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Abbreviations: PD, Parkinson's disease; 6-OHDA, 6-hydroxydopamine; ChAT, choline acetyltransferase; MDA, malondialdehyde; TNF, tumor necrosis factor; DMV, dorsal motor nucleus of the vagus; SNpc, substantia nigra pars compacta; PPP, percentage of positive pixels; ICSMCs, isolated colonic smooth muscle cells; ES, electrical stimuli; MFB, medial forebrain bundle; GI, gastrointestinal

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Abstract

Parkinson's disease (PD) 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 radiological assay. Electrically and carbachol-induced cholinergic contractions were recorded *in vitro* from longitudinal and circular muscle colonic preparations, while acetylcholine levels were assayed in their incubation media. Choline acetyltransferase (ChAT), HuC/D (pan-neuronal marker), muscarinic M2 and M3 receptors. 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; increased expression of colonic muscarinic M2 and M3 receptors. The majority of such alterations were detected also 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 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 the hypothesis 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 MPTP (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; 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, neurotransmitters 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 alpha 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 an overexpression of human α-synuclein in colonic myenteric ganglia (Wang et al., 2008; Wang et al., 2012). However, as no consistent nigrostriatal degeneration is associated with synuclein overexpression in these animals, at least in their 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 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), while, in the same model, a decreased acetylcholine content was found in the muscular layer of rat stomach (Zheng et al., 2011a). 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, while the evaluation of cholinergic neuron density and muscarinic receptor expression in the colonic neuromuscular layer were carried out by means of molecular approaches.

Methods

Animals

Male Sprague-Dawley rats, 200–250 g body weight, were used throughout the study. The animals were fed standard laboratory chow and tap water *ad libitum* and were not employed for at least one week after their delivery to the laboratory. They were housed, three in a cage, in temperature-controlled rooms on a 12-h 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 of sodium thiopental (i.p.) and placed into a stereotaxic frame (Stoelting, Wood Dale, IL, USA). 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 (mm) relative to bregma and dural surface: (i) AP= -4.0, ML= -0.8, DV= -8.0 (9 µg /3 µL); (ii) AP= -4.4, ML= -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 min to avoid leaks. At the end of the process, wounds were clipped and the animal 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, while colonic specimens were dissected and processed for functional experiments and other assays as described below.

Immunohistochemistry of tyrosine hydroxylase in brain sections

Serial coronal sections (40 μm), including striatum and substantia nigra pars compacta (SNpc) 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 SNpc, as previously described (Blandini et al., 2004). Briefly, sections containing the striatum and SNpc were postfixed in cold, 4% neutral buffered formaldehyde (NBF; 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 min at room temperature. Sections were incubated overnight at 4°C with a mouse anti-TH antibody (1:2000; Chemicon International, Temecula, CA, USA), then rinsed in TBS and incubated for 60 min at room temperature, with a goat biotinylated anti-mouse IgG antibody (1:1000; Vector Laboratories, Burlingame, CA, USA). 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 tetra-hydrochloride (DAB Substrate Kit for Peroxidase, Vector Laboratories). After rinsing with TBS, sections were dehydrated in ascending alcohol concentrations, cleared in xylene (Carlo Erba, Milan, Italy) 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 brain of sham-operated animals.

The total number of dopaminergic cells in SNc of both hemispheres was counted using stereological analysis. Unbiased stereological estimation was made using the optical fractionator method (West et al., 1991) by the STEREO INVESTIGATOR software on a Neurolucida computer-controlled microscopy system (Microbrightfield Inc., VT, USA). The boundaries of SNc at all levels in the rostro-caudal 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 x 75 μm) were placed at the intersections of a grid (frame size 208,65 x 161,6 μm) that had been randomly placed over the section. Cells were marked 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% as cut-off level for the striatal lesion and 95% for the lesion in the SNc.

Radiological assessment of colonic transit

The radiological assessment of overall *in vivo* colonic transit was performed as previously described (Vegezzi et al., 2014). Briefly, after 4 and 8 weeks from 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 h later. This time frame was previously shown to allow the detection of contrast medium in radiographs of the caecal and colorectal regions. Focus-film distance was manually fixed at 100 cm and exposure values were 65 kVp – 4.5 mAs (exposure time: 0.01 sec). Total body dorso-ventral (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 labelled caecum and colorectum, the intensity of labelling, the organ profile, and the homogeneity of labelling 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 8 rats per group.

Recording of colonic contractile activity in vitro

Contractile activity of colonic longitudinal 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 distal colon, of approximately 3 mm width and 20 mm length, 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, Besozzo, VA, Italy). Krebs solution had the following composition (mM): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.5 (pH 7.4 ± 0.1). Each preparation was allowed to equilibrate for at least 30 min, with intervening washings at 10-min intervals. A pair of coaxial platinum electrodes was 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 were applied as follows: 10-s single trains (ES), consisting of square wave pulses (0.5 ms, 30 mA). At 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 2 or 3 stimulations). Frequency–response curves (from 1 to 20 Hz) were constructed under the

different *in vitro* experimental conditions reported below. 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, 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), in order 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 (TTX, 1 μM) and stimulated with carbachol (CCh, 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 above. After equilibration, aliquots of Krebs solution (200 μL) were collected at -300, -180, -60, +60, +180 and +300 s with respect to the onset of ES (0.5 ms, 30 mA, 10 Hz). At the end of the 10-s period of ES application, one additional aliquot was collected, in order to evaluate the amount of electrically induced acetylcholine release, as

