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Parkinson's disease and gastrointestinal dysfunctions: morphological, neurochemical and functional modifications of the enteric nervous system associated with central dopaminergic impairment

Coordinatore:

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RECOMMENDATION LETTERS

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Dear Sir or Madam,

RE: Carolina Pellegrini PhD proposal,

Carolina Pellegrini during her doctoral program has developed research in the field of gastrointestinal alterations occurring in the presence of neurodegenerative pathologies. In particular, she has characterized the intestinal neuromotor dysfunctions occurring in the presence of central nigrostriatal denervation (an experimental model related to Parkinson's disease). In addition, the research program developed by Miss. Pellegrini has fostered significant progress in understanding the mechanisms underlying the link between alterations in the central nervous system and gastrointestinal dysfunctions, characterized by the onset and development of an inflammatory response, suggesting that the immune system plays a pivotal role in the pathology.

During her time in my laboratory at the Manchester Collaborative Centre of Inflammation Research (MCCIR), Miss. Pellegrini has greatly expanded her knowledge in the field of immunology and inflammation. This and the results here produced have contributed to successfully move her research forward.

Therefore, I have got an excellent opinion about the work developed by Carolina Pellegrini and I have no doubts that Carolina will continue to be a successful researcher in the future. I consider that Miss Pellegrini Thesis highly fulfils the standards to obtain a PhD degree.

Should you need any further information, please do not hesitate to contact me.

Dr Gloria Lopez-Castejon, PhD
MCCIR
University of Manchester

UNIVERSITY OF DEBRECEN
FACULTY OF MEDICINE
DEPARTMENT OF MEDICAL CHEMISTRY

October 20, 2015

To whom it may concern:

Dr. Carolina Pellegrini, during her PhD program, has conducted interesting research activity in a project concerning the pharmacological and molecular characterization of pathways regulating the neuromuscular functions of the lower digestive tract in an experimental model of Parkinson's disease. In addition, Dr. Carolina Pellegrini took part in the implementation of innovative research strategies, in order to extend currant knowledge in the field of enteric neuro-immune alterations occurring in the presence of neurodegenerative diseases. Based on my personal experience, I believe that Dr. Pellegrini has obtained important results about the pathophysiological mechanisms underlying gastrointestinal dysfunctions associated with Parkinson's disease. Therefore, I recommend that the thesis presented by Carolina Pellegrini be accepted.

Sincerely,

Gyorgy Hasko, MD, PhD
Scientific Advisor
Department of Medicinal Chemistry

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INDEX

Abstract

Parkinson's disease (PD) is frequently associated with gastrointestinal (GI) symptoms, mostly represented by abdominal distension, constipation and defecatory dysfunctions. Despite GI dysfunctions have a major impact on the clinical picture of PD, there is currently a lack of information on the neurochemical, pathological and functional correlates of GI dysmotility associated with PD. Moreover, there is a need of effective and safe pharmacological therapies for managing GI disturbances in PD patients.

The present research project has been undertaken to investigate the relationships between PD and related GI dysfunctions by means of investigations in an animal model of PD induced by intranigral injection of 6-hydroxydopamine (6-OHDA). The use of the 6-OHDA experimental model of PD in the present program has allowed to pursue the following goals: 1) to examine the impact of central dopaminergic denervation on colonic excitatory cholinergic and tachykininergic neuromotility by means of molecular, histomorphologic and functional approaches; 2) to elucidate the role of gut inflammation in the onset and progression of colonic dysmotility associated with PD, characterizing the degree of inflammation and oxidative damage in colonic tissues, as well as identifying the immune cells involved in the production of pro-inflammatory cytokines in the gut; 3) to evaluate the impact of chronic treatment with L-DOPA plus benserazide on colonic neuromuscular activity both in control and PD animals.

The results suggest that central nigrostriatal dopaminergic denervation is associated with an impaired excitatory cholinergic neurotransmission and an enhanced tachykininergic control, resulting in a dysregulated smooth muscle

motor activity, which likely contributes to the concomitant decrease in colonic transit rate. These motor alterations might result from the occurrence of a condition of gut inflammation associated with central intranigral denervation. The treatment with L-DOPA/BE following central dopaminergic neurodegeneration can restore colonic motility, likely through a normalization of the cholinergic enteric neurotransmission, and it can also improve the colonic inflammation associated with central dopaminergic denervation.

STATE OF THE ART

PARKINSON'S DISEASE

Parkinson's disease (PD) is the second most common neurological disorder in the general population, expected to impose an increasing social and economic burden on societies with an incidence rate of 8-18 per 100,000 person-years (de Lau and Breteler 2006). The pathological hallmarks of PD include: a) degeneration of dopaminergic neurons of the nigrostriatal pathway, projecting from the substantia nigra pars compacta (SNpc) to the striatum; b) presence of eosinophilic inclusions, designated as Lewy bodies (LB) and neurites (LN), consisting of aggregates of phosphorylated α-synuclein (α-syn) in the surviving neurons (Blandini, Nappi et al. 2000, Kalia and Lang 2015). Central dopaminergic denervation of the striatum triggers a cascade of functional alterations within the basal ganglia circuitry - the neural area responsible for correct execution of voluntary movements - that leads to the appearance of typical PD motor symptoms (tremor, rigidity and bradykinesia) (Blandini, Nappi et al. 2000). Furthermore, besides the dopaminergic system, other neurotransmitter pathways are involved in PD, such as noradrenergic, serotonergic and cholinergic systems (Jellinger 1991).

Treatment of PD is based on replacing deficient dopamine by oral administration of its precursor, L-3,4-dihydroxyphenylalanine (L-DOPA), in combination with a peripheral inhibitor of DOPA-decarboxylase (Cotzias, Papavasiliou et al. 1969). However, long-term L-DOPA treatment is associated with a variety of adverse effects, such as "on-off" fluctuations, freezing episodes, lack of responsiveness and dyskinesia. These complications are thought to be associated with fluctuations of L-DOPA plasma concentrations, which can be exacerbated by alterations of gastric emptying, often observed in PD patients (Obeso, Olanow et al. 2000, Nyholm and Lennernas 2008).

Although PD is considered a typical movement disorder, PD patients experience also several non-motor symptoms, including cognitive and mood disorders, olfactory deficiencies, sleep disturbances, and functional gastrointestinal (GI) disturbances. Indeed, symptoms such as dysphagia, nausea, abdominal distension and severe constipation occur frequently in PD patients, to such an extent that they are now considered an integral part of the clinical picture of this neurodegenerative disease (Natale, Pasquali et al. 2008).

Clinical features

The classical motor symptoms of PD, recognized since James Parkinson's initial description in the 19th century and later redefined by Jean-Martin Charcot, include bradykinesia, muscular rigidity, tremor, as well as postural and gait impairment (Blandini, Nappi et al. 2000). Empirical clinical observations suggest two major patterns of PD: tremor-dominant PD, with a relative lack of other motor symptoms, and non-tremor-dominant PD, characterized by rigidity and postural instability, gait disorder (Marras and Lang 2013).

PD is also associated with non-motor symptoms, including olfactory dysfunction, cognitive impairment, psychiatric symptoms, sleep disorders, autonomic dysfunction, pain, fatigue and -above all- GI dysfunctions (Khoo, Yarnall et al. 2013). In particular, GI abnormalities, including mainly delayed gastric emptying, constipation and anorectal dysfunction, often precede the onset of motor symptoms by many years, and they seem to be associated with an increased risk of developing PD in otherwise healthy people (Edwards, Quigley et al. 1994, Hardoff, Sula et al. 2001, Cersosimo, Raina et al. 2013, Sung, Park et al. 2014). Indeed, the pathogenic process of PD seems

to occur during the pre-motor phase, involving both peripheral and central nervous system (Siderowf and Lang 2012).

Pathological features

The main pathological feature of PD is the loss of dopaminergic neurons within the SNpc projecting to the striatum, that has been shown to be the cause of motor symptoms of disease, bradykinesia and rigidity (Dickson, Braak et al. 2009). Neuronal loss in PD occurs also in other brain regions, including the locus ceruleus, nucleus basalis of Meynert, pedunculo pontine nucleus, raphe nucleus, dorsal motor nucleus of the vagus (DMV), amygdala, and hypothalamus, thus suggesting that the neurodegenerative process involves different neuronal circuits (Dickson 2012). In addition, Lewy pathology, another hallmark of parkinsonian brain, has been detected in the spinal cord and peripheral nervous system, including the vagus nerve, sympathetic ganglia, cardiac plexus, salivary glands, adrenal medulla, cutaneous nerves, sciatic nerve and enteric nervous system (ENS) (Masters, Kril et al. 2011, Goedert, Spillantini et al. 2013) (Wakabayashi and Takahashi 1997, Braak, de Vos et al. 2006). Another feature of PD pathology is represented by neuroinflammation, since the occurrence of an inflammatory condition in the brain of PD patients, mediated by resident astrocytes and microglia, has been identified (Tansey and Goldberg 2010). In particular, it has been observed that activated microglia release trophic factors, such as brain-derived neurotrophic factor and glial-derived neurotrophic factor, but also reactive oxygen and nitrogen species as well as pro-inflammatory cytokines [Interleukin (IL)-1β and tumor necrosis factor (TNF)], that contribute to neuroinflammation in the parkinsonian brain (Phani, Loike et al. 2012).

Interestingly, Devos et al. (Devos, Lebouvier et al. 2013) observed that PD is associated with a gut inflammatory condition, characterized by cytokine patterns similar to those observed in the presence of inflammatory bowel diseases (Matsuda, Koide et al. 2009). In particular, the authors found an increase in the expression of pro-inflammatory cytokines (IL-1β, TNF, interferon (IFN)-γ, IL-6), and glial markers [*glial fibrillary acidic protein* (GFAP) and sex determining region Y(SRY)-box containing gene 10 (Sox-10)] in colonic biopsies from PD patients at different stages of the disease, suggesting additional pathological features of PD, but, most importantly, strengthering the role of peripheral inflammation in the initiation and/or progression of the neurodegenerative process (Devos, Lebouvier et al. 2013).

Pathogenesis

Current evidence regarding the pathophysiological mechanisms underlying the onset of PD stem from epidemiological findings as well as genetic and pathological observations (Trinh and Farrer 2013). Braak and colleagues hypothesized that the neurodegenerative process could start peripherally, in particular in the ENS, and affect progressively the central nervous system (CNS) in a caudal-to-rostral direction. In particular, the spreading of PD pathology seems to be mediated by a prion-like transmission of α-synuclein among neurons, thus suggesting the presence of LBs in the ENS as the possible earliest signs of the disease (Figure 1) (Del Tredici, Rub et al. 2002, Hawkes, Del Tredici et al. 2007, Visanji, Brooks et al. 2013).

In the above context, nerve pathways connecting the brain to the gut appears to be pivotally involved in the pathogenesis of PD. In this regard, increasing evidence suggests that the DMV, known to provide most of parasympathetic innervation to the

GI tract, is one of the CNS sites affected by PD pathology at its early stages (Jellinger 1987, Del Tredici, Rub et al. 2002).

Conversely, several evidence support hypothesis that the dopaminergic neurodegeneration starts in the CNS and spread progressively, through sympathetic and parasympathetic fibers, in the peripheral neuronal circuits, including the ENS (Braak, Rub et al. 2003, Braak, Ghebremedhin et al. 2004, Zheng and Travagli 2007, Zheng, Wang et al. 2011).

Therefore, at present, whether the ENS represents the starting site of neurodegenerative process or a direct consequence of central neurodegeneration, remains still unclear and largely debated.

Over the last years, different genetic mutations have been associated with PD, including α-syn non A4 component of amyloid precursor (SNCA), alanine 53 to threonine (A53T) in the α-syn gene, as well as other polymorphisms in the parkin, LRKK2 (leucine-rich repeat kinase 2), PINK1 [phosphatase and tensin homolog (PTEN)-induced putative kinase 1] and DJ-1 (protein deglycase-1) genes (Kalia and Lang 2015). However, central dopaminergic neurodegeneration related to genetic mutations have been found to be rare and associated with atypical PD clinical features (i.e. prominent cognitive impairment, ophthalmologic abnormalities, pyramidal signs, or ataxia) (Kalia and Lang 2015).

