Drugs and Solutions. Atropine sulfate, guanethidine monosulfate, carbachol chloride, N^{ω}-nitro-L-arginine methylester, 6-hydroxy dopamine, and ascorbic acid were purchased from Sigma Chemicals Co. (St. Louis, MO). Tetrodotoxin, L-732,138, GR159897, and SB218795 were purchased from Tocris (Bristol, UK). Mouse anti-TH antibody was purchased from Chemicon International. Biotinylated anti-mouse IgG antibody and nickel-intensified 3,3′-diaminobenzidine tetra-hydrochloride (DAB Substrate Kit for Peroxidase) were purchased

TABLE 1

Effects of atropine (1 μM) on electrically and carbachol-evoked cholinergic mechanical responses in isolated colonic tissues

ES-evoked cholinergic responses were recorded in tissues maintained in Krebs' solution containing L-NAME (100 μM), guanethidine (10 μM), L-732,138 (10 μM), GR159897 (1 μM), and SB218795 (1 μM). Carbachol-induced motor responses were recorded in tissues maintained in Krebs' solution containing tetrodotoxin (1 μM). The effects of atropine were expressed as percentage of reductions versus contractions recorded in the absence of atropine. Each number represents the mean ± S.E.M. value obtained from five to six animals (statistics: one-way analysis of variance followed by post-hoc analysis with Student-Newman-Keuls test).

Colonic Layer	Percentage of Reduction of Contractile Response to ES at 10 Hz			Percentage of Reduction of Contractile Response to 1 μM Carbachol		
	Control	6-OHDA, 4 Weeks	6-OHDA, 8 Weeks	Control	6-OHDA, 4 Weeks	6-OHDA, 8 Weeks
Longitudinal	93.4 ± 4.1	96.2 ± 5.6	98.7 ± 6.9	96.5 ± 8.1	89.9 ± 6.4	98.8 ± 4.9
Circular	97.0 ± 3.8	91.1 ± 5.5	99.2 ± 5.8	91.9 ± 6.0	99.7 ± 5.5	95.9 ± 7.1

from Vector Laboratories (Burlingame, CA). Xylene was purchased from Carlo Erba.

Statistical Analysis. The results are presented as the mean ± S.E.M. unless otherwise stated. The significance of differences was evaluated by Student's t test for paired or unpaired data or one-way analysis of variance followed by post-hoc analysis with Student-Newman-Keuls or Bonferroni tests. Data regarding colonic transit time were analyzed by Kruskal-Wallis test followed by Dunn's post-test. P values <0.05 were considered significantly different. All statistical procedures were performed by commercial software (GraphPad Prism, version 3.0; GraphPad Software Inc., San Diego, CA).

Results

Immunohistochemical Analysis of TH in Brain. The unilateral injection of 6-OHDA into the MFB caused a virtually complete loss of dopaminergic striatal terminals (98%) and dopaminergic nigral neurons (95%) of the right (injected) hemisphere, both at week 4 and at week 8. Animals bearing lesions of less than 98% in the striatum and 95% in the SNc were excluded from the study. Sham-operated rats did not display differences in TH immunoreactivity between hemispheres both at week 4 and at week 8 (Fig. 1).

In Vivo Colonic Transit. Radiologic findings in the cecum and colorectum of control and 6-OHDA rats were compared and scored at weeks 4 and 8 from the induction of nigrostriatal denervation (n = 8 animals for each treatment group). Both 10 and 12 hours after gavage with $BaSO_4$, the scores of control and 6-OHDA rats, calculated for cecum radiographs, were higher than colorectal values, indicating that, at those times, the medium had reached both the more proximal portion of the large bowel (cecum radiographs) and the more distal region of the large intestine (colorectal radiographs) (Fig. 2).

In both cecal and colorectal areas, total scores estimated for 6-OHDA animals were lower than those estimated for controls. In particular, after 8 weeks from 6-OHDA injection, a significant reduction of total scores was obtained in both the cecum and colorectum (Fig. 2).