previously described by Yajima et al., (2011). In order to better appreciate the alterations of electrically evoked acetylcholine release, the variations of acetylcholine concentrations in the bathing fluid upon application of electrical stimulation were calculated for each group as percentage of the values at end of the 10-s stimulation period over the baseline values assessed at -60 s. Aliquots were stored at -80°C, in order to determine acetylcholine content (Choline/Acetylcholine Assay Kit, Abcam). Acetylcholine release was expressed as choline concentration normalized to the weight of 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). Briefly, sections were incubated overnight at 4°C with primary antibodies against the pan-neuronal HuC/D (A-21271, Molecular Probes, Eugene, USA) and ChAT (Mab AP144P, Chemicon, Temecula, USA), 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 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 40X objective using the Image Analysis System 'L.A.S. software v4.5' (Leica Microsystems, Cambridge, UK): 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 percentage of positive pixels [PPP]. 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 (SMCs) were explanted from tunica muscularis, as described by Ippolito et al. (2015) Briefly, colonic specimens from controls and 6-OHDA rats at week 4 were washed repeatedly with cold, sterile PBS, and the muscular layers were separated from mucosa and submucosa. The specimens of colonic muscular tissue were then minced and incubated in complete DMEM growth 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 DMEM 10% foetal bovine serum and used until the fifth passage. Care was taken to verify that ICSMCs displayed and maintained a SMC 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 RIPA lysis buffer (Cole Palmer homogenizer). Homogenates were spun by centrifugation at 20,000 r.p.m. for 15 min 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, USA). Equivalent amounts of protein lysates (50 µg for both tissues and ICSMCs) were separated by 8% SDS-PAGE for immunoblotting. After transfer onto a PVDF membrane, the blots were blocked and incubated overnight with a rabbit anti-M2 antibody (MR002; Alomone Labs; Jerusalem, Israel) or a rabbit anti-M3 (87199; ABCAM; Cambridge, UK) antibody. After repeated washings with TBS-T, appropriate secondary peroxidase-conjugated antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) were added for 1 hr at room temperature. Immunoreactive bands were then visualized by incubation with

chemiluminescent reagents (Immobilon reagent; Millipore, Billerica, MA, USA), and examined by Kodak Image Station 440 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 sulphate, guanethidine monosulphate, carbachol chloride, N^{ω}-nitro-L-arginine methylester, 6-hydroxy dopamine and ascorbic acid were purchased from Sigma Chemicals Co., St. Louis, MO, USA. Tetrodotoxin, L-732,138, GR159897 and SB218795 were purchased from Tocris, Bristol, UK. Mouse anti-TH antibody was purchased from Chemicon International, Temecula, CA, USA. Biotinylated anti-mouse IgG antibody and nickel-intensified 3,3′-diaminobenzidine tetra-hydrochloride (DAB Substrate Kit for Peroxidase) were purchased from Vector Laboratories, Burlingame, CA, USA. Xylene was purchased from Carlo Erba, Milan, Italy.

Statistical analysis

The results are presented as mean ± SEM unless otherwise stated. The significance of differences was evaluated by Student t test for paired or unpaired data or one-way analysis of variance (ANOVA) 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 from GraphPad Software Inc., San Diego, CA, USA).

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 8. Animals with lower percentage of lesion were excluded from the study. 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 immuno-reactivity between hemispheres, both at week 4 and 8 (Figure 1).

In vivo colonic transit

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

In vitro colonic contractile activity

In colonic longitudinal muscle preparations from 6-OHDA rats, maintained in standard Krebs solution, electrically evoked motor responses were decreased both at week 4 (significant

difference at 20 Hz) and 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 group of treatment) (Figure 3A). Likewise, in colonic circular muscle preparations from 6-OHDA rats after 4 or 8 weeks (n=8 animals for each group of treatment), the electrically evoked contractile activity was significantly reduced at all tested frequencies (Figure 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 week 4 and 8, in comparison with controls (n=8 animals for each group of treatment) (Fig. 4A, 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 atropine-sensitive contractions (Table 1), which were abolished by atropine, which were significantly enhanced in preparations from rats at both 4 and 8 weeks after 6-OHDA injection, as compared to control (n=8 animals for each group of treatment) (Fig. 5A, 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 8, as compared with controls (Figure 6A). Upon exposure of control colonic strips to ES, acetylcholine release into Krebs

solution increased by +50% versus the baseline value assessed at -60 s, whereas in preparations from 6-OHDA animals, the percentage of evoked acetylcholine release was lower as compared with controls (+30% vs baseline at week 4 and +28% vs baseline at week 8, respectively) (n=8 animals for each group of treatment) (Figure 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±0.38 $x10^{-3}$ mm^2; 6-OHDA rats at week 4, 2.75±0.29 $x10^{-3}$ mm^2; 6-OHDA rats at week 8, 2.44±0.25 $x10^{-3}$ mm^2. With regard for neurons, a strong, cytoplasmic and/or nuclear HuC/D immunostaining was detected in myenteric neurons of distal colon 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 8, as compared to controls. By contrast, a significant decrease in ChAT immunopositivity was detected in the myenteric ganglia of 6-OHDA rats at both week 4 and 8 (-61.0% and -36.1% *versus* controls, respectively) (n=6 animals for each group of treatment) (Figure 7).

Western blot analysis of muscarinic M_2 and M_3 receptor expression

Western blot analysis revealed the constitutive expression of both muscarinic M2 and M3 receptors in colonic neuromuscular tissues from control rats (Figure 8A). In colonic tissues obtained from rats at week 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 group of treatment) (Figure 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 group of treatment) (Figure 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 impairment of 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 morphological approach. Overall, our results provide convincing evidence that the induction of nigrostriatal denervation, which reflects one of the main pathological 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 radiological analysis documented that four weeks after neurotoxin injection the rats displayed a caecum total score comparable to control animals, whereas colorectum score values were lower than control, 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 caecum 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 h after barium gavage was higher than after 10 h, indicating a delay in colorectum filling. These findings suggest that the model of 6-OHDA-induced nigrostriatal degeneration is suitable for the assessment of bowel dysmotility associated with PD. Consistently, several evidence indicate 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 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).