Another typical feature of PD pathology is central and peripheral neuroinflammation (Hirsch, Vyas et al. 2012). For instance, the increase in oxidative stress in PD, which causes an excessive generation of cytotoxic free radicals and oxidation of dopamine and its metabolites, is associated with an increase in cell death in the SNpc (Sherer and Greenamyre 2005). Moreover, the condition of enteric neuroinflammation, observed in PD patients at different stages of the disease, could represent a crucial feature of PD

pathology (Devos, Lebouvier et al. 2013). Current evidence supports a pivotal role played by neuroinflammation in PD pathogenesis, although the mechanisms underlying the onset and development of the inflammatory condition in PD remain still largely undetermined.

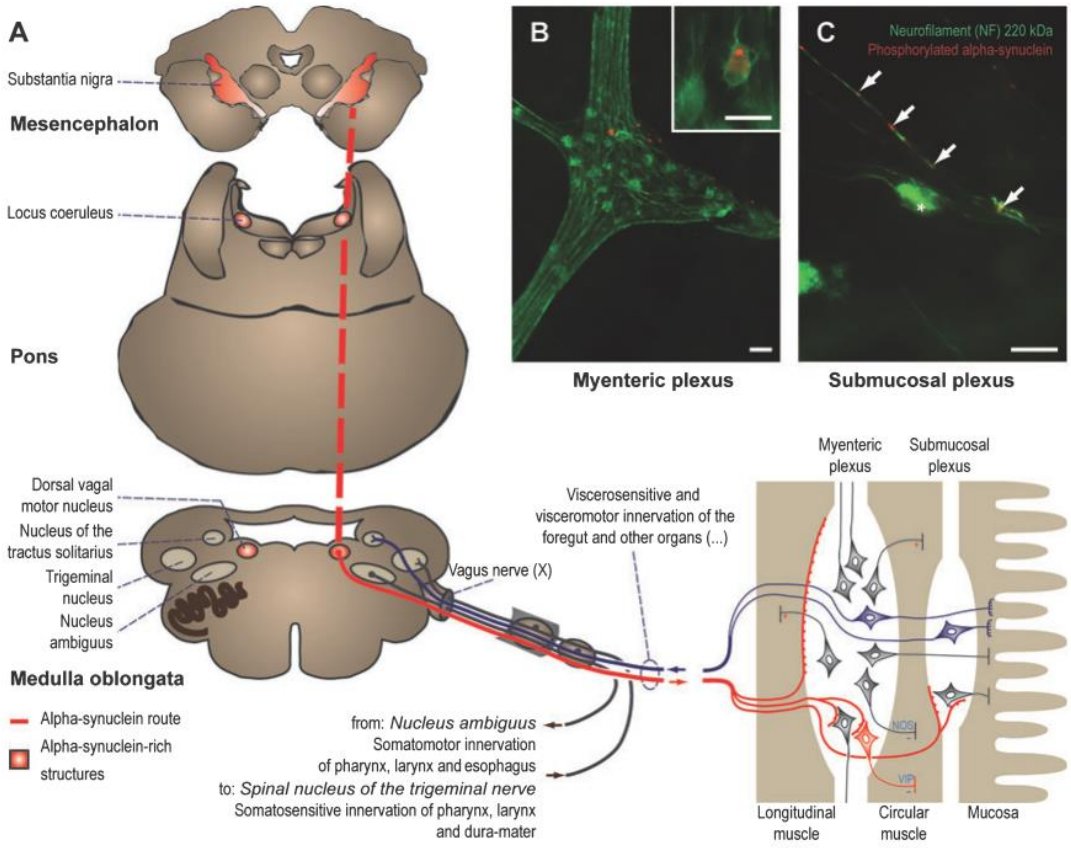


Fig. 1. (A) Within the brainstem, under physiological conditions the dorsal motor nucleus of the vagus nerve, locus coeruleus and substantia nigra contain low amounts of non-aggregated α-syn. Interestingly, vagal efferent axons (red), which are the only ones to degenerate in PD, are differentiated from the afferent fibers (blue) by selective α-syn expression. A putative retrograde and ascending pathway following α-syn structures can be drawn, running from the ENS towards the CNS.
(B) Whole mount of colonic myenteric and submucosal plexus from an end-stage PD patient. In the myenteric pleaxus double labeling with antibodies against neurofilament and phosphorylated α-syn reveals some Lewy neurites (arrow) in most of myenteric ganglia, and occasional Lewy bodies. (C) In the submucosal plexus, although no intrinsic submucosal neurons seem to be affected, the same immunolabeling shows degenerative changes within fibers, that are likely extrinsic in nature (arrows).
Lebouvier T et al. European Journal of Neuroscience, 2009.

THE ENTERIC NERVOUS SYTEM

Structure of the enteric nervous system

The ENS is considered a neuronal semiautonomous network that regulates digestive functions (GI motility, gastric acid secretion; movement of fluid across the lining epithelium, changing local blood flow, modifying nutrient handling, interaction with immune and endocrine systems of the gut, maintaining the integrity of epithelial barrier between the gut lumen and cells and tissues within the gut wall) and cooperates with an integrated neuronal network involving sympathetic and parasympathetic systems and CNS (Toumi, Neunlist et al. 2003, Furness 2012). However, the ENS, referred as the 'brain of the gut', maintains the ability of controlling gut functions even when it is completely disconnected from CNS (Bayliss and Starling 1901).

The enteric neural network consists of two major plexuses: myenteric (or Auerbach's) plexus and submucous (or Meissner's) plexus (Figure 2). The myenteric plexus runs between the longitudinal and circular muscle layers for the whole length of the gut. It primarily provides motor innervation to both muscle layers, secretomotor innervation to the mucosa, and several projections to submucosal ganglia and enteric ganglia of gallbladder and pancreas (Kirchgessner and Gershon 1990, Furness 2012). In addition, a substantial number of neuronal projections from myenteric neurons reach the sympathetic ganglia (Furness, Callaghan et al. 2014).

The submucous plexus, located in the submucosa between the circular muscle layer and the muscularis mucosa, has an important role in the control of secretion. It provides neuronal innervations to the glandular epithelium, muscularis mucosa, intestinal endocrine cells, and submucosal blood vessels. Enteric glial cells are also an integral component of the ENS. They resemble astrocytes of the CNS and can produce

interleukins and express MHC class II antigens in response to immune/inflammatory stimuli, suggesting a pivotal role played by enteric glia in the pathophysiology of intestinal inflammatory responses (Gabella 1979).

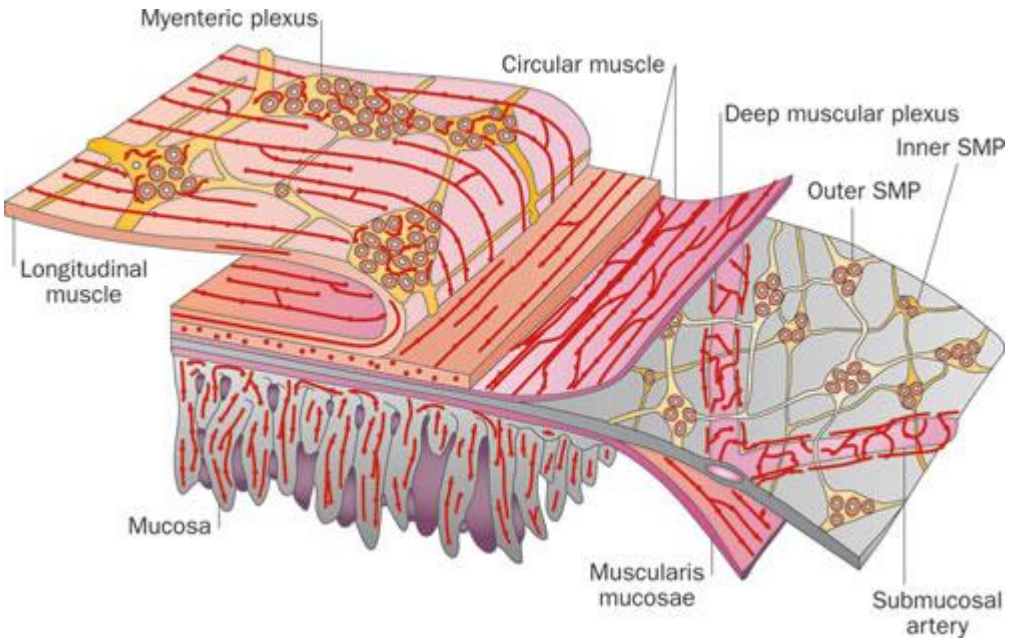


Figure 2. The enteric nervous system. The myenteric plexus between the longitudinal and circular layers of the external musculature and the submucosal plexus that has outer and inner components. Nerve fiber bundles connect the ganglia and form plexuses that innervate the longitudinal muscle, circular muscle, muscularis mucosae, intrinsic arteries and the mucosa.

Furness JB. The enteric nervous system and neurogastroenterology *Nature Reviews Gastroenterology and Hepatology* 9, 286-294 (May 2012)

Enteric neurons

Neurons of the ENS have been classified in intrinsic afferent neurons, interneurons, and motor neurons. I*ntrinsic afferent neurons* are type II neurons (multipolar with many long, smooth processes) located both in the myenteric and submucous plexuses. They control intrinsic motor and secretomotor reflexes, projecting circumferentially to interneurons in the surrounding myenteric and submucous plexuses. Electrophysiologically, intrinsic afferent neurons behave as *sensory neurons*. They receive slow synaptic inputs that regulate their excitability, followed by a prominent

after-hyperpolarization. Therefore they have been designated as AH neurons. They are all cholinergic and may also contain substance P.

Interneurons, interposed between primary afferent neurons and motor or secretomotor neurons, form multisynaptic pathways along the digestive tract, allowing the propagation of peristaltic waves. They are designated as ascending or descending, as they regulate motor reflexes orally or anally, respectively. *Motor neurons* display type I morphologic features (many club-shaped processes and a single long, slender axon); and those endowed with excitatory functions project locally or orally to the circular muscle, and their main neurotransmitters are acetylcholine and substance P; those exerting inhibitory functions project caudally and employ vasoactive intestinal polypeptide (VIP) and nitric oxide (NO) as mediators.

Chemistry of enteric neurotransmitters

Table showing the enteric neurotransmitters involved in the control of digestive functions

Type of neuron	Primary transmitter	Secondary transmitters, modulators	Other neurochemical markers
Enteric excitatory muscle motor neuron	ACh	Tachykinin, enkephalin (presynaptic inhibition)	Calretinin, γ-aminobutyric acid
Enteric inhibitory muscle motor neuron	Nitric oxide	VIP, ATP or ATP-like compound, carbon monoxide	PACAP, opioids
Ascending interneuron	ACh	Tachykinin, ATP	Calretinin, enkephalin
ChAT, NOS descending interneuron	ATP, ACh	ND	Nitric oxide, VIP
ChAT, 5-HT descending interneuron	ACh	5-HT, ATP	ND
ChAT, somatostatin descending interneuron	ACh	ND	Somatostatin
Intrinsic sensory neuron	ACh, CGRP, tachykinin	ND	Calbindin, calretinin, IB4 binding
Interneurons supplying secretomotor neurons	ACh	ATP, 5-HT	ND
Noncholinergic secretomotor neuron	VIP	PACAP	NPY (in most species)
Cholinergic secretomotor neuron	ACh	ND	Calretinin
Motor neuron to gastrin cells	GRP, ACh	ND	NPY
Motor neurons to parietal cells	ACh	Potentially VIP	ND
Sympathetic neurons, motility inhibiting	Noradrenaline	ND	NPY in some species
Sympathetic neurons, secretion inhibiting	Noradrenaline	Somatostatin (in guinea pig)	ND
Sympathetic neurons, vasoconstrictor	Noradrenaline, ATP	Potentially NPY	NPY
Intestinofugal neurons to sympathetic ganglia	ACh	VIP	Opioid peptides, CCK, GRP

Abbreviations: 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; CCK, cholecystokinin; ChAT, choline acetyltransferase; CGRP, calcitonin gene-related peptide; GRP, gastrin releasing peptide; ND, not determined; NPY, neuropeptide Y; NOS, nitric oxide synthase; PACAP, pituitary adenylyl-cyclase activating peptide; VIP vasoactive intestinal peptide.