In Vitro Colonic Contractile Activity. In colonic longitudinal muscle preparations (from 6-OHDA rats) maintained in standard Krebs' solution, electrically evoked motor

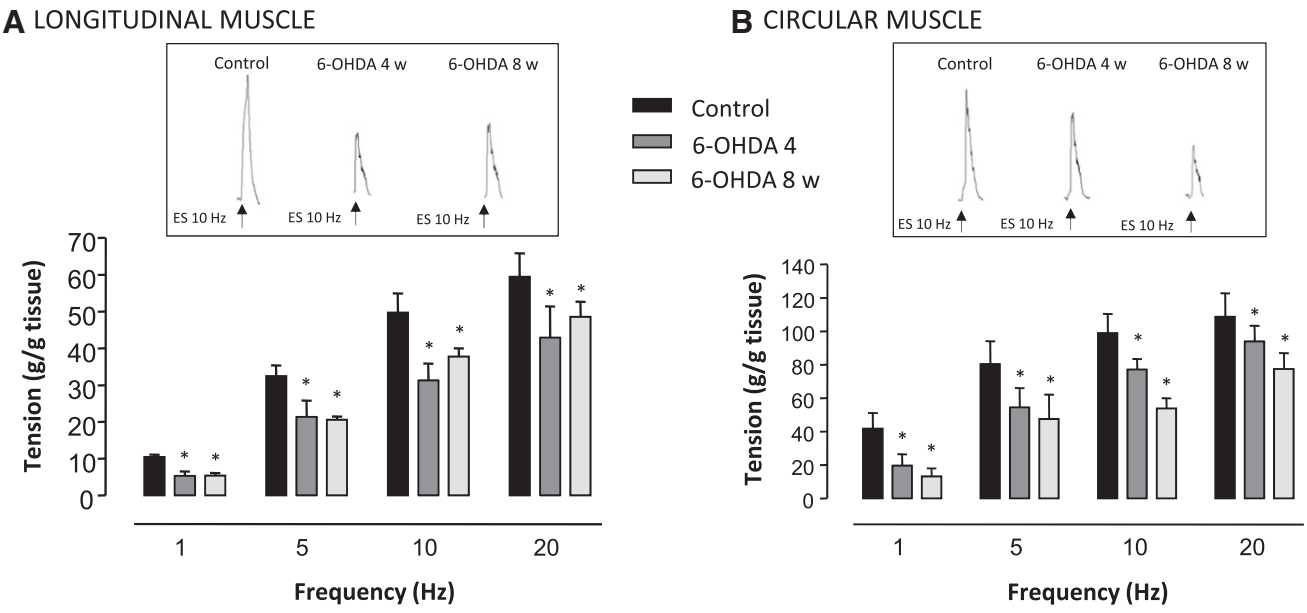

Fig. 4. Effects of ES (1–20 Hz) on the mechanical activity of colonic longitudinal (A) and circular (B) smooth muscle preparations isolated from control animals or rats 4 and 8 weeks after nigrostriatal denervation with 6-OHDA. Colonic preparations were maintained in Krebs' solution containing L-NAME (100 μM), guanethidine (10 μM), L-732,138 (10 μM), GR159897 (1 μM), and SB218795 (1 μM) to record cholinergic contractions. Tracings in the inset on the top of each panel display contractile responses to ES recorded at a frequency of 10 Hz. Each column represents the mean ± S.E.M. obtained from eight animals. Statistics: one-way analysis of variance followed by post-hoc analysis with Student-Newman-Keuls test. *$P < 0.05$, significant difference versus control.