In order 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, where 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, while 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 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 to 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 their 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., 2011b). 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 these findings suggest that the impairment of colonic cholinergic motility, following central 6-OHDA-induced denervation, depends on an impairment of acetylcholine release from post-junctional myenteric motor neurons. However, the possibility that the central dopaminergic neurodegeneration could affect also other classes of myenteric cholinergic neurons (i.e. intrinsic primary afferent neurons and/or interneurons) cannot be ruled out.

Besides an impaired neurogenic cholinergic motor activity in the colon of 6-OHDA rats, we observed also 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 up-regulation 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 M_2 and M_3 receptors in specimens of colonic neuromuscular layer as well as in ICSMCs by western blot assays, and found that both receptor subtypes were indeed up-regulated 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 colon of patients with diverticular disease, where cholinergic denervation and related motor abnormalities of isolated colonic muscle were associated with an up-regulation of muscular muscarinic M_3 receptors (Golder et al., 2003). Overall, it is conceivable that lowering of colonic transit in 6-OHDA rats depends, at least in part, on the impairment of cholinergic enteric neurotransmission.

Beside 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 (VIP) levels and a concomitant decrease in nNOS expression in the myenteric plexus of 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 support the notion that colonic dysmotility associated with central dopaminergic denervation results from alterations occurring at 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 CNS 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 DMV, which is regulated by brainstem dopaminergic circuitries and represents a prominent target of PD-related neurodegenerative processes (Braak et al., 2004; Braak et al., 2003; Zheng et al., 2011b; Zheng et al., 2007). In this regard, the vagus nerve has been proposed to play a crucial role in the regulation of inflammatory responses, a function referred also as the "cholinergic anti-inflammatory pathway" (Matteoli et al., 2013). In particular, there is evidence that the vagus nerve exerts tonic anti-inflammatory actions and contributes to the maintenance of intestinal homeostasis (Matteoli et al., 2013), while vagotomy confers an increased susceptibility to the development of inflammatory bowel diseases (Ghia et al., 2006). In addition, (Toti et al., 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 (Fornai M, unpublished data). Of interest, our preliminary observations are consistent with the findings of a previous study, showing an increase in pro-inflammatory 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 nigro-striatal 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 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.

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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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Footnotes

Conflict of interest: The authors declare that they have no conflict of interest

Ethical approval: 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

M.F. and C.P. contributed equally to the manuscript

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Legends for figures

Figure 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.

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

Figure 3. Effects of electrical stimulation (ES, 1-20 Hz) on the mechanical activity of colonic longitudinal (A) and circular (B) smooth muscle preparations isolated from control animals, or rats after 4 and 8 weeks from 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 the frequency of 10 Hz. Each column represents the mean±S.E.M value obtained from 8 animals. Statistics: one-way ANOVA followed by post-hoc analysis with Student-Newman-Keuls test. *$P<0.05$; significant difference vs control.

Figure 4. Effects of electrical stimulation (ES, 1-20 Hz) on the mechanical activity of colonic longitudinal (A) and circular (B) smooth muscle preparations isolated from control animals, or rats after 4 and 8 weeks from nigrostriatal denervation with 6-OHDA. Colonic preparations were maintained in Krebs solution containing L-NAME (100 μM), guanethidine (10 μM), L-732138 (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 the frequency of 10 Hz. Each column represents the mean±S.E.M value obtained from 8 animals. Statistics: one-way ANOVA followed by post-hoc analysis with Student-Newman-Keuls test. *P<0.05; significant difference vs control.

Figure 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 after 4 and 8 weeks from 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 Cch at the concentration of 10 μM. Each point represents the mean±S.E.M value obtained from 8 animals. Statistics: one-way ANOVA followed by post-hoc analysis with Student-Newman-Keuls test. *P<0.05; significant difference vs control.

Figure 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 s with respect to the onset of electrical stimulation (ES, 10 Hz). One additional aliquot was collected at the end of the 10-s period of ES application, in order to evaluate the electrically induced acetylcholine release. Statistics: one-way ANOVA followed by post-hoc analysis with Student-Newman-Keuls test. *P<0.05, significant difference vs control values. (B) Percent increments of acetylcholine levels in response to ES, calculated over the respective values assessed at -60 s, in Krebs solution incubating longitudinal muscle preparations from control animals or rats after 4 and 8 weeks from nigrostriatal denervation with 6-OHDA. Each column represents the mean±S.E.M value obtained from 8 animals.

Statistics: Student t test for paired data or one-way ANOVA followed by with Bonferroni's post-test. [a]P<0.05 vs the respective value at -60 s.

Figure 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, E) without changes in neuron density (G). Myenteric neurons of control colon contain abundant amounts of ChAT staining, which is significantly decreased in 6-OHDA rats (B, D, F, 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 6 animals. Statistics: one-way ANOVA followed by post-hoc analysis with Student-Newman-Keuls test. [a]P<0.05 vs controls.

Figure 8. Western blot analysis of muscarinic M2 and M3 receptors in the colonic neuromuscular layer (A) and isolated colonic smooth muscle cells (ICSMCs) (B) from control rats and animals after 4 and 8 weeks from nigrostriatal denervation with 6-OHDA. Each column represents the mean±S.E.M value obtained from 5 to 6 animals. Statistics: Student t test for unpaired data or one-way ANOVA followed by post-hoc analysis with Student-Newman-Keuls test. *P<0.05; significant difference vs control.