Furness JB.Nat. Rev. Gastroenterol. Hepatol, 2012.

Gastrointestinal motility

Gastric motility

Motility of the proximal stomach differs from the distal portion. The motor activity of proximal stomach, referred as a reservoir, maintains a continuous contractile tone (tonic contraction), while the distal portion contracts phasically, in order to propel gastric contents toward the gastroduodenal junction. The gastric smooth musculature is innervated by both enteric excitatory and inhibitory motor neurons. Motor neurons are controlled by both efferent vagal nerves and intramural microcircuits of the ENS. Vagal efferent nerve fibers release acetylcholine at nicotinic postsynaptic receptors on both excitatory and inhibitory enteric motor neurons. Excitatory motor neurons release acetylcholine at postjunctional muscarinic receptors and substance P at neurokinin-1 receptors on gastric muscle cells. Relaxation is mediated by release of NO, ATP, and VIP from the inhibitory motor innervation of musculature. The relative balance of excitatory and inhibitory inputs to gastric musculature adjusts the volume and pressure of the reservoir to the amount of solid and/or liquid contents, while maintaining constant compressive forces on contents. Integrative interactions between brainstem and ENS in the form of vago-vagal reflexes determine the overall gastric motor activity (Grundy, Al-Chaer et al. 2006). Of note, the rhythmic contractile waves that pass caudally over the stomach to propel its contents are generated in the smooth muscle through the activity of interstitial cells of Cajal, that function as pacemaker cells able to generate bioelectrical slow wave potentials that lead smooth muscle contractions (Sanders 1996).

Intestinal motility

Intrinsic ENS microcircuitries, regulating intestinal motility, are arranged to generate four fundamental motor patterns: interdigestive migrating motor complex (MMC) pattern; postprandial pattern of mixing movements; power propulsion; neurally programmed motor quiescence (Grundy, Al-Chaer et al. 2006). The ENS cooperate also with extrinsic nerve pathways, involving vagus nerve, splanchnic nerves, and pelvic nerves, to regulate intestinal motility. Indeed, sympathetic and parasympathetic extrinsic neurons influence smooth muscle functions indirectly by acting on neurons in the ENS. Smooth muscle cells are innervated by a complex network of excitatory and inhibitory motor neurons. VIP, PACAP, noradrenaline, opioids, and NO mediate inhibitory stimuli, while substance P, acetylcholine, and serotonin mediate excitatory stimuli, thus creating an overall neuronal electrical activity in the gut characterized by slow waves and spike bursts (Kunze and Furness 1999, Huizinga, Robinson et al. 2000, Olsson and Holmgren 2001, Hansen 2003). In addition, interstitial cells of Cajal, located at the myenteric and submucosal borders of the circular muscles layers, take contact with each other, smooth muscle cells and nerve terminals contributing to initiate rhythmic electrical activity. Interstitial cells of Cajal have been shown to express receptors both the inhibitory VIPergic and nitrergic, and excitatory tachykininergic and cholinergic transmitters (Huizinga, Robinson et al. 2000).

Enteric nervous and intestinal system interactions

Increasing evidence supports the concept that crosstalks between enteric nervous and immune systems play a crucial role in the regulation of immune responses (Steinman 2004). In particular, neuro-immune interplays act as counter-regulatory mechanism that dampen inflammation trying to restore intestinal homeostasis, and they appear also to be involved in the regulation of GI motility (Sternberg 2006).

In this setting, it has been appreciated that, under both physiological and pathological conditions, there is a complex multidirectional interactions between ENS-extrinsic autonomic nervous system, and intestinal immune and smooth muscle cells (Figure 3) (Bauer 2008). For instance, enteric glial cells modulate neuromuscular transmission, GI motility and secretion, but also intestinal barrier functions and gut immune homeostasis, thus suggesting their pivotal role in interplays among enteric neurons, immune cells and intestinal epithelium (Figure 1 and 3) (Ruhl 2005, Neunlist, Van Landeghem et al. 2008).

Substance P and calcitonin gene related peptide (CGRP), released from enteric neurons, activate peritoneal mast cells that release pro-inflammatory mediators into the peritoneal cavity diffusing into blood vessels and increasing mucosal permeability. In turn, luminal bacteria and/or bacterial products enter the GI wall and activate resident macrophages, triggering intracellular signaling pathways and thereby leading to the transcription of inflammatory molecules, cytokines, chemokines and adhesion molecules (Boeckxstaens and de Jonge 2009).

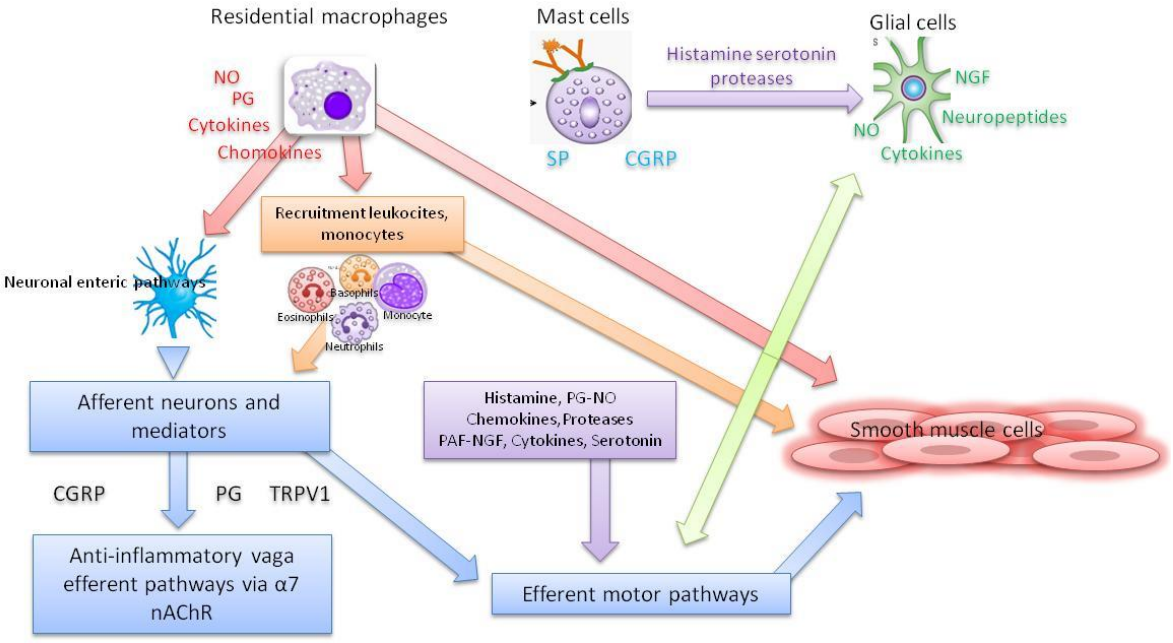


Figure 3. Complex interplays between residential macrophages, mast cells, glial cells and the recruitment of inflammatory cells, the activation of neuronal reflex pathways. NO: Nitric oxide; PG: Prostaglandins; SP: Substance P; CGRP: Calcitonin gene-related peptide; NGF: Neuropeptides, growth factors; PAF: Platelet-activating factor; TRPV: Transient receptor potential channel of the vanilloid subtype; nAChR: Nicotinic acetylcholine receptor.

Modified from De Winter and De Man. Interplay between inflammation, immune system and neuronal pathways: Effect on gastrointestinal motility. World J Gastroenterol 2010.

Recently, it has been observed that GI peristaltic activity is also regulated by macrophages of the muscularis externa, which represent a distinct macrophage population arranged in layers between the serosa and longitudinal muscle and the longitudinal and circular muscles, running from the stomach to distal colon both in humans and mice Mikkelsen et al., 2010). In particular, Muller et al. (2014) have shown that, under homeostatic conditions, intestinal macrophages stimulate enteric neuronal activity via release of bone morphogenetic protein 2 (BMP2). This factor, released from muscularis externa macrophages, sustains the development of ENS and constitutively activates enteric neurons, thus regulating colonic contractility. In turn, enteric neurons produce the macrophage growth factor CSF1, which sustains the

maintenance of muscularis externa macrophages, thus suggesting the interaction among enteric neurons and macrophages as a neuro-immune bridge involved in the regulation of digestive functions (Figure 4). Therefore, macrophages and enteric neurons provide mutual support and ensure the homeostatic gut function. In this context, and commensal microbiota can influence bidirectional neuro-immune crosstalks (Figure 4).

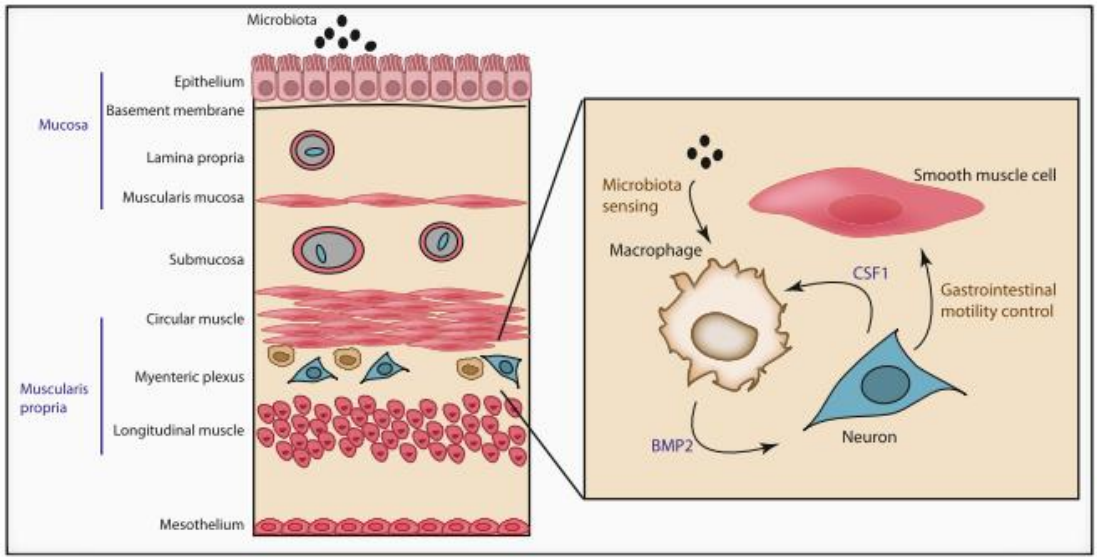


Figure 4. Crosstalk between muscularis mucosae macrophages and enteric neurons
In the gut, muscularis propria macrophages, arranged in layers between the longitudinal and circular muscles and the outer and inner circular muscles, establish bidirectional interactions with enteric neurons. The development of intestinal macrophages is sustained by neuronal-derived CSF1 and, conversely macrophages support neuronal activity via BMP2 secretion. Then, enteric neurons control gastrointestinal motility acting on smooth-muscle cells. Changes in microbiome composition and intestinal homeostasis can influence muscularis propria macrophages, which accordingly may tune intestinal peristalsis acting on this network.
Barrett, K.E., Barman, S.M., Boitano, S., Brooks, H. Ganong's Review of Medical Physiology, 23rd Edition (2010).

ROLE SYMPATHETIC AND PARASYMPATHETIC NERVOUS SYSTEMS IN THE CONTROL OF INTESTINAL INFLAMMATION

Several evidence supports the idea that an integrated neural network, consisting of intrinsic enteric and extrinsic sympathetic and parasympathetic innervations, plays a pivotal role in the regulation of the intestinal immune response (Willemze et al., 2015). Indeed, the interplay between autonomic nervous system and immune cells, referred also as "the inflammatory reflex", acts as counter-regulatory mechanisms able to dampen inflammation and restore homeostasis (Willemze et al., 2015).