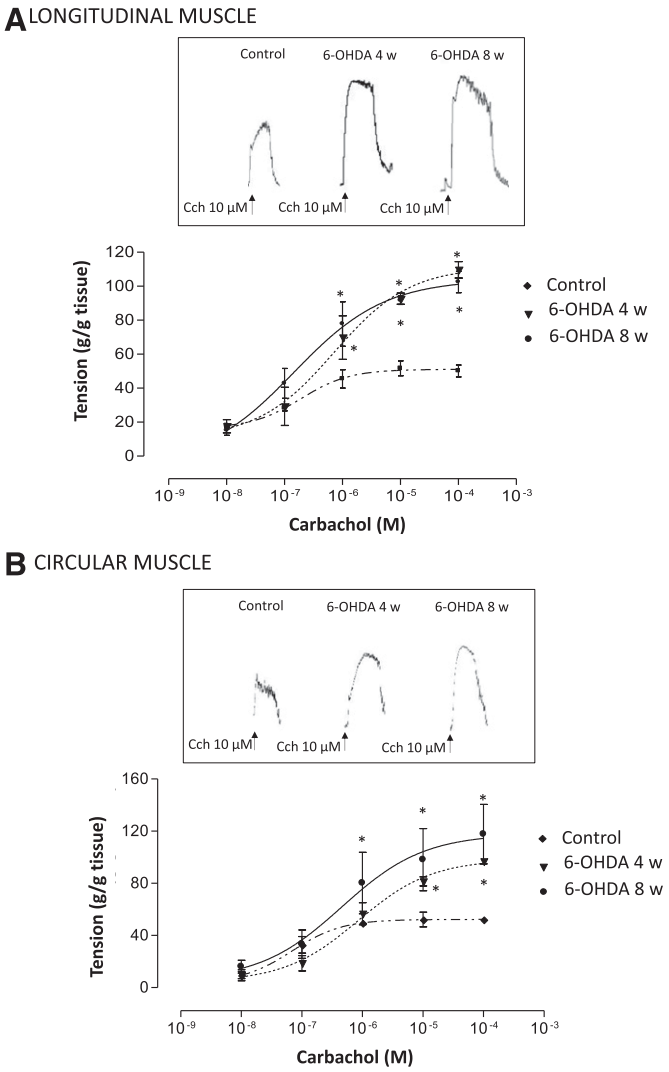

Fig. 5. Effects of increasing concentrations of carbachol (Cch; 0.01–100 μM) on the contractile activity of colonic longitudinal (A) and circular (B) smooth muscle preparations isolated from control animals or rats 4 and 8 weeks after nigrostriatal denervation with 6-OHDA. Colonic preparations were maintained in Krebs' solution containing tetrodotoxin (1 μM). Tracings in the inset on the top of each panel display contractile responses to carbachol at a concentration of 10 μM. Each point represents the mean ± S.E.M. value obtained from eight animals. Statistics: one-way analysis of variance followed by post-hoc analysis with Student-Newman-Keuls test. *$P < 0.05$, significant difference versus control.

responses were decreased both at week 4 (significant difference at 20 Hz) and at week 8 (significant difference at all tested frequencies) after 6-OHDA injection, as compared with contractions recorded from control preparations ($n = 8$ animals for each treatment group) (Fig. 3A). Likewise, in colonic circular muscle preparations from 6-OHDA rats after 4 or 8 weeks ($n = 8$ animals for each treatment group), the electrically evoked contractile activity was significantly reduced at all tested frequencies (Fig. 3B).

In colonic longitudinal and circular muscle preparations maintained in Krebs' solution containing L-NAME, guanethidine, and NK receptor antagonists, the application of electrical stimulation elicited contractile responses, which were abolished by atropine (Table 1) or tetrodotoxin, while being slightly affected by hexamethonium, indicating the recruitment of postganglionic cholinergic motor neurons (not shown). Under these conditions, electrically evoked cholinergic contractions were decreased at both weeks 4 and 8 in comparison with controls ($n = 8$ animals for each treatment group) (Fig. 4, A and B).

The exposure of colonic longitudinal and circular muscle preparations to carbachol (0.001–100 μM), in the presence of tretrodotoxin (1 μM), elicited concentration-dependent contractions (Table 1), which were abolished by atropine and were significantly enhanced in preparations from rats at both 4 and 8 weeks after 6-OHDA injection, as compared with control ($n = 8$ animals for each treatment group) (Fig. 5, A and B).

Acetylcholine Release. Acetylcholine concentrations in aliquots of Krebs' solution collected upon ES were almost suppressed by incubation with tetrodotoxin (1 μM). In aliquots of medium collected under resting conditions, acetylcholine concentrations, assessed for colonic longitudinal muscle preparations from 6-OHDA rats, were lower at both week 4 and week 8, as compared with controls (Fig. 6A). Upon exposure of control colonic strips to ES, acetylcholine release into Krebs' solution increased by +50% versus the baseline value assessed at −60 seconds, whereas in preparations from 6-OHDA animals, the percentage of evoked acetylcholine release was lower as compared with controls (+30% versus baseline at week 4 and +28% versus baseline at week 8, respectively; $n = 8$ animals for each treatment group) (Fig. 6B).

Immunohistochemical Analysis of HuC/D and ChAT. Preliminarily to neuronal counting, the area of myenteric ganglia was quantitatively estimated, and no significant changes were found among the experimental groups, as shown by the following mean values: controls, $2.82 \pm 0.38 \times 10^{-3}$ mm^2; 6-OHDA rats at week 4, $2.75 \pm 0.29 \times 10^{-3}$ mm^2; 6-OHDA rats at week 8, $2.44 \pm 0.25 \times 10^{-3}$ mm^2. With regard to neurons, strong cytoplasmic and/or nuclear HuC/D immunostaining was detected in myenteric neurons of distal colons from controls and rats with 6-OHDA–induced nigrostriatal denervation. The total number of HuC/D immunoreactive myenteric neurons did not change in 6-OHDA rats at both week 4 and week 8, as compared with controls. By contrast, a significant decrease in ChAT immunopositivity was detected in the myenteric ganglia of 6-OHDA rats at both weeks 4 and 8 (−61.0 and −36.1% versus controls, respectively; $n = 6$ animals for each group of treatment) (Fig. 7).