Tables

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

Colonic layer	Percentage of reduction of contractile response to ES at 10 Hz			Percentage of reduction of contractile response to carbachol 1 μM		
	Control	6-OHDA 4 w	6-OHDA 8 w	Control	6-OHDA 4 w	6-OHDA 8 w
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

Electrically (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 reductions versus contractions recorded in the absence of atropine. Each number represents the mean±S.E.M value obtained from 5-6 animals. Statistics: one-way ANOVA followed by post-hoc analysis with Student-Newman-Keuls test.

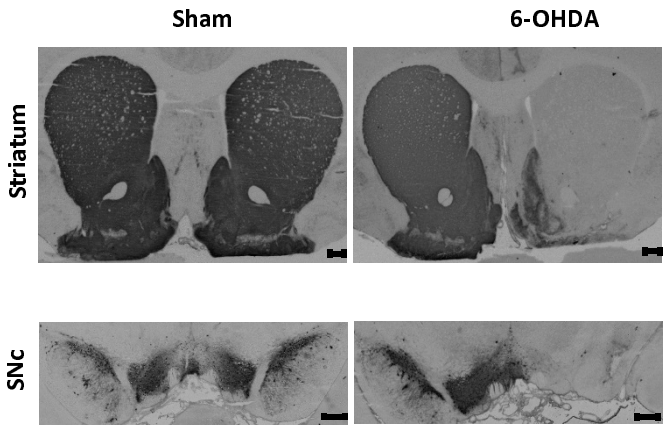


Figure 1

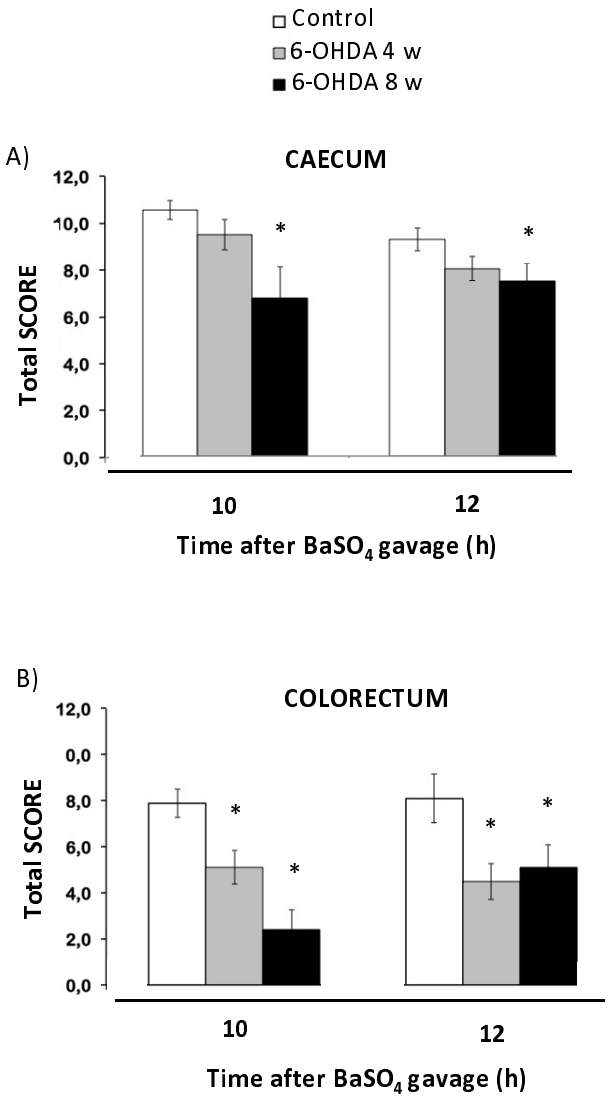

Control
6-OHDA 4 w
6-OHDA 8 w
A)
CAECUM
Total SCORE
12,0
10,0
8,0
6,0
4,0
2,0
0,0
*
*
10
12
Time after BaSO4 gavage (h)
B)
COLORECTUM
Total SCORE
12,0
0,0
8,0
6,0
4,0
2,0
0,0
*
*
*
*
10
12
Time after BaSO4 gavage (h)