With regard to sympathetic nervous system, it innervates all primary and secondary immune organs, including spleen and gut-associated lymphoid tissues, releasing catecholamines able to modulate immune functions (Cohen, 2001; Bellinger et al., 2008). In addition, sympathetic innervation modulates directly the intestinal immune response, since sympathetic fibers extend throughout the lamina propria, forming a dense plexus around the epithelial crypts, and ramification throughout the villi in the ileum (Bellinger et al., 2001). In particular, a double-label immunocytochemistry demonstrated that noradrenergic fibers course adjacent to the epithelial cells in close proximity to intraepithelial lymphocytes and macrophages in the lamina propria (Bellinger et al., 2001). In addition, T and B lymphocytes, natural killer cells and macrophages express α and β adrenergic receptor, corroborating a modulator role for sympathetic nervous pathway in the immune response (Sanders et al., 1997; Fuchs et al., 1988).

Of note, sympathetic nervous system can modulate differentially the immune response, exercising both an anti-inflammatory and pro-inflammatory effect mediated by α and β adrenergic receptors expressed on the surface of immune cells (Bellinger et

al., 2008). Furthermore, in a previous report by Ariki and Husband (1998) it has been demonstrated that peripheral sympathetic nervous system influences the migration and accumulation *in vivo* of both naive and memory/effector lymphocytes in intestinal lamina propria, suggesting a key role played by sympathetic nervous system in the intestinal immune responses (Ariki and Husband, 1998).

In the past decade, the parasympathetic system has been also proposed as a crucial regulator of inflammatory responses (Matteoli and Boeckxstaens, 2013). In particular, the vagus nerve, referred as the "cholinergic anti-inflammatory pathway", exerts tonic anti-inflammatory actions and contributes to the maintenance of immune homeostasis (Borovikova et al., 2000; Ghia et al., 2006). Tracey and colleagues (2000) were the first to suggest that vagal efferents regulated inflammatory responses, directly inhibiting TNF production by macrophage via activation of α7 nicotinic receptors (nAChRs). In particular, nicotinic receptor activation on macrophage surface reduced NF-κB activation and pro-inflammatory cytokine production in experimental sepsis (Wang et al., 2004).

Recent evidence suggests that the vagal innervation of the gastrointestinal tract holds a relevant role in the modulation of intestinal immune response. Indeed, vagal, parasympathetic preganglionic fibres, originating from DMV, innervate primary and secondary immune organs and synapse with postganglionic neurons within the myenteric plexus. In particular, vagal innervation has the highest density in the duodenum and the lowest density in the distal part of the ileum. The large intestine receives parasympathetic innervation from two distinct sources: the vagus nerve that innervates the proximal colon, and the parasympathetic spinal nerves originating in the sacrum that provide neural input to the distal colon (Berthoud et al., 1990).

Several studies displayed that vagal electrical stimulation potently reduces intestinal inflammation restoring intestinal homeostasis, whereas vagotomy confers an increased susceptibility to the development of inflammatory bowel diseases (Willemze et al., 2015).

However, despite vagus nerve does not innervate directly the distal portion of the colon, it has been observed that vagal nerve stimulation attenuates the severity of tissue lesions and inflammation in distal colon of rats with experimental colitis (Meregnani et al., 2011), suggesting that vagal-parasympathetic nerve pathway regulates mucosal inflammation and drives innate immune cells into a tolerogenic phenotype (Pabst and Mowat, 2012).

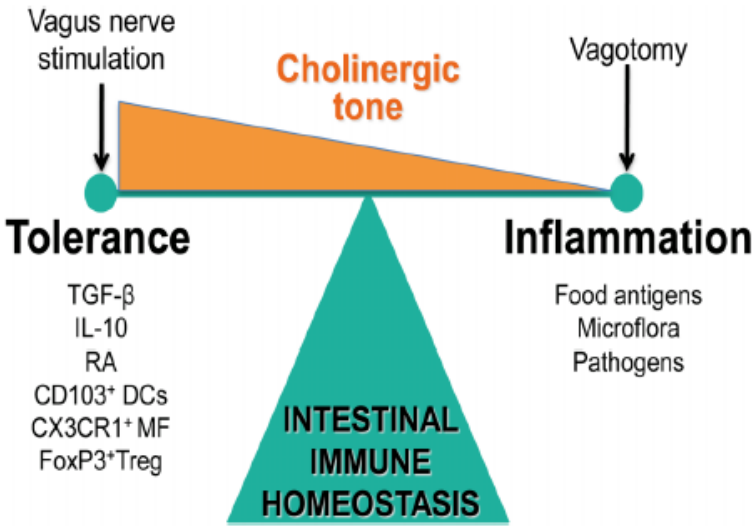


Figure 5. Vagal pathway regulates intestinal immune homeostasis. The cholinergic tone determines immune homeostasis either shifting the balance towards immune tolerance or inflammation.
G. Matteoli and G. Boeckxstaens. The vagal innervation of the gut and immune homeostasis. *Gut* 2013.

GASTROINTESTINAL DYSFUNCTIONS IN PATIENTS WITH PARKINSON'S DISEASE

Most PD patients experience GI dysfunctions, including delayed gastric emptying, dysphagia, chronic constipation and anorectal dysfunction, which occur in the advanced disease, but also at early stages, resulting in a deterioration of the quality of life and consistent burden for health care costs (Pfeiffer 2003). GI disturbances, which often precede the onset of motor symptoms by many years, seem to be associated with an increased risk of developing PD in otherwise healthy people (Cersosimo, Raina et al. 2013). Furthermore, the presence of α-syn inclusions (LBs) in neurons of myenteric and submucosal plexuses in the GI tract, and DMV of PD patients led Braak to postulate the hypothesis that the ENS could be pivotally involved in the pathophysiology of neurodegenerative process, and represent an early target of PD neurodegeneration (Wakabayashi and Takahashi 1997, Braak, de Vos et al. 2006, Hawkes, Del Tredici et al. 2007). In this context, a number of clinical investigations have attempted to unravel the pathophysiological mechanisms underlying GI alterations occurring in PD patients.

Stomach

Upper GI motor abnormalities are reported in up to 100% of patients with PD both at early and advanced stages of the disease, and account for symptoms such as early satiety, bloating, and nausea (Djaldetti, Baron et al. 1996, Marrinan, Emmanuel et al. 2014).

Current clinical evidence suggests that the presence of α-syn inclusions in the myenteric neurons, as well as alterations of neuro-hormonal brain-gut axis could play a role in the pathogenesis of gastric dysmotility associated with PD (Pellegrini et al., 2015).

Braak and colleagues (2006) observed α-syn immunoreactive inclusions in neurons of the gastric myenteric and submucosal plexuses, that were associated with a damaged enteric neural network, suggesting the accumulation of α-syn in the ENS as a key step in the pathogenesis of gastric motor abnormalities associated with central nigrostriatal neurodegeneration (Braak, de Vos et al. 2006, Suzuki, Kurita et al. 2006, Tanaka, Kato et al. 2012).

Interestingly, studies in PD patients have documented alterations of hormone pathways involved in the control of gastric motor functions (Sanger and Lee 2008, Khoo, Rayner et al. 2010). In particular, a significant association has been observed between PD and polymorphisms of cholecystokinin (CCK) gene, that produced by I cells located in the mucosal epithelium of small intestine and neurons of the autonomic nervous system and CNS, and is known to inhibit gastric emptying via CCK receptors located in the CNS and peripheral endings of the vagus nerve (Raybould and Tache 1988, Fujii, Harada et al. 1999). In addition, a significant decrease in ghrelin levels, that stimulates gastric motility and several brain functions, including food intake and energy balance, were observed in patients with both PD and idiopathic REM sleep behavior disorder, a condition regarded as a putative early stage of PD (Wren, Seal et al. 2001, Williams, Grill et al. 2003, Unger, Moller et al. 2011). Despite these interesting findings, the pathophysiological mechanisms underlying gastric motor dysfunctions associated with PD remain still unclear.

Small bowel

Intestinal dysmotility and infrequent bowel movements represent the main bowel disturbances in patients with PD (Pellegrini et al., 2015) (Pfeiffer 2003). However, scarce information are available about the pathophysiology of small bowel

dysfunctions associated with PD. Although the presence of α–syn accumulation has been detected in duodenal and ileal biopsies from PD patients at different stages of the disease, no significant changes in the number of myenteric neurons or alterations of NO, VIP, dopamine and catecholamine neuronal density have been detected (Annerino, Arshad et al. 2012, Hilton, Stephens et al. 2014).

Over the last years, research efforts have been focused on the role played by enteric microbiota in GI dysfunctions associated with PD (Naseer, Bibi et al. 2014, Severance, Yolken et al. 2014). In particular, it the occurrence of both small intestine bacterial overgrowth (SIBO) and malabsorption syndrome, characterized by increased bacterial density in the small bowel, with frequent impairment of GI motility, have been observed in PD patients (Zietz, Lock et al. 2000, Gabrielli, Bonazzi et al. 2011). However, the actual involvement of intestinal microbial flora, as well as the possible role of the brain-gut-microbiota axis in the pathogenesis of GI abnormalities in PD is unclear and open to further investigations.

Large bowel

Chronic constipation is the most widely recognized gut disorder associated with PD (Dillmann, Kratz et al. 2003). PD patients with constipation are characterized by infrequent bowel movements, impairment of propulsive colonic motility and prolonged colonic transit time as well as reduced rectal contractions and abnormalities in the motor activity of anal sphincter (Pfeiffer 2011). The pathogenic mechanisms underlying constipation associated with PD are presently unclear. Most of clinical investigations have evaluated changes in the colonic submucosal and myenteric neurons of PD patients at different stage of the disease. Singaram et al., (1995) observed that the number of dopamine immunopositive neurons in the myenteric

plexus were reduced, as well as the dopamine content in the muscularis externa, suggesting an impairment of dopaminergic neurotransmission in PD human colon (Singaram, Ashraf et al. 1995). However, subsequent studies, thet analyzed nitrergic, VIP-ergic, dopaminergic and noradrenergic neuronal markers in colonic biopsies from PD patients, did not reveal significant variations in the density of inhibitory myenteric neurons, suggesting that colonic inhibitory pathways are not significantly affected in the presence of PD (Lebouvier, Chaumette et al. 2008, Annerino, Arshad et al. 2012, Corbille, Coron et al. 2014).

Although no relationships between colonic LBs, LNs and bowel neuromuscular functions during PD have been mechanistically established, two recent studies have observed the presence of aggregated α-syn in colonic myenteric cholinergic and substance P-containing neurons of PD patients at an early stage of the disease, suggesting that such alterations could take part to the onset and development of colonic dysmotility in PD (Shannon, Keshavarzian et al. 2012, Sharrad, Gai et al. 2013).

Over the last years, several lines of evidence underscored the role played by central and peripheral neuroinflammation in the pathophysiology of PD (Gonzalez, Elgueta et al. 2014). Indeed, an increment of pro-inflammatory cytokine levels, glial cell activation and increased oxidative stress were observed in brain tissues and cerebrospinal fluid of PD patients, thus suggesting that central neuroinflammation may contribute to substantia nigra degeneration and accelerate the disease progression (Blum-Degen, Muller et al. 1995, Reale, Iarlori et al. 2009, Hirsch, Vyas et al. 2012). Interestingly, the occurrence of neuroinflammatory condition has been also observed in colonic tissues obtained from PD patients at different stages of the disease (Devos, Lebouvier et al. 2013, Clairembault, Kamphuis et al. 2014). In particular, both Devos

et al. (2013) and Clairembault et al. (2014) detected a significant increase in pro-inflammatory cytokine levels (TNF, IL-1β) and enteric glial activation (SOX-10) in colonic biopsies from PD patients (Devos, Lebouvier et al. 2013, Clairembault, Kamphuis et al. 2014).

Overall, current knowledge points out that enteric inflammation could play a key role in the pathogenesis of bowel dysfunctions associated with PD, even though these alterations have not been linked yet to the pathogenesis of colonic motor dysfunctions.