Western Blot Analysis of Muscarinic M2 and M3 Receptor Expression. Western blot analysis revealed the constitutive expression of both muscarinic M2 and M3 receptors in colonic neuromuscular tissues from control rats (Fig. 8A). In colonic tissues obtained from rats at weeks 4 and 8 after treatment with 6-OHDA, a significant increase in the expression of both receptor subtypes was detected ($n = 5$–6 animals for each treatment group) (Fig. 8A). In ICSMCs from control rats, western blot analysis confirmed the basal expression of both muscarinic M2 and M3 receptors. At week 4 after nigrostriatal denervation by 6-OHDA, the expression of both receptor subtypes in ICSMCs significantly increased ($n =$ 5–6 animals for each treatment group) (Fig. 8B).

Discussion

PD is associated with alterations of gut motor functions (Cloud and Greene, 2011; Pfeiffer, 2011; Pellegrini et al., 2015), which have been proposed to result both from an early

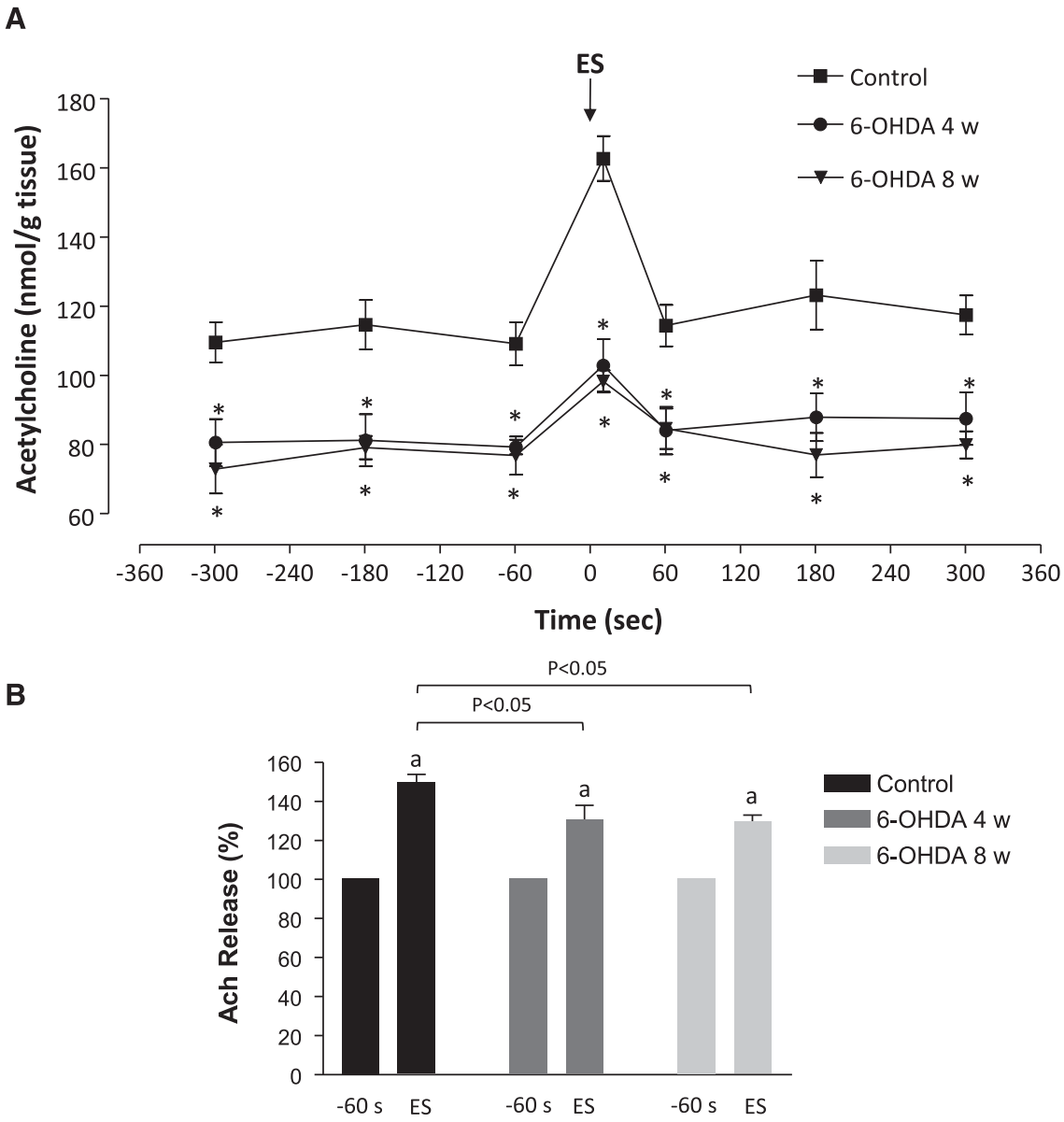

Fig. 6. (A) Acetylcholine content in aliquots of Krebs' solution incubating colonic longitudinal muscle preparations from control animals or rats after 4 and 8 weeks from nigrostriatal denervation with 6-OHDA. Aliquots were collected at −300, −180, −60, +60, +180, and +300 seconds with respect to the onset of ES (10 Hz). One additional aliquot was collected at the end of the 10-second period of ES application to evaluate the electrically induced acetylcholine release. Statistics: one-way analysis of variance followed by post-hoc analysis with Student-Newman-Keuls test. $^{*}P < 0.05$, significant difference versus control values. (B) Percent increments of acetylcholine levels in response to ES, calculated over the respective values assessed at −60 seconds, in Krebs' solution incubating longitudinal muscle preparations from control animals or rats 4 and 8 weeks after nigrostriatal denervation with 6-OHDA. Each column represents the mean ± S.E.M. obtained from eight animals. Statistics: Student's *t* test for paired data or one-way analysis of variance followed by Bonferroni's post-test. $^{a}P < 0.05$ versus the respective value at −60 seconds. Ach, acetylcholine.

impairment of the ENS and as a consequence of central nigrostriatal degeneration associated with dopaminergic denervation. In this context, our purpose was to evaluate the impact of nigrostriatal dopaminergic denervation on the patterns of colonic motility and related cholinergic control. Indeed, current data on the abnormalities of colonic cholinergic neuromotor control in PD are scarce and inconsistent. Thus, we aimed at characterizing the alterations occurring in the 6-OHDA model by a multidisciplinary functional, molecular, and morphologic approach. Overall, our results provide convincing evidence that the induction of nigrostriatal denervation, which reflects one of the main pathologic hallmarks of PD, is associated with significant alterations of colonic excitatory cholinergic neurotransmission, resulting in abnormal patterns of in vivo transit and in vitro contractility.

The radiologic analysis documented that 4 weeks after neurotoxin injection, the rats displayed a cecum total score comparable to control animals, whereas colorectum score values were lower than those of controls, suggesting a delay in the transit of contrast medium within the large bowel. A further delay was observed in rats 8 weeks after 6-OHDA injection. In this case, the cecum total score was significantly lower than in control animals, indicating a slow transit along the small intestine. However, in these animals, the colorectum score calculated 12 hours after barium gavage was higher than after 10 hours, indicating a delay in colorectum filling. These