Figure 2

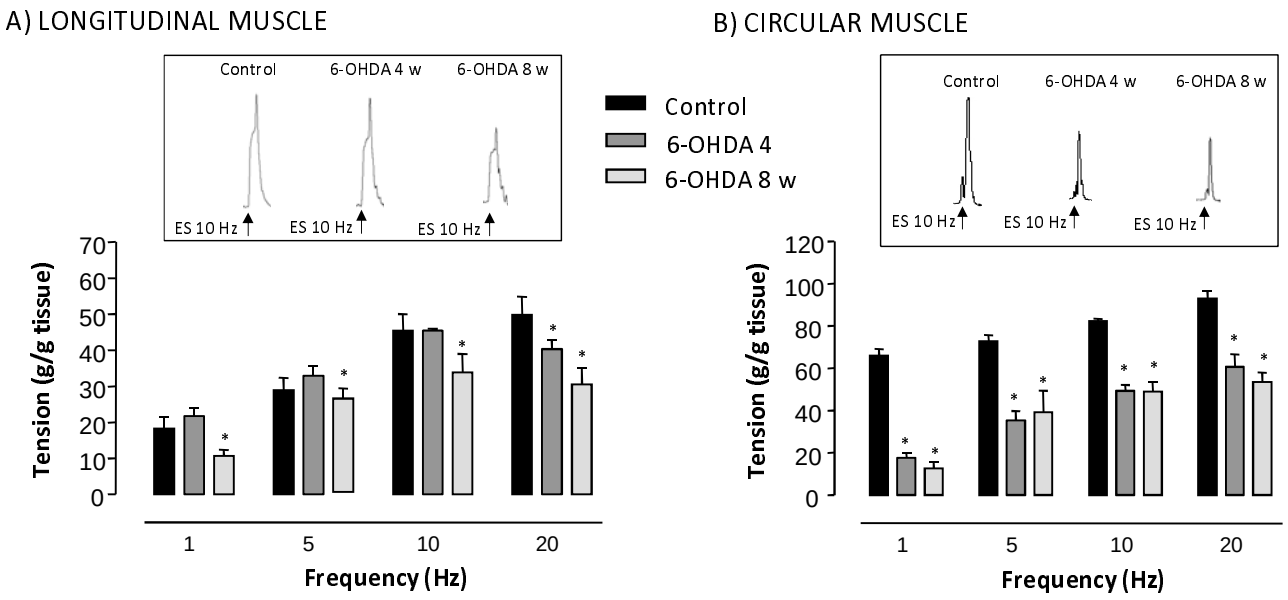

A) LONGITUDINAL MUSCLE
B) CIRCULAR MUSCLE
Control
6-OHDA 4 w
6-OHDA 8 w
ES 10 Hz
Control
6-OHDA 4
6-OHDA 8 w
Tension (g/g tissue)
Frequency (Hz)
1
5
10
20
0
10
20
30
40
50
60
70
80
100
120

Figure 3

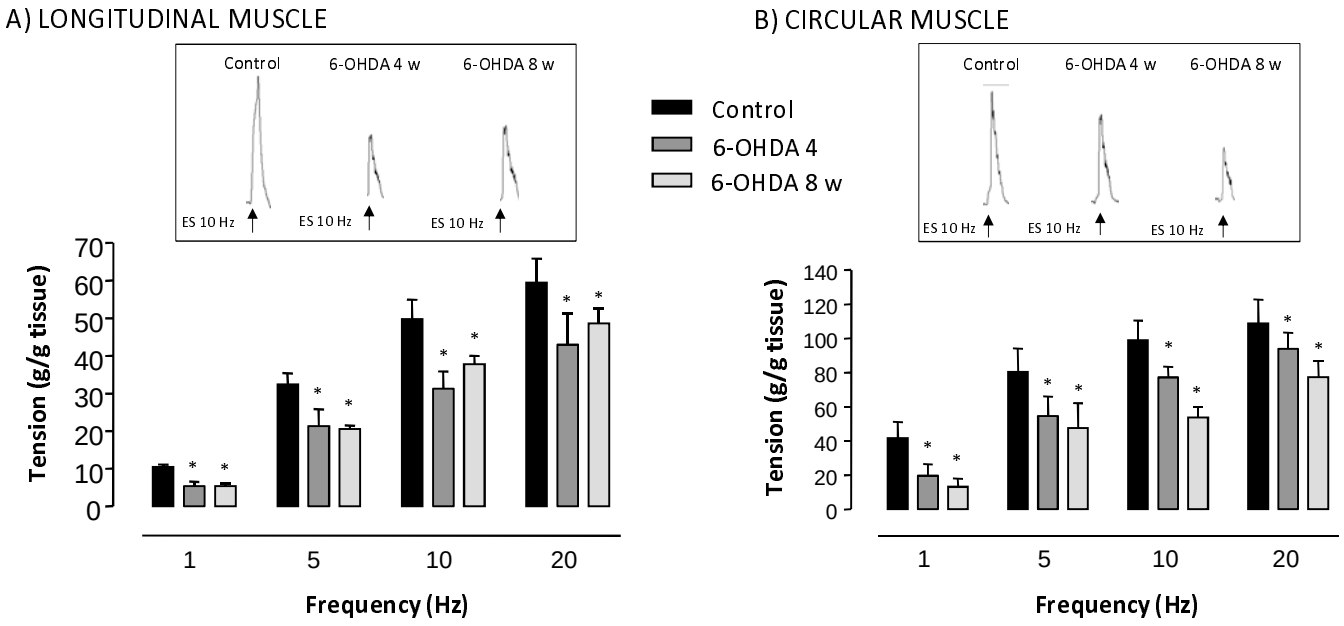
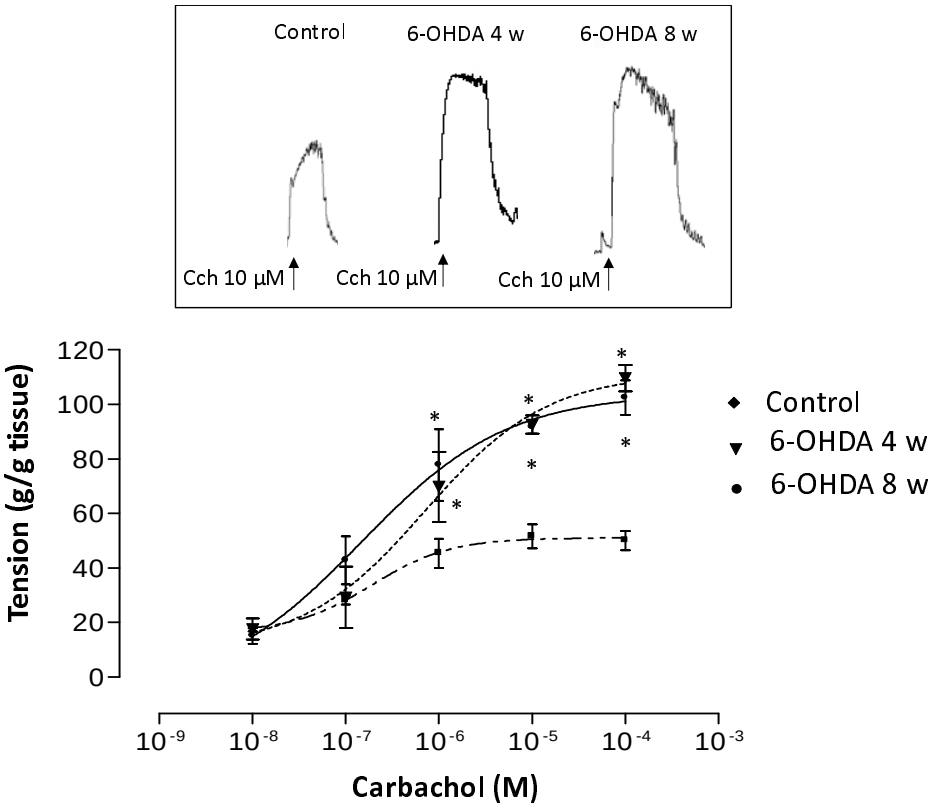