GASTROINTESTINAL DYSFUNCTIONS IN EXPERIMENTAL MODELS OF PARKINSON'S DISEASE

The implementation of animal models of PD have allowed a better characterization of the pathophysiological mechanisms underlying GI motor dysfunctions associated with central nigrostriatal neurodegeneration. Current pre-clinical models of PD can be divided in accordance with two different approaches: 1) peripheral induction of PD by systemic administration of neurotoxins; 2) induction of nigrostriatal denervation by central injection of neurotoxins. In a recent review, {Pellegrini, 2015 #111} described the main features of PD models employed in studies on the evaluation of GI dysfunctions.

Gastrointestinal alterations in models of peripheral PD induced by systemic administration of neurotoxins

In PD models induced by systemic injection of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), rotenone or salsolinol, GI motor alterations have been ascribed to direct effects of neurotoxins on enteric neurons (Banach, Zurowski et al. 2005, Anderson, Noorian et al. 2007, Pan-Montojo, Anichtchik et al. 2010).

Upper gastrointestinal tract

In the upper GI tract, scarce and conflicting results are available about the alterations of gastric motility in experimental PD induced by peripheral injection of toxins. Indeed, no differences were detected in either liquid or solid gastric emptying or overall GI transit time in PD induced by MPTP and rotenone in mice (Anderson, Noorian et al. 2007, Tasselli, Chaumette et al. 2013). By contrast, two studies by (Drolet, Cannon et al. 2009, Greene, Noorian et al. 2009) showed a significant slowing of gastric emptying in PD rats 6 months after intraperitoneal rotenone injection, suggesting that nigrostriatal denervation can influence gastric motor functions on long-term.

Lower gastrointestinal tract

With regard for small bowel, no significant alterations of transit were observed in MPTP mice (Anderson, Noorian et al. 2007). Conversely, two subsequent reports described a delay of *in vivo* colonic transit and constipation in rotenone- and MPTP-induced neurodegeneration in mice (Greene, Noorian et al. 2009, Natale, Kastsiushenka et al. 2010). In addition, functional *in vitro* experiments showed an increase in contractile activity and an impaired relaxation in the proximal colon from MPTP mice, suggesting a decreased inhibitory control of colonic motility (Anderson, Noorian et al. 2007, Greene, Noorian et al. 2009).

Interestingly, neurochemical and molecular studies have shown that PD induced by peripheral injection of neurotoxins is associated with an impairment of the dopaminergic pathway throughout the whole GI tract. In particular, Tian et al. (Tian, Chen et al. 2008) observed a significant decrease in myenteric neurons expressing

tyrosine hydroxylase (TH), a specific marker of dopaminergic neurons. Likewise, mice treated with MPTP displayed a significant decrease in the number of dopamine transporter (DAT) and TH-positive neurons in both myenteric and submucosal plexus of duodenum and ileum (Anderson, Noorian et al. 2007). These findings are in keeping with a subsequent report by Natale et al. (Natale, Kastsiushenka et al. 2010), who observed a significant reduction of dopamine content in the duodenum of MPTP-treated mice, thus suggesting an impairment of small bowel dopaminergic neurotransmission. However, the molecular mechanisms underlying GI motor abnormalities in PD remain still unclear and opened to further investigations.

Gastrointestinal alterations in experimental Parkinson's disease induced by central administration of neurotoxins

Several studies have provided evidence about GI alterations occurring in experimental PD induced by central injection of neurotoxins, that allow to evaluate the impact of nigrostriatal denervation on GI motor functions (Pellegrini et al., 2015).

Upper gastrointestinal tract

A delayed gastric emptying and alterations of gastric motor activity *in vivo* were observed in rats with PD induced by intranigral 6-OHDA injection (Zheng, Wang et al. 2011) (Zhu, Zhao et al. 2012, Toti and Travagli 2014, Vegezzi, Al Harraq et al. 2014). These findings have been corroborated by *in vitro* functional studies showing a reduced gastric motility in 6-OHDA-treated rats (Zheng, Wang et al. 2011, Zheng, Zhang et al. 2013, Zheng, Song et al. 2014). However, scarce evidence is available about the contribution of putative changes in specific nerve pathways on gastric motor abnormalities associated with central neurodegeneration. In this respect, (Song, Zheng

et al. 2014) revealed an enhanced relaxant effect mediated by exogenous noradrenaline in 6-OHDA rats through β_1 adrenoceptors located on smooth muscle cells. Moreover, an involvement of enteric dopamine in gastric dysmotility associated with central dopaminergic neurodegeneration has been highlighted by Zheng et al. (Zheng, Song et al. 2014), who observed that the application of exogenous dopamine to gastric smooth muscle preparations isolated from 6-OHDA rats resulted in a greater inhibition of spontaneous contractile activity, likely driven by D_2 receptors (Zheng, Song et al. 2014). However, conclusive evidence supporting alterations of excitatory or inhibitory motor pathways, including cholinergic and tachykininergic systems or nitrergic pathways, are currently lacking.

Neurochemical and molecular investigations have confirmed the involvement of dopaminergic pathways in the gastric dysfunctions during PD. In particular, two studies by (Tian, Chen et al. 2008) and (Zhu, Zhao et al. 2012) showed an increased immunoreactivity for both TH and dopamine transporter (DAT) in gastric neurons after bilateral and unilateral 6-OHDA injection. Further evidence was provided also by Zheng et al., (Zheng, Song et al. 2014), who observed an increased expression of dopaminergic D_2 receptors along with a marked increment of dopamine content in the gastric muscularis externa of 6-OHDA rats (Zheng, Wang et al. 2011, Zheng, Song et al. 2014), suggesting an enhancement of gastric dopaminergic inhibitory neurotransmission. However, despite these interesting findings, the actual involvement of dopaminergic pathways in the alterations of gastric motor activity during PD remains unclear and should be explored further.

Current evidence suggests also an impairment of the nitrergic network in the gastric myenteric plexus under experimental PD. In particular, a loss of inhibitory nitrergic control might result in an altered gastric antrum relaxation, with consequent

impairment of gastric emptying (Zhu, Zhao et al. 2012, Zheng, Song et al. 2014). With regard for changes in gastric cholinergic neurons in models of PD induced by intranigral injection of toxins, conflicting findings have been reported. In general, it appears that bilateral intranigral injection of 6-OHDA in rats is associated with an impairment of enteric cholinergic neurotransmission, which could take a significant part in the alterations of gastric motor functions, since a reduced density of myenteric cholinergic neurons, resulting in a significant decrease of acetylcholine concentration in the gastric muscularis externa, was observed in this PD model (Zheng, Wang et al. 2011, Zheng, Song et al. 2014).

Lower gastrointestinal tract

Scarce evidence are available about alterations of intestinal motility occurring in the presence of central dopaminergic denervation. Indeed, only one study have shown a delayed transit in the distal region of small bowel, suggesting that central dopaminergic denervation is associated with an impaired motility in this gut region (Vegezzi, Al Harraq et al. 2014).

Current data suggest that in the presence of central dopaminergic neurodegeneration small bowel transit is impaired, likely as a consequence of alterations in chemical coding of enteric inhibitory neurons (Vegezzi et al., 2014). The majority of studies, aimed at investigating the neuronal pathways involved in intestinal dysmotility associated with PD induced by central toxins, have examined the alterations of dopaminergic, nitrergic and VIPergic pathways in intestinal tissues from rats with 6-OHDA-induced nigrostriatal denervation. In particular, a decrease in the density of nNOS positive neurons, along with an increased percentage of VIP-immunoreactive neurons, as well as dopaminergic neuronal markers (TH and DAT) in the myenteric plexus of distal ileum, was observed in 6-OHDA rats (Tian, Chen et al. 2008, Colucci,

Cervio et al. 2012). By contrast, no significant alterations were detected in the number of cholinergic enteric neurons in the small bowel (Colucci, Cervio et al. 2012).

In the central 6-OHDA model, an altered pattern of colonic longitudinal muscle contraction and a reduced peak pressure have been detected, suggesting that central dopaminergic neurodegeneration is associated with an impairment of colonic motility resulting in a reduced efficiency of peristaltic reflex (Zhu, Zhao et al. 2012) (Colucci, Cervio et al. 2012). Neurochemical and molecular studies, aimed at indentifying the enteric neuronal pathways involved in colonic dysmotility associated with PD, have shown alterations of inhibitory enteric nerve pathways. In particular, increments of VIPergic and dopaminergic neurons, in concomitance with a decrease in nitrergic neurons, were detected in the proximal colon (Blandini, Balestra et al. 2009, Colucci, Cervio et al. 2012, Zhu, Zhao et al. 2012). In addition, Colucci et al. (Colucci, Cervio et al. 2012) showed that dopamine D_2 receptors were mostly expressed in enteric cholinergic and dopaminergic neurons, and that their immunoreactivity was markedly reduced in myenteric neurons of both proximal and distal colon (Zhu, Zhao et al. 2012). With regard for the colonic excitatory cholinergic system, no changes in the density of ChAT-positive neurons, as well as in ChAT protein and mRNA expression levels in the proximal colon from 6-OHDA rats have been detected (Colucci, Cervio et al. 2012, Zhu, Zhao et al. 2012).

Overall, central nigrostriatal neurodegeneration is associated with an impairment of colonic motility, leading to a reduced efficiency of peristaltic reflex. Such alterations seem to result from a rearrangement in the chemical coding of enteric inhibitory neurons, since a loss of neurons in the colonic myenteric plexus has not been detected. However, the role played by specific enteric excitatory nerve pathways in colonic

dysmotility associated with PD remains still unclear and deserves further investigations.

EXPERIMENTAL SECTION

Introduction

It is widely recognized that patients with PD experience non-motor symptoms, including functional GI disturbances, such as dysphagia, abdominal distension and severe constipation, which impair their quality of life (Pfeiffer 2011). Despite GI dysfunctions have a major impact on the clinical picture of PD, there is currently a lack of information on the neurochemical, pathological and functional correlates of GI dysmotility associated with PD. Moreover, there is a need of effective and safe pharmacological therapies for managing GI disturbances in PD patients.

Recent observations suggest that GI motor dysfunctions associated with PD might depend on an impairment of the enteric nervous network. Indeed, the ENS, that is deputed to the local control of GI motility, appears to be involved in PD, as confirmed by the presence of LB in enteric neurons of PD patients (Braak, de Vos et al. 2006). A reduced density of enteric dopaminergic neurons has been reported also in the ENS of PD patients, suggesting an involvement of inhibitory dopaminergic nerve pathway in the pathophysiology of PD-related GI dysfunctions (Singaram, Ashraf et al. 1995). Moreover, the occurrence of colonic neuroinflammatory conditions in PD patients strengthens the role of peripheral inflammation in the initiation and/or progression of the disease, paving the way to new hypotheses about the pathophysiological mechanisms underlying GI disturbances in PD (Devos, Lebouvier et al. 2013). However, it remains to be established whether other excitatory or inhibitory GI nerve pathways are involved in PD, and whether enteric neuroinflammation can play a role in the pathogenesis of GI dysmotility in PD. Based on the above background, the present research project has been undertaken to investigate the relationships between PD and related GI dysfunctions by means of investigations in an animal model of PD. In particular, based on the notion that chronic constipation occur in up to 80% of

patients with PD (Natale, Pasquali et al. 2008, Petrovitch, Abbott et al. 2009), the present research project has investigated the enteric nerve pathways involved in colonic dysmotility associated with central dopaminergic impairment induced by 6-OHDA. As already discussed in the state-of-the-art section, this model is particularly suitable for a wide spectrum of neurochemical and functional investigations, which may provide relevant information on GI dysfunctions resulting from altered CNS/ENS cross-talk. In particular, the injection of 6-OHDA into SNpc or into medial forebrain bundle, that conveys efferent fibers from nigral cell bodies to the striatum, leads to massive anterograde degeneration of the nigrostriatal pathway within 2-3 days. Indeed, 6-OHDA accumulates in nigrostriatal dopaminergic neurons, promoting hydrogen peroxide formation, as well as in mitochondria, where it inhibits the complex I activity (Blandini and Armentero 2012, Blesa, Phani et al. 2012).