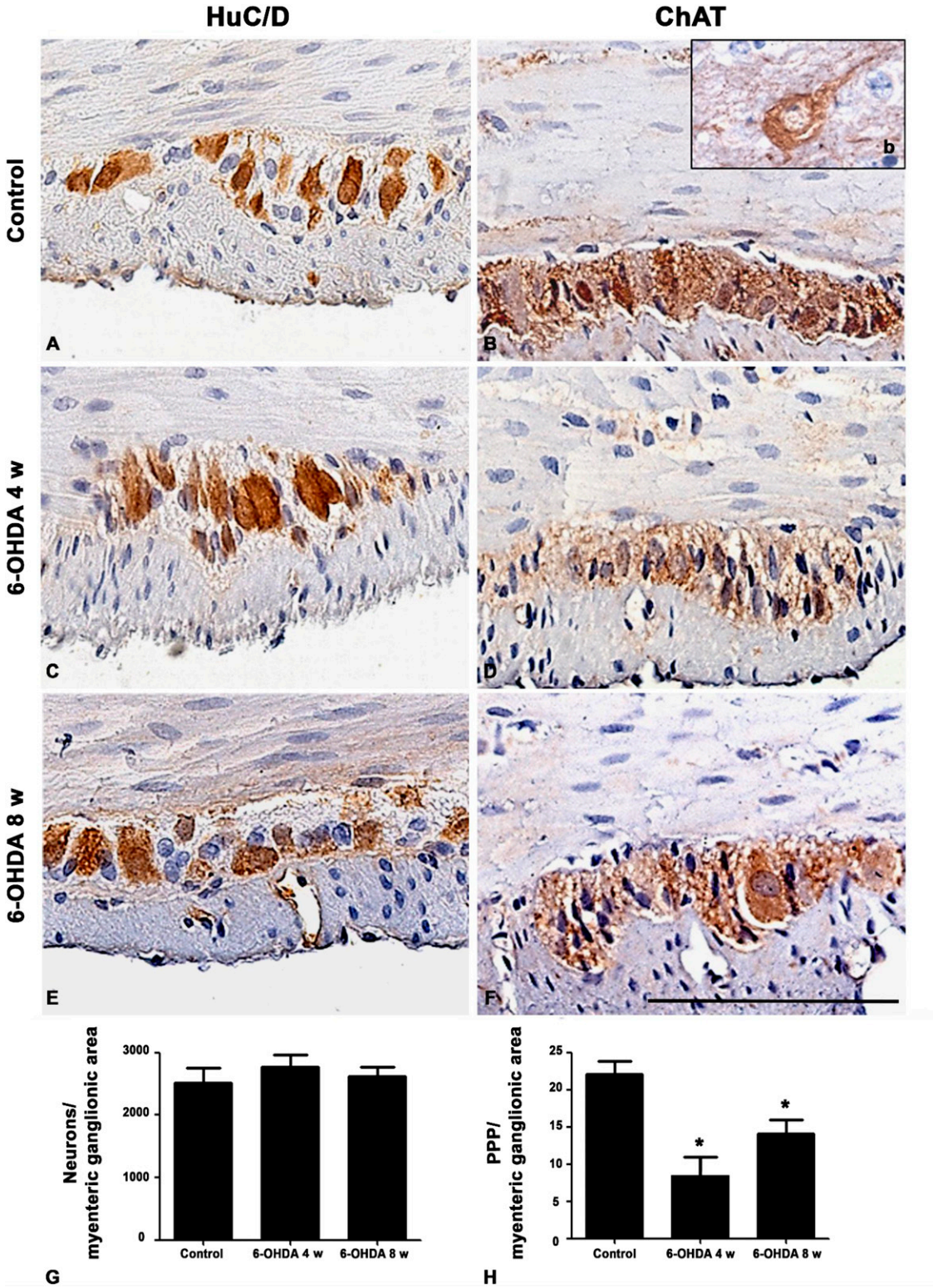

Fig. 7. Representative pictures of HuC/D and ChAT immunostaining of rat colonic specimens. Myenteric ganglia from control and 6-OHDA rats show HuC/D immunoreactive neurons (A, C, and E) without changes in neuron density (G). Myenteric neurons of control colons contain abundant amounts of ChAT staining, which is significantly decreased in 6-OHDA rats (B, D, F, and H). ChAT immunostaining was validated in the rat central nervous system, which is regarded as a positive control tissue (b). Scale bar = 100 μm. (G and H) The column graphs display the mean values of neuron density (neurons/mm^2) ± S.E.M. obtained from six animals. Statistics: one-way analysis of variance followed by post-hoc analysis with Student-Newman-Keuls test. *$P < 0.05$ versus controls.

findings suggest that the model of 6-OHDA–induced nigrostriatal degeneration is suitable for the assessment of bowel dysmotility associated with