A) LONGITUDINAL MUSCLE
Control
6-OHDA 4 w
6-OHDA 8 w
ES 10 Hz
ES 10 Hz
ES 10 Hz
Tension (g/g tissue)
70
60
50
40
30
20
10
0
1
5
10
20
Frequency (Hz)
Control
6-OHDA 4
6-OHDA 8 w
B) CIRCULAR MUSCLE
Control
6-OHDA 4 w
6-OHDA 8 w
ES 10 Hz
ES 10 Hz
ES 10 Hz
Tension (g/g tissue)
140
120
100
80
60
40
20
0
1
5
10
20
Frequency (Hz)

Figure 4

A) LONGITUDINAL MUSCLE



B) CIRCULAR MUSCLE

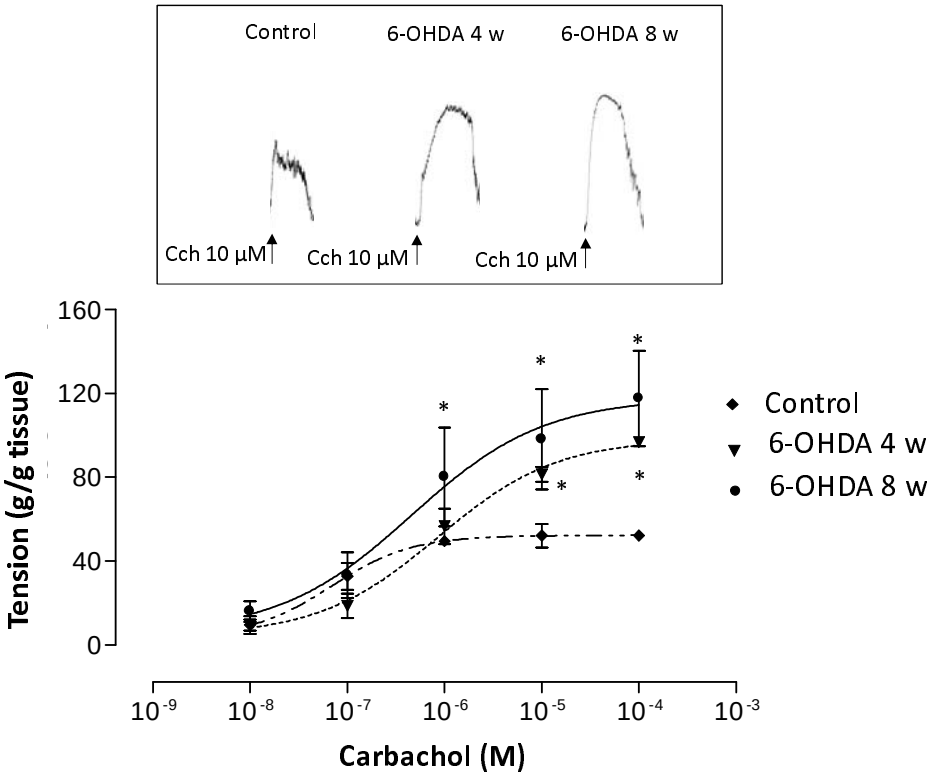
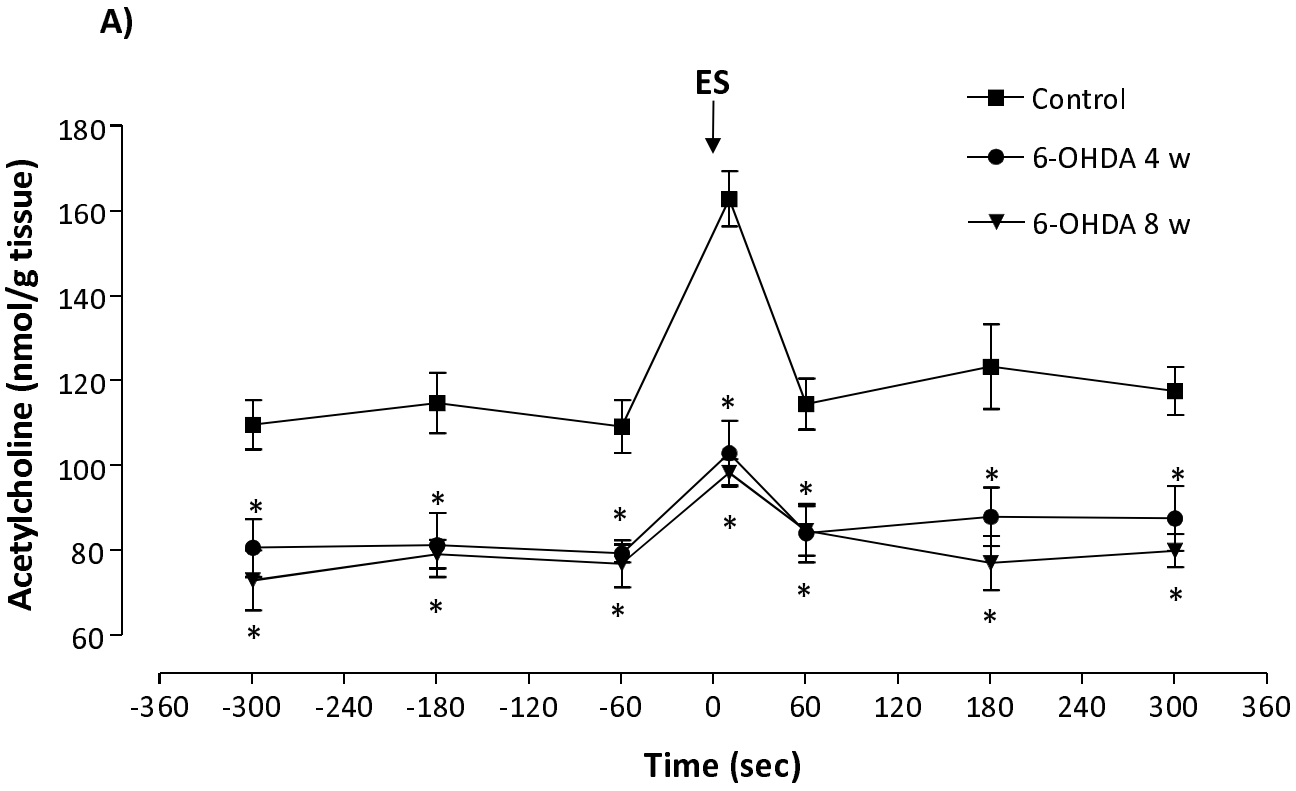