The use of the 6-OHDA experimental model of PD in the present program has allowed to pursue the following goals: 1) to examine the impact of central dopaminergic denervation on colonic excitatory cholinergic and tachykininergic neuromotility by means of molecular, histomorphologic and functional approaches; 2) to elucidate the role of gut inflammation in the onset and progression of colonic dysmotility associated with PD, characterizing the degree of inflammation and oxidative damage in colonic tissues, as well as identifying the immune cells involved in the production of pro-inflammatory cytokines in the gut; 3) to evaluate the impact of chronic treatment with L-DOPA plus benserazide on colonic neuromuscular activity both in control and PD animals.

Materials and Methods

Animals

Albino 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 and sacrifice

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, Katritsis et al. 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 sacrificed 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, Armentero 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 were counted using stereological analysis. Unbiased stereological estimation was made using the optical fractionator method (West, Slomianka 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, Katritsis et al. 1998). TH-positive cells in the SNc were counted in every fourth sections, 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. Guard volumes (3 µm from the top and 3 µm from the bottom of the section) were excluded from both surfaces to avoid the problem of lost caps. Schaeffer's estimated coefficient of error ranged between 0.05 and 0.12 for the SNc of sham-operated animals. For SNc of 6-OHDA animals, it ranged between 0.4 and 0.9, reflecting the scarceness of TH-positive cells in this area. Results represent the percentage of the number of TH-positive neurons in the injected SNc with respect to the intact side (neuron survival).

Radiological assessment of colonic transit

The radiological assessment of overall *in vivo* colonic transit was peformed as described by Vegezzi et al. (Vegezzi, Al Harraq 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. Each rat was housed in a restrainer (Plexiglas tube-shaped cage closed at the extremities), adjusted to the size of the animal, so that it could easily accommodate and not move, escape or turn around, to avoid the use of any anaesthesia. The analysis of radiographic images was carried out according to the scoring proposed by (Cabezos, Vera 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 and scored, to obtain an overall value ranging from 0 to 12.

Recording of colonic contractile activity in vitro

The contractile activity of colonic longitudinal smooth muscle was recorded as previously described (Antonioli, Fornai et al. 2014). After sacrifice, 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.

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), and 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) or atropine (1 μM) , in order to examine the patterns of colonic contractions driven by excitatory nerve cholinergic and tachykininergic pathways, respectively.

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).

In the fourth series, colonic tachykininergic contractions where evoked by direct pharmacological activation of tachykininergic NK_1 receptors located on smooth muscle cells. For this purpose, colonic preparations were maintained in Krebs solution containing tetrodotoxin (TTX, 1 μM), NK_2 receptor antagonist (1 μM), and NK_3 receptor antagonist (1 μM) and stimulated with exogenous substance P (SP, 0.1 – 10 μM).

Measurement of acetylcholine release from colonic longitudinal muscle preparations

Longitudinal muscle strips of colon, containing the Auerbach 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. 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, Inoue et al. 2011), with some modifications. Variations in acetylcholine release upon application of electrical stimulation were expressed 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 from formalin-fixed full-thickness colonic samples were processed for immunostaining, as described by Ippolito et al. (Ippolito, Segnani et al. 2015). Briefly, sections were incubated overnight at 4°C with primary antibodies against 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). Sections 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 ganglionic area. ChAT expression was evaluated as percentage of the total ganglionic tissue area examined (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. (Ippolito, Segnani 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, Han 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).

Immunohistochemistry of SP and NK_1

Sections from formalin-fixed full-thickness colonic samples were processed for routine staining (haematoxylin and eosin) and immunoperoxidase, as previously described (Ippolito et al., 2014). Briefly, sections were incubated overnight at 4°C with primary antibodies against SP (code n. Sc-21715, Santa Cruz Biotech, California, USA) and NK_1 (code n orb 11133 Biorbyt Limited, Cambridge, United Kingdom), and then exposed to appropriate biotinylated immunoglobulins, peroxidase-labeled streptavidin complex, and 3.3'-diaminobenzidine tetrahydrochloride (DakoCytomation, Glostrup, Denmark). SP- and NK_1-immunostained tissues 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. SP and NK_1 expression was evaluated as percentage of positive pixels (PPP) calculated on the total area examined of myenteric ganglia (MG) and the whole *tunica muscularis*, respectively.

Histology and Immunohistochemistry of GFAP

Sections from formalin-fixed full-thickness colonic samples were processed for routine staining (haematoxylin and eosin) and immunoperoxidase, as previously described (Ippolito et al., 2014). Briefly, sections were incubated overnight at 4°C with primary antibodies against GFAP (code n. Z0334, Dakocytomation, Glostrup, Denmark), and then exposed to appropriate biotinylated immunoglobulins, peroxidase-labeled streptavidin complex, and 3.3'-diaminobenzidine tetrahydrochloride

(DakoCytomation, Glostrup, Denmark). GFAP- immunostained tissues 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. GFAP expression was evaluated as percentage of positive pixels (PPP) calculated on the total area examined of myenteric ganglia (MG).

Immunohistochemistry of eosinophils and mast cells

Sections from formalin-fixed full-thickness colonic samples were processed for routine staining (haematoxylin and eosin) and immunoperoxidase, as previously described (Ippolito et al., 2014). Briefly, sections were incubated overnight at 4°C with primary antibodies c-Kit (code n. PC34, Calbiochem, Darmstadt, Germany), and then exposed to appropriate biotinylated immunoglobulins, peroxidase-labeled streptavidin complex, and 3.3'-diaminobenzidine tetrahydrochloride (DakoCytomation, Glostrup, Denmark). The density of eosinophils and mast cells was evaluated in *tunica mucosa/submucosa* and *tunica submucosa*, respectively: cells were counted in 3 different sections for each rat and at least 20 randomly selected microscopic fields were examined in each section (objective, 40x). The values from all the fields examined for each rat were averaged and expressed as cell number per mm^2 of *tunica mucosa/submucosa* (eosinophils) or *tunica submucosa* (mast cells) areas, which were calculated by the Image Analysis System 'L.A.S. software v.4'. These values were used to calculate the mean values for each experimental groups. Data were given as mean ± S.E.M. (n=6 animals/group).

Isolation peritoneal macrophages

Rat peritoneal macrophages were harvested as previously described (Pinhal-Enfi et al., 2003). Briefly, controls and 6-OHDA rats 4 and 8 weeks after nigrostriatal lesion rats were sacrificed by cervical dislocation and peritoneal macrophages were harvested and cultured as a monolayer in RPMI 1640 medium (Cellgro, Mediatech Inc., Herndon, VA), supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA), 2 mM L-glutamine, 100 IU/ml Penicillin, and 100 μg/ml Streptomycin (Irvine Scientific, Santa Ana, CA). Monolayers were washed 4 hours after plating to remove any non-adherent cells. The cultures were shown to be >98% pure macrophages, as assessed by non-specific esterase staining and staining with the macrophage-specific F4/80 mAb (data not shown).

RNA Extraction and Real-Time Quantitative PCR Analysis (RT-qPCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Life Technologies, Thermo Fisher Scientific Inc., CA, USA) according to the manufacturer's instructions. The concentration of isolated RNA was determined with a spectrophotometer and finally two micrograms of RNA were used for reverse transcription (RT) procedure (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Thermo Fisher Scientific Inc., CA, USA). RT was performed in a mixture of 2 μL RT buffer, 0.8 μL dNTP, 2 μL random primer, 1 μL reverse transcriptase and nuclease-free water (Amresco LLC, Solon, USA) up to 10 μL. PCR cycles (Veriti 96-Well Thermal Cycler, Applied Biosystems, Thermo Fisher Scientific Inc., CA, USA) were set as follows: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min. Ten-fold dilution of cDNA was used for RT-qPCR procedure. The qPCR reactions were performed with the following primers: TNF-α 5'-CCTCACACTCAGATCATCTTC-3' (sense) and 5'-

GCTACGGGCTTGTCACTCG-3' (anti-sense); iNOS 5'-CCTTGTTCAGCTACGCCTTC-3' (sense) and 5'-CCAGGCCAAATACCGCATAC-3' (anti-sense); Arginase-1 5'-GGACATCGTGTACATCGGCT-3' (sense) and 5'-GGGCCTTTTCTTCCTTCCCA-3' (anti-sense); TGF-β 5'-GACGTCACTGGAGTTGTCC-3' (sense) and 5'-TTCATGTCATGGATGGTGC-3' (anti-sense), 18S 5'-GGGAGCCTGAGAAACGGC-3' (sense) and 5'-GGGTCGGGAGTGGGTAATTTT-3' (anti-sense). SYBR Green (Applied Biosystems, Thermo Fisher Scientific Inc., CA, USA) qPCR reactions were run in LightCycler 480 II Real-Time PCR Instrument (Roche, Basel, Switzerland). Expression values were normalized to the housekeeping gene 18S expression.

Evaluation of tissue malondialdehyde levels (MDA)

MDA concentration in specimens of colonic neuromuscular tissues was evaluated to obtain a quantitative estimation of membrane lipid peroxidation, and was performed as previously described (Antonioli, Fornai et al. 2007). Colonic tissues were weighed, minced by forceps, homogenized in 2 ml of cold buffer (20 mM Ripa buffer, pH 7.4) by a polytron homogenizer (QIAGEN), and spun by centrifugation at 1600g for 10 min at 4°C. Colonic MDA concentrations were determined with a kit for colorimetric assay (Calbiochem, San Diego, CA), and the results were expressed as nmol of MDA per milligram of colonic tissue.

Evaluation of tissue TNF levels

TNF levels in colonic neuromuscular tissues were measured with enzyme-linked immunosorbent assay kits (Abcam), as previously described (Antonioli, Fornai et al. 2007). For this purpose, colonic tissue samples, stored previously at -80°C, were weighed, thawed, and homogenized in 0.4 ml of PBS, pH 7.2/20 mg of tissue at 4°C,

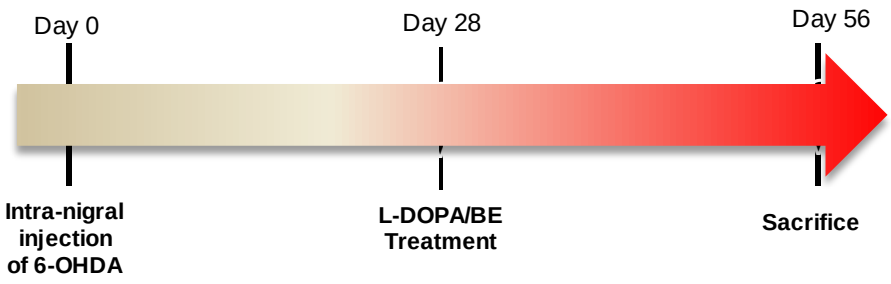
and centrifuged at 10,000g for 5 min. Aliquots (100 µL) of the supernatants were then used for assay. Tissue TNF levels were expressed as picogram per gram of tissue.

Evaluation of tissue IL-1β levels

IL-1β levels in colonic neuromuscular tissues were measured with enzyme-linked immunosorbent assay kits (Abcam), as previously described (Antonioli, Fornai et al. 2007). For this purpose, colonic tissue samples, stored previously at -80°C, were weighed, thawed, and homogenized in 0.4 ml of PBS, pH 7.2/20 mg of tissue at 4°C, and centrifuged at 10,000g for 5 min. Aliquots (100 µL) of the supernatants were then used for assay. Tissue IL-1β levels were expressed as picogram per gram of tissue.

Treatment with L-DOPA/BENSERAZIDE (BE) and recording of colonic contractile activity in vitro

6-OHDA or control animals were treated orally with L-DOPA/BE (6/15 mg/Kg/day) or their vehicle for 28 days, starting 28 days after intra-nigral injection.



At the end of drug treatment, colonic longitudinal and circular muscle preparations were isolated, set up in organ baths, and connected to isometric transducers (as described above) to record neurogenic contractions (g/g tissue) elicited by electrical stimulation (ES, 1-20 Hz) under the following conditions: 1) Krebs solution containing

guanethidine (10 μM), L-NAME (100 μM) and L-732,138 (NK_1 receptor antagonist, 10 μM), GR159897 (NK_2 receptor antagonist, 1 μM) and SB218795 (NK_3 receptor antagonist, 1 μM) to record cholinergic contractile responses; 2) Krebs solution containing L-NAME, guanethidine and atropine (1 μM) to obtain contractions driven primarily by tachykinins. Myogenic contractions elicited by carbachol (CCh, 0.01 – 100 μM) or exogenous substance P (SP, 0.1 – 10 μM) in the presence of tetrodotoxin (1 μM) were also recorded.