PD. Consistently, evidence indicates that a decreased rate of bowel movements and severe constipation represent the most widely recognized clinical signs of enteric dysfunction in PD patients (Pfeiffer, 2011). In

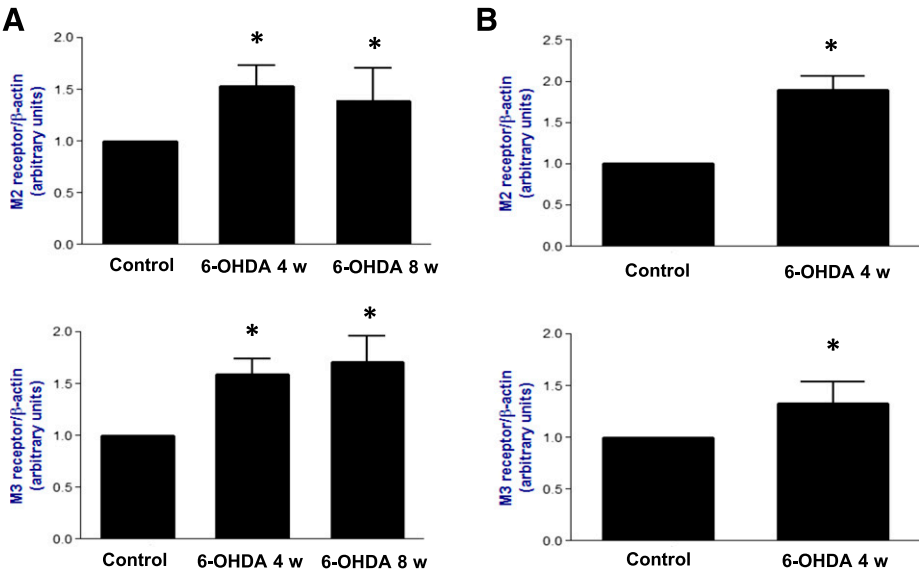

Fig. 8. Western blot analysis of muscarinic M2 and M3 receptors in the colonic neuromuscular layer (A) and ICSMCs (B) from control rats and animals 4 and 8 weeks after nigrostriatal denervation with 6-OHDA. Each column represents the mean ± S.E.M. value obtained from five to six animals. Statistics: Student's *t* test for unpaired data or one-way analysis of variance followed by post-hoc analysis with Student-Newman-Keuls test. $^*P < 0.05$, significant difference versus control.

addition, our in vivo results are in keeping with previous data, showing a reduced efficiency of in vitro peristalsis in colonic preparations from rats with 6-OHDA–induced nigrostriatal denervation (Colucci et al., 2012).