Figure 5


A)
ES
Control
6-OHDA 4 w
6-OHDA 8 w
Acetylcholine (nmol/g tissue)
180
160
140
120
100
80
60
-360
-300
-240
-180
-120
-60
0
60
120
180
240
300
360
Time (sec)

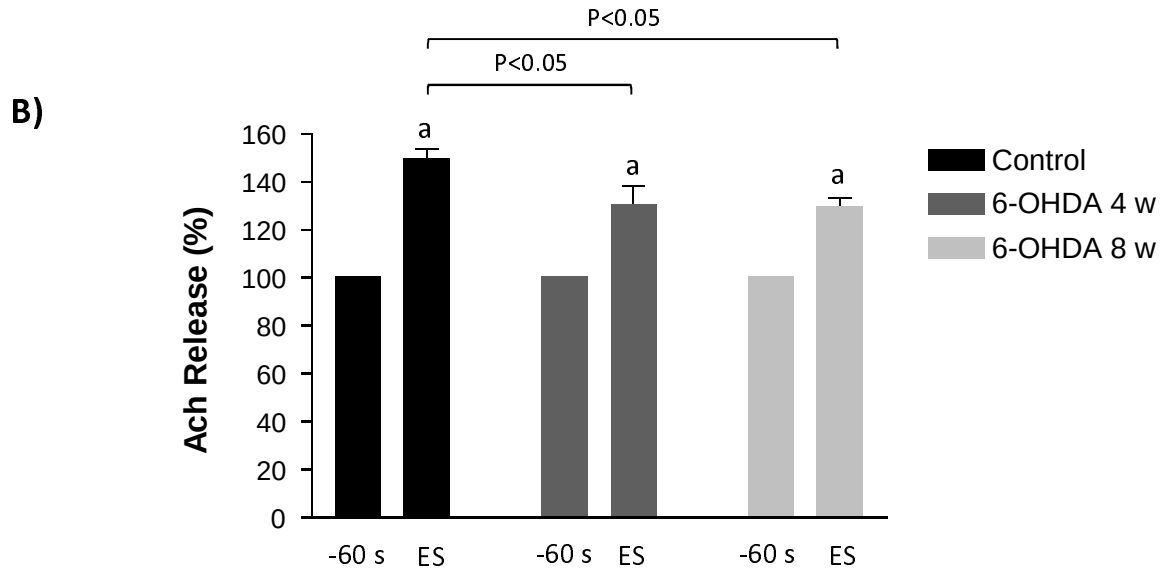

B)
P<0.05
P<0.05
a
a
a
Ach Release (%)
160
140
120
100
80
60
40
20
0
-60 s
ES
-60 s
ES
-60 s
ES
Control
6-OHDA 4 w
6-OHDA 8 w