Measurement of acetylcholine release from colonic longitudinal muscle preparations in 6-OHDA rats treated with L-DOPA/BE

As described above longitudinal muscle strips of colon, containing the Auerbach 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. 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, Inoue et al. 2011), with some modifications. Variations in acetylcholine release upon application of electrical stimulation were expressed 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.

Drugs and solutions

Atropine sulphate, guanethidine monosulphate, carbachol chloride, N^{ω}-nitro-L-arginine methylester, substance P, 6-hydroxydopamine, ascorbic acid, dihydroxy-l-phenylalanine (L-DOPA) and benserazide (Sigma Chemicals Co., St. Louis, MO, USA). Tetrodotoxin, L-732,138, GR159897 and SB218795 (Tocris, Bristol, UK); mouse anti-TH antibody (Chemicon International, Temecula, CA, USA), biotinylated anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA), nickel-intensified 3,3′-diaminobenzidine tetra-hydrochloride (DAB Substrate Kit for Peroxidase, Vector Laboratories), xylene (Carlo Erba, Milan, Italy)

Statistical analysis

The results are presented as mean ± SEM. The significance of differences was evaluated by Student t test for unpaired data or one-way analysis of variance (ANOVA) followed by post-hoc analysis with Student-Newman-Keuls test, and 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 the brain

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. 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 rate

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. Both 10 and 12 h after gavage with $BaSO_4$, the scores of control and 6-OHDA rats, estimated for caecum radiographs, were higher than colorectal values, indicating that the contrast medium had reached the large bowel (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 recording of colonic motility

During equilibration in standard Krebs solution, most colonic preparations displayed rapid spontaneous activity, which was low in amplitude and generally stable throughout the experiment. Electrically evoked responses consisted of phasic contractions followed, in some cases, by after-contractions of variable amplitude (not shown).

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 (Figure 3A). Likewise, in colonic circular muscle preparations from 6-OHDA rats after 4 or 8 weeks, the electrically evoked contractile activity was significantly reduced at all tested frequencies (Figure 3B).

Study on colonic cholinergic pathway

In vitro recording of colonic motility

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. Under these conditions, electrically evoked cholinergic contractions were decreased at both week 4 and 8, as compared with controls (Figure 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, which were significantly enhanced in preparations from rats at both 4 and 8 weeks after 6-OHDA injection, as compared to controls (Figure 5A, 5B).

Acetylcholine release from colonic longitudinal muscle preparations

In aliquots of Krebs solution collected from incubation baths 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. After application of ES to colonic preparations from 6-OHDA animals, acetylcholine release was lower as compared with controls, since it increased by +30% at week 4 and +28% at week 8 versus the baseline values assessed at -60 s (Figure 6B).

ChAT expression in the colonic myenteric plexus

A strong, cytoplasmic and/or nuclear HuC/D immunostaining was detected in myenteric neurons of colon from both 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) (Figure 7).

Expression of muscarinic M_2 and M_3 receptors in neuromuscular tissues and isolated smooth muscle cells

Western blot analysis revealed the basal 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 (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 (Figure 8B).

Study on colonic tachykininergic pathway

In vitro recording of colonic motility

In longitudinal (Figure 9A) and circular (Figure 9B) colonic preparations, maintained in Krebs solution containing L-NAME (100 μM), guanethidine (10 μM), atropine (1 μM), GR159897 (NK_2 receptor antagonists, 1 μM), and SB218795 (NK_3 receptor antagonists, 1 μM), electrically evoked L-732.138-sensitive tachykininergic contractions were enhanced both at 28 days and 56 days, in comparison with controls (Figure 9).

The exposure of longitudinal (Figure 10A) and circular (Figure 10B) colonic preparations to exogenous substance P (0.001-10 μM) in the presence of tretrodotoxin (1 μM), evoked concentration-dependent contractile effects, which appeared enhanced in rats at 28 and 56 days after 6-OHDA injection, in comparison with controls (Figure 10).

SP and tachykininergic NK_1 receptor expression in the colonic myenteric plexus

The SP immunopositivity displayed by colonic myenteric ganglia was significantly increased in rats with 6-OHDA-induced nigrostriatal denervation at both weeks 4 and 8 as compared to controls (Figure 11). Likewise, the NK_1 receptor expression was significantly enhanced in 6-OHDA rats both at 4 and 8 weeks following central nigrostriatal denervation (Figure 11).

Colonic neuroinflammation in 6-OHDA rats

GFAP expression in colonic myenteric ganglia and analysis of inflammatory cells in tunica mucosa/submucosa

The GFAP immunopositivity displayed by glial cells of control colonic myenteric ganglia was significantly increased in rats with 6-OHDA-induced nigrostriatal denervation at both weeks 4 and 8 (Figure 12).

Eosinophils, which were frequently found within the *tunica mucosa* and *submucosa* of normal colon, were significantly increased in both groups of 6-OHDA rats, which displayed also sporadic eosinophils in the *tunica muscularis* and along the myenteric ridge. Likewise mast cells, which were occasionally detected in controls within the perivascular connective tissue of the *tunica mucosa*, *submucosa* and *serosa*, were increased in density in 6-OHDA-treated rats at both time points (Figure 13).

Pro-inflammatory polarization of peritoneal macrophages

As previously described, peritoneal macrophages from control rats expressed higher levels of iNOS mRNA than Arginase-1 mRNA (Li et al., 2012). In peritoneal macrophages obtained by 6-OHDA rats at 4 and 8 weeks the iNOS/Argenase-1 expression ratio (reminiscent of macrophage M1/M2 polarization) is significantly increased in comparison with control rats (12.34±2.4 and 15.57±3.7, respectively) (Figure 14).

Assessement of MDA, TNF and IL-1β levels in colonic neuromuscular tissues

MDA levels in colonic specimens from control rats accounted for 18.8±2.4 nmol/mg (Figure 15A). 6-OHDA-induced nigrostriatal denervation was associated with a significant increase in the oxidative stress of colonic tissues at both week 4 and 8

(57.5±15.8 and 61.3±10.6 nmol/mg tissue, respectively) (Figure 15A).

TNF levels in colonic tissues from control animals accounted for 64.7±7.2 pg/mg tissue (Figure 15B). In colonic tissues from rats with 6-OHDA-induced nigrostriatal denervation, TNF levels were increased at both week 4 and 8 (208.6±23.7 and 267.35±27.4 pg/mg, respectively), as compared with controls (Figure 15B).

IL-1β levels in colonic tissues from control animals accounted for 60.02±16.2 pg/total proteins (Figure 15C). In colonic tissues from rats with 6-OHDA-induced nigrostriatal denervation, IL-1β levels were increased at both week 4 and 8 (229.6±76.3 and 255.4±33.1 pg/total proteins, respectively), as compared with controls (Figure 15C).

Effects of L-DOPA/Benserazide on colonic motor activity

In vitro recording of colonic motility

In control animals, treatment with L-DOPA/BE did not modify all patterns of stimulated motor activity, as compared with drug vehicle.

In colonic longitudinal muscle preparations from 6-OHDA-treated rats, maintained in standard Krebs solution, electrically evoked motor responses were restored as compared with controls (Figure 16). Likewise, in colonic circular muscle preparations from rats treated with 6-OHDA, the electrically evoked contractile activity was significantly increased at all tested frequencies, with values comparable to controls (Figure 17).

In vitro recording of cholinergic excitatory colonic neuromuscular functions

In control animals, treatment with L-DOPA/BE did not affect all patterns of stimulated motor activity, as compared with drug vehicle. In 6-OHDA animals, treatment with L-DOPA/BE was associated with a restoration of ES-evoked neurogenic cholinergic

motor responses as compared with controls (Figure 18 and 19), while the changes in carbachol-evoked myogenic contractions were unaffected in comparison with 6-OHDA rats treated with drug vehicle (Figure 20 and 21).

Acetylcholine release from colonic longitudinal muscle preparations

Under resting conditions, the treatment of control animals with L-DOPA/BE did not modify the acetylcholine release from longitudinal muscle preparations, in comparison with rats treated with drug vehicle (Figure 22). In 6-OHDA rats the release of acetylcholine from colonic longitudinal preparations was reduced as compared with controls (Figure 22). The administration of L-DOPA/BE to 6-OHDA rats resulted in an increase of acetylcholine release as compared with 6-OHDA animals treated with drug vehicle (Figure 22).

Upon exposure of control colonic strips to ES, acetylcholine release into Krebs solution was enhanced versus the baseline value assessed at -60 s. The application of ES to colonic preparations from 6-OHDA animals resulted in a slight increase in acetylcholine release, as compared with baseline levels (Figure 22). Similar findings were obtained in control animals treated with L-DOPA/BE, as well as in 6-OHDA rats treated with L-DOPA/BE (Figure 22).

In vitro recording of tachykininergic excitatory colonic neuromuscular functions

Treatment of 6-OHDA animals with L-DOPA/BE did not produce any effect on the alterations of both ES- and SP-evoked tachykininergic motor responses in 6-OHDA animals (Figure 23, 24 and 25, 26).

Assessement of TNF, IL-1β and ***MDA levels***

L-DOPA/BE treatment did not reduce the oxidative stress (MDA levels) in 6-OHDA rats (Figure 27A), while TNF and IL-1β levels were significantly reduced -66% and -57%, respectively, as compared to 6-OHDA rats treated with drug vehicle (Figure 27B and 27C).

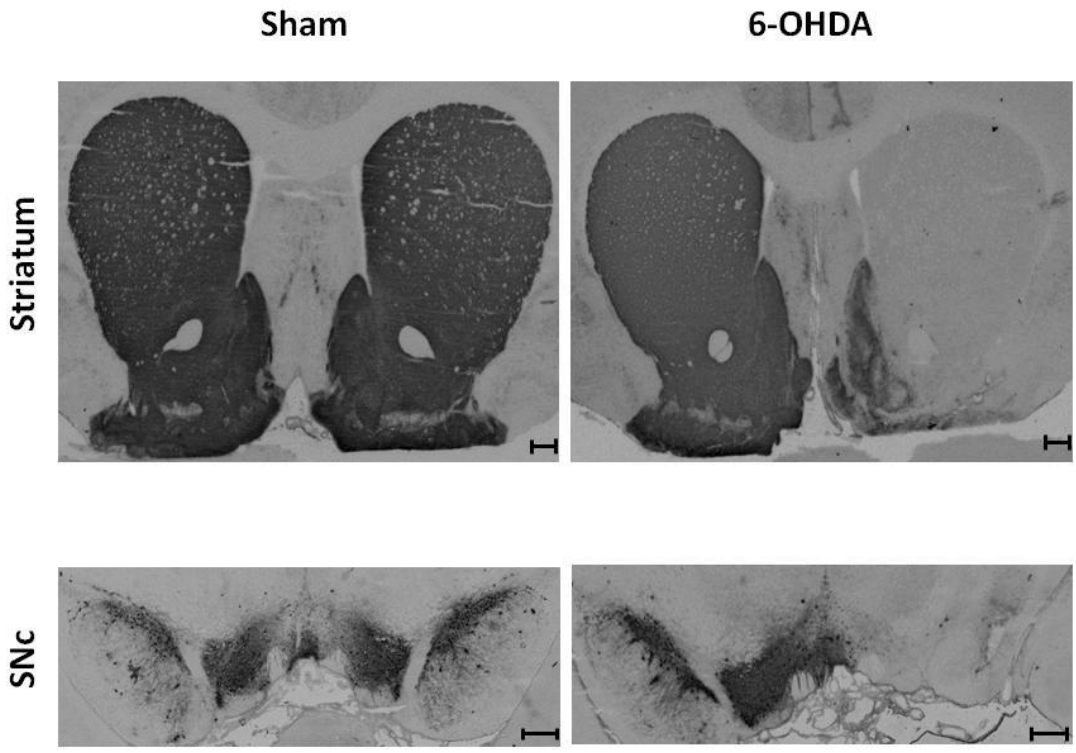


Figure 1. Representative images of dopaminergic (TH+) striatal terminals and SNc cell bodies of both sham-operated and 6-OHDA-lesioned animals. Scale bar: 500 μm.
SNc: substantia nigra pars compacta