To verify whether the changes of in vivo propulsive colonic motility might depend on underlying alterations of enteric neurotransmission, we focused on the patterns of in vitro excitatory cholinergic motor activity. Our results showed that electrically evoked cholinergic contractions of colonic muscle from 6-OHDA rats were decreased, indicating an altered excitatory control of colonic motility. These findings provide the first demonstration that central nigrostriatal denervation is associated with a significant impairment of excitatory cholinergic motility in the large bowel, and they add new knowledge to the pathophysiological mechanisms underlying the occurrence of intestinal alterations in PD (Zhu et al., 2012). We then went on to assess whether these motor abnormalities could depend on changes in the density of myenteric nerves. To pursue this aim, we carried out immunohistochemical assays in which myenteric ganglia were labeled with the neuronal marker HuC/D and ChAT, a specific marker of cholinergic neurons. In these experiments, colonic tissues from 6-OHDA rats displayed a significant decrease in immunopositivity for ChAT, whereas the overall density of HuC/D+ myenteric neurons did not vary, suggesting that nigrostriatal denervation leads to a reduced expression of ChAT in myenteric cholinergic neurons, likely resulting in an impairment of colonic cholinergic neurotransmission. Of note, Toti and Travagli (2014) recently described a reduced density of ChAT+ myenteric neurons in the upper GI tract, without a concomitant variation of total neuronal density in 6-OHDA rats. In line with this picture, evidence of unchanged overall density of myenteric neurons was previously obtained in patients with PD, suggesting that PD-related GI dysmotility is not associated with a loss of neurons in the myenteric plexus but rather with alterations in the chemical coding of specific enteric neurons (Annerino et al., 2012).

To test the hypothesis that the decrease in colonic ChAT would translate into a hampered enteric cholinergic neurotransmission, we assessed the levels of acetylcholine released from in vitro colonic preparations into incubation medium. Our results showed that, in 6-OHDA rats, the acetylcholine output from colonic neuromuscular strips was significantly decreased (both under basal conditions and in response to electrical stimulation), as compared with controls. Therefore, it appears that the decrease in myenteric ChAT expression, which follows central nigrostriatal denervation, results in an impairment of acetylcholine release from colonic myenteric neurons. In line with this view, evidence of altered cholinergic neurotransmission has been previously observed in the stomach of 6-OHDA rats where nigrostriatal denervation led to a reduced acetylcholine content in the muscularis externa and a significant delay in gastric emptying (Zheng et al., 2011). In addition, a correlation between the amount of acetylcholine released from myenteric neurons and the magnitude of electrically evoked contractions has been previously demonstrated in isolated distal colonic preparations from rats with experimental colitis (Poli et al., 2001). Therefore, taken together with a reduced ChAT localization in myenteric ganglionic neurons (as observed in our experiments), all of these findings suggest that the impairment of colonic cholinergic motility, following central 6-OHDA–induced denervation, depends on an impairment of acetylcholine release from postjunctional myenteric motor neurons. However, the possibility that the central dopaminergic neurodegeneration could also affect other classes of myenteric cholinergic neurons (i.e., intrinsic primary afferent neurons and/or interneurons) cannot be ruled out.

In addition to an impaired neurogenic cholinergic motor activity in the colon of 6-OHDA rats, we also observed an enhancement of colonic myogenic responses elicited by direct activation of muscarinic receptors with carbachol. Based on our results, supporting a decrease in the release of acetylcholine from myenteric cholinergic neurons of 6-OHDA animals, we hypothesized that this finding resulted from an upregulation of muscular muscarinic receptors occurring as a compensatory response to the impairment of cholinergic neurotransmission. To address this issue, we examined the expression of muscarinic M2 and M3 receptors in specimens of the colonic neuromuscular layer as well as in ICSMCs by western blot assays, and found that both receptor subtypes were indeed upregulated in the colon of 6-OHDA rats. Of

note, compensatory increments of muscarinic receptor density, as a consequence of cholinergic denervation, have been previously described in the colons of patients with diverticular disease, where cholinergic denervation and related motor abnormalities of isolated colonic muscle were associated with an upregulation of muscular muscarinic M3 receptors (Golder et al., 2003). Overall, it is conceivable that the lowering of colonic transit in 6-OHDA rats depends, at least in part, on the impairment of cholinergic enteric neurotransmission.