Figure 6

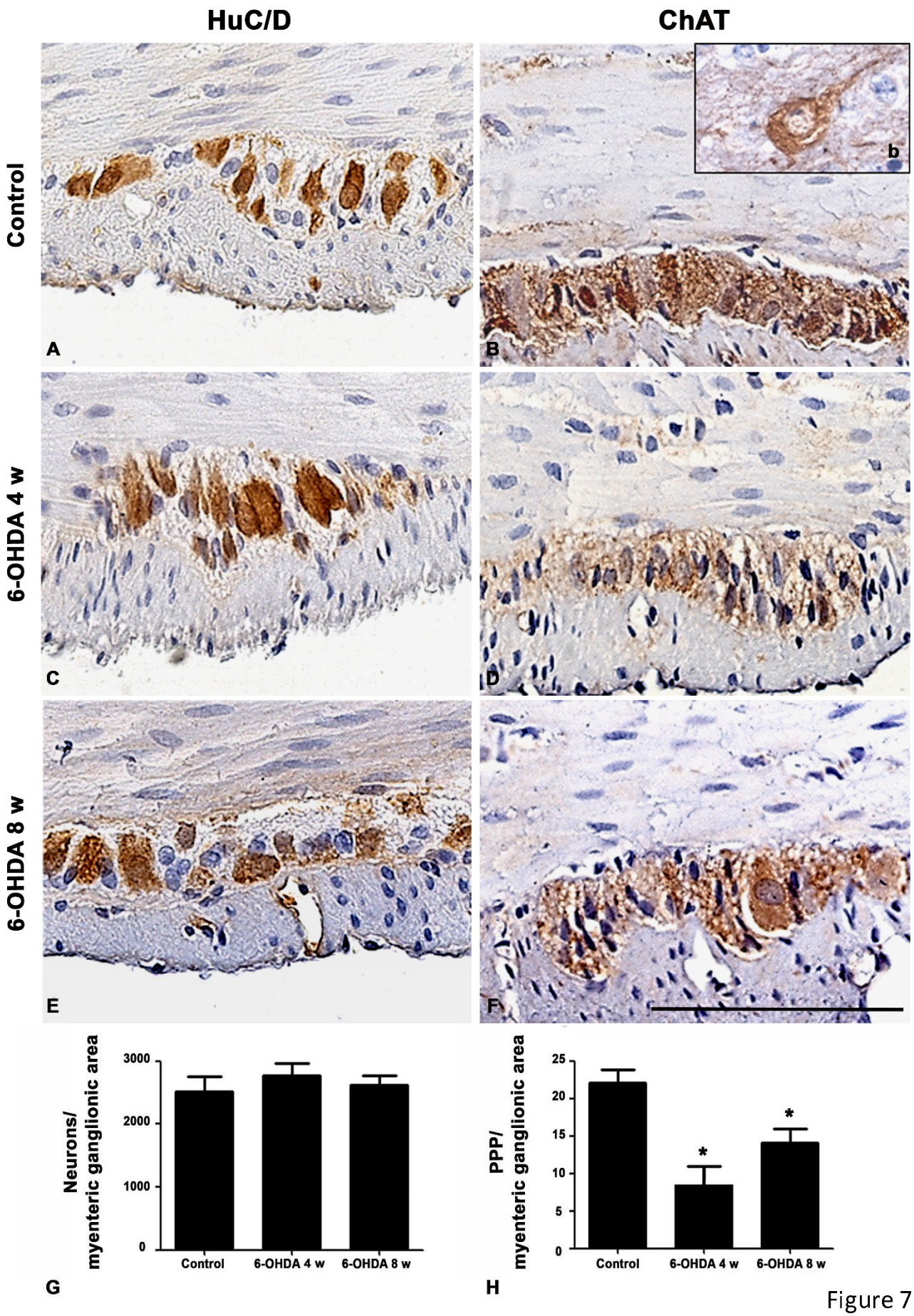

HuC/D
ChAT
Control
6-OHDA 4 w
6-OHDA 8 w
A
B
b
C
D
E
F
Neurons/ myenteric ganglionic area
3000
2000
1000
0
Control
6-OHDA 4 w
6-OHDA 8 w
G
PPP/ myenteric ganglionic area
25
20
15
10
5
0
*
*
Control
6-OHDA 4 w
6-OHDA 8 w
H

Figure 7

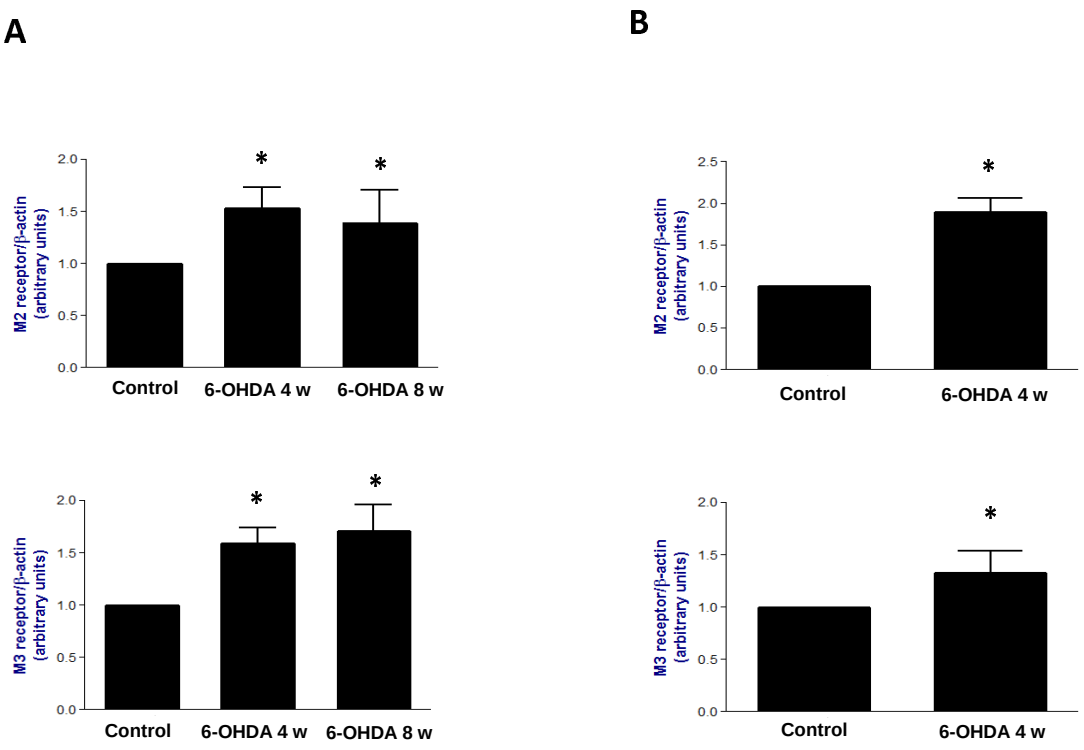

A
B
M2 receptor/β-actin (arbitrary units)
M3 receptor/β-actin (arbitrary units)
Control
6-OHDA 4 w
6-OHDA 8 w
*

Figure 8