In addition to the alterations of the cholinergic pathway observed in the present study, it has been previously appreciated that central dopaminergic neurodegeneration could affect different neuromotor systems. For instance, Colucci et al. (2012) observed a significant increase in vasoactive intestinal polypeptide levels and a concomitant decrease in neuronal nitric oxide synthase expression in the myenteric plexus of the distal ileum and proximal colon of 6-OHDA rats. In addition, Levandis et al. (2015) recently found that rats with central denervation induced by 6-OHDA displayed an altered colonic dopaminergic motor control, characterized by a loss of inhibitory effects mediated by dopamine D2 receptors on peristalsis, along with a reduced receptor expression and increased dopamine levels. Therefore, the overall current knowledge supports the notion that colonic dysmotility associated with central dopaminergic denervation results from alterations occurring at the level of different neuromotor pathways.

The changes in colonic cholinergic neurotransmission, as highlighted by the present investigations, lend further support to the available knowledge about the existence of a close link between brain and gut. In this regard, increasing evidence suggests that the DMV, which is known to provide most of the parasympathetic innervation to the GI tract (Jellinger, 1987), is one of the central nervous system sites affected by PD pathology at its early stage (Del Tredici et al., 2002). Indeed, neurochemical changes affecting the ENS, after central dopaminergic denervation, have been shown to depend on alterations of the DMV, which is regulated by brainstem dopaminergic circuitries and represents a prominent target of PD-related neurodegenerative processes (Braak et al., 2003, 2004; Zheng and Travagli, 2007; Zheng et al., 2011). In this regard, the vagus nerve has been proposed to play a crucial role in the regulation of inflammatory responses, a function also referred to as the "cholinergic anti-inflammatory pathway" (Matteoli and Boeckxstaens, 2013). In particular, there is evidence that the vagus nerve exerts tonic anti-inflammatory actions and contributes to the maintenance of intestinal homeostasis (Matteoli and Boeckxstaens, 2013), whereas vagotomy confers an increased susceptibility to the development of inflammatory bowel diseases (Ghia et al., 2006). In addition, Toti and Travagli (2014) recently found a reduced expression of ChAT in the DMV of rats with 6-OHDA–induced nigrostriatal denervation. Based on this knowledge, it is conceivable that 6-OHDA–induced nigrostriatal denervation might impair the DMV–vagus nerve anti-inflammatory pathway, and that this alteration might result in a condition of mild chronic bowel inflammation, leading to persistent dysfunctions in the enteric neuromuscular compartment. In keeping with this hypothesis, in preliminary experiments, we found that the levels of two inflammatory parameters (tumor necrosis factor and malondialdehyde), were increased in colonic tissues from 6-OHDA rats, thus suggesting that experimental nigrostriatal denervation is associated with inflammatory activity and related oxidative stress in the colonic wall (M. Fornai, unpublished data). Of interest, our preliminary observations are consistent with the findings of a previous study, which showed an increase in proinflammatory cytokine levels and markers of glial cell activation in colonic biopsies from PD patients (Devos et al., 2013). However, the possible link between DMV–vagus nerve impairment, bowel inflammation, and development of colonic dysmotility in the setting of nigrostriatal dopaminergic neurodegeneration requires further confirmation by means of specific experimental approaches, which could represent a logical continuation of the ongoing research on this topic.

In conclusion, our results indicate that central nigrostriatal dopaminergic denervation is associated with an impaired excitatory neurotransmission characterized by a loss of myenteric neuronal ChAT positivity and a decrease in acetylcholine release, resulting in a dysregulated smooth muscle motor activity, which is likely to contribute to the concomitant decrease in colonic transit. Overall, the present findings provide a translational basis for better understanding the mechanisms underlying bowel dysfunctions in PD patients.

Authorship Contributions

Participated in research design: Fornai, Colucci, Blandizzi, Barocelli, Ballabeni, Blandini, Bernardini.

Conducted experiments: Fornai, Antonioli, Colucci, Pellegrini, Vegezzi, Al Harraq, Segnani, Ippolito, Levandis, Cerri.

Contributed new reagents or analytic tools: Levandis, Cerri, Vegezzi, Al Harraq.

Performed data analysis: Antonioli, Blandizzi, Segnani, Ballabeni, Blandini.

Wrote or contributed to the writing of the manuscript: Fornai, Blandizzi, Bernardini, Pellegrini, Ippolito, Barocelli.

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