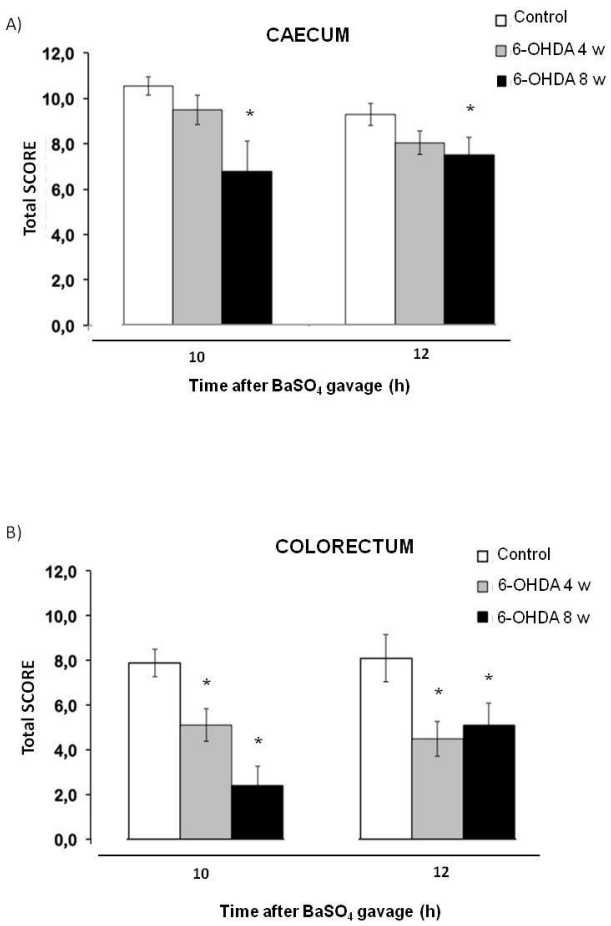


Figure 2. Radiographic contrast study of large bowel luminal content transit in control rats and animals after 4 and 8 weeks from nigrostriatal denervation with 6-OHDA. Total score was assessed 10 and 12 hours after barium sulphate gavage, in the caecum (A) and colorectum (B). Each column represents the mean±S.E.M score value obtained from 8 animals. *$p<0.05$, significant difference vs control.

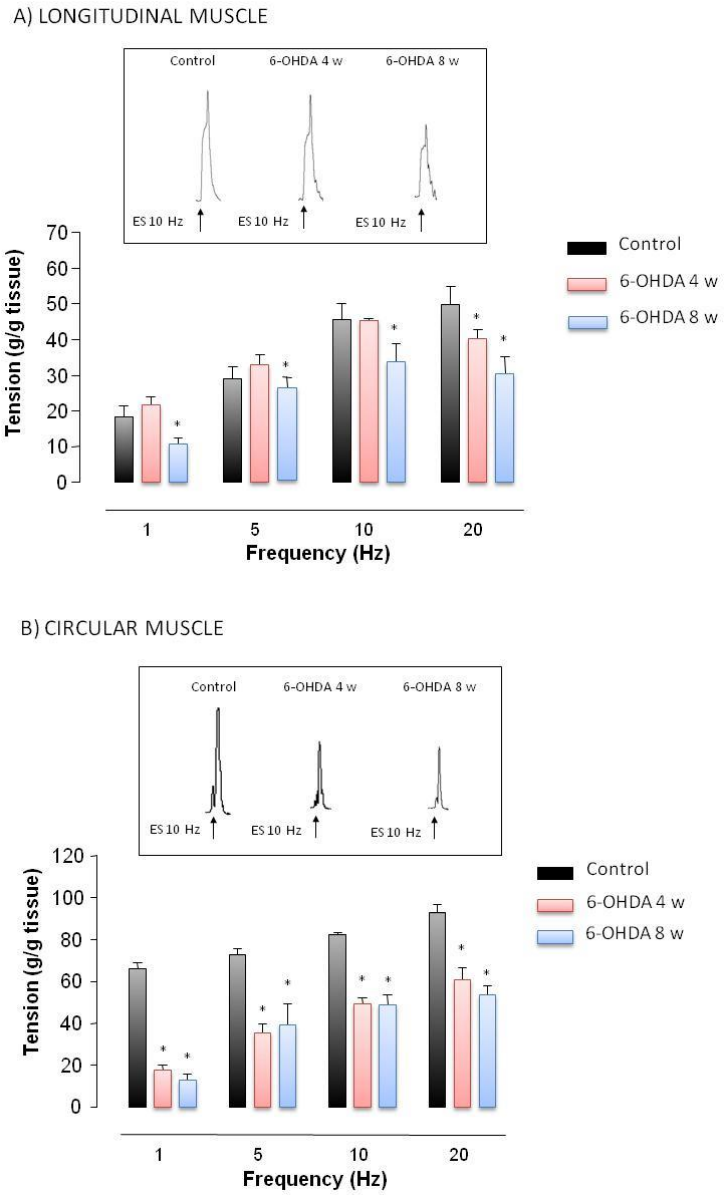


Figure 3. Effects of electrical stimulation (ES, 1-20 Hz) on the motor activity of colonic longitudinal (A) and circular (B) smooth muscle preparations isolated from control animals, or rats after 4 and 8 weeks from nigrostriatal denervation with 6-OHDA. Colonic preparations were maintained in standard 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. *P<0.05; significant difference vs control.

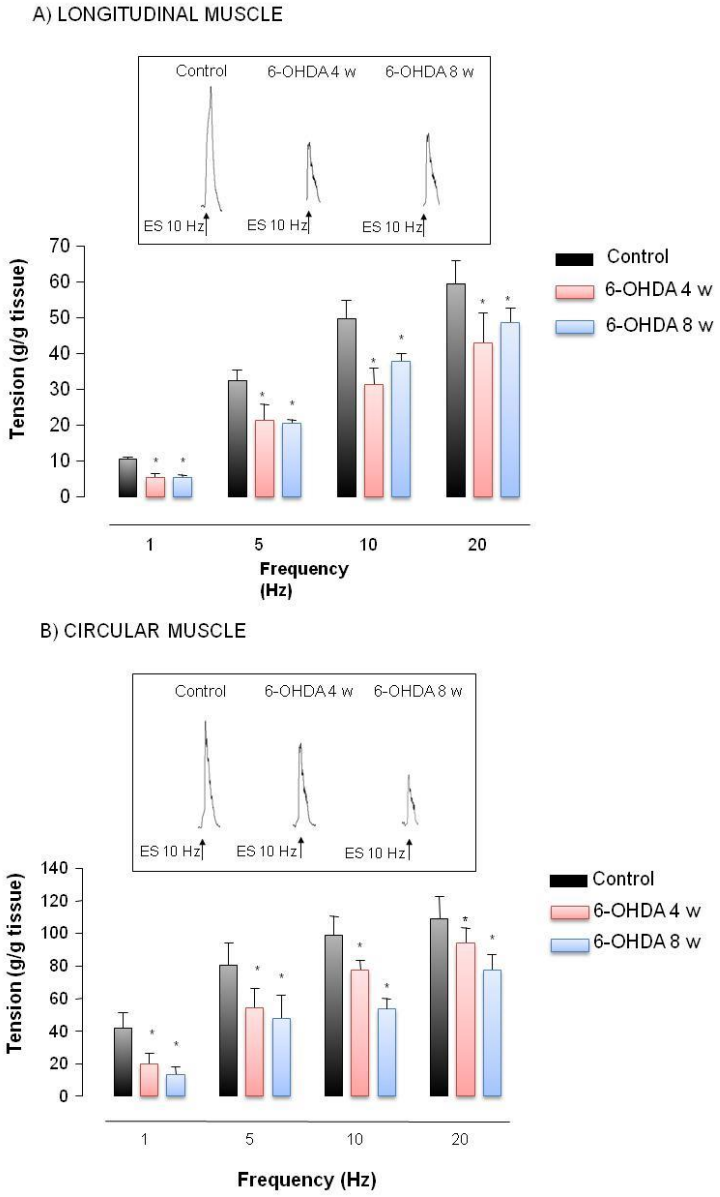


Figure 4. Effects of electrical stimulation (ES, 1-20 Hz) on the motor activity of colonic longitudinal (A) and circular (B) smooth muscle preparations isolated from control animals, or rats after 4 and 8 weeks from nigrostriatal denervation with 6-OHDA. Colonic preparations were maintained in Krebs 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. *P<0.05; significant difference vs control.

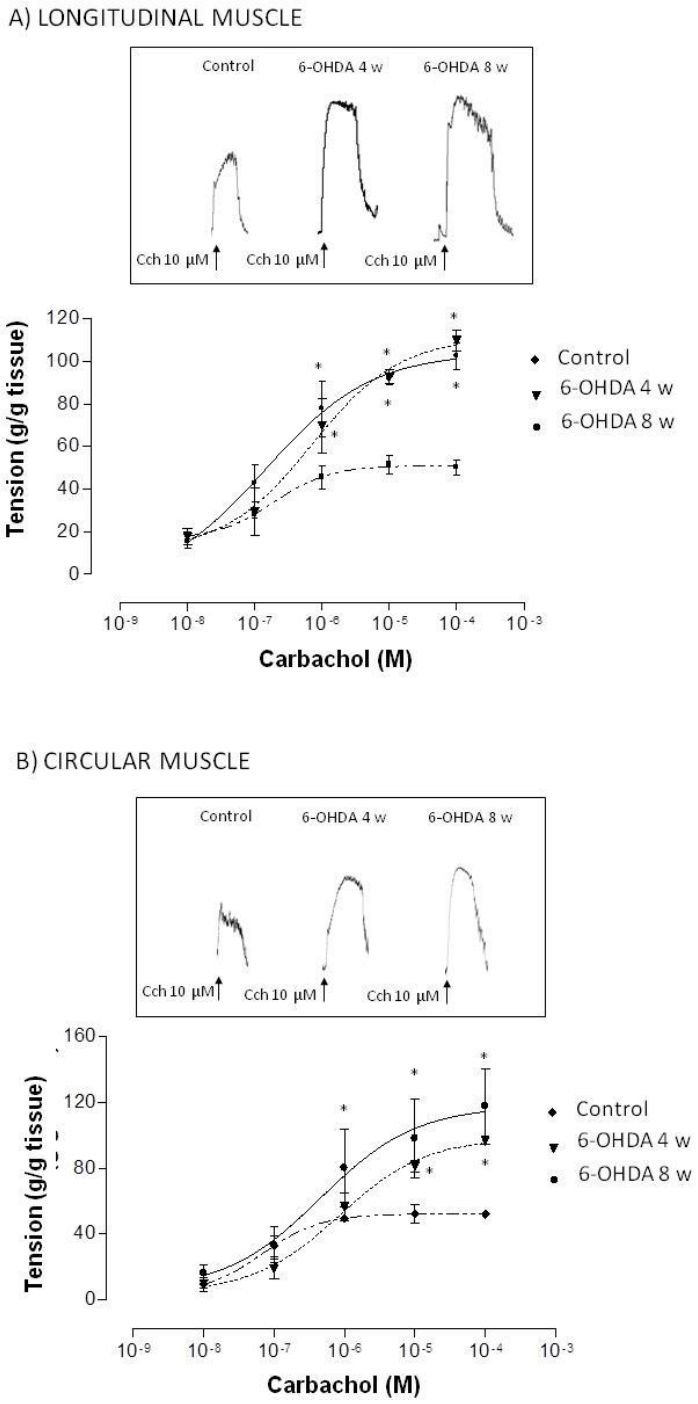


Figure 5. Effects of increasing concentrations of carbachol (Cch, 0.01-100 µM) on the contractile activity of colonic longitudinal (A) and circular (B) smooth muscle preparations isolated from control animals, or rats after 4 and 8 weeks from nigrostriatal denervation with 6-OHDA. Colonic preparations 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. *P<0.05; significant difference vs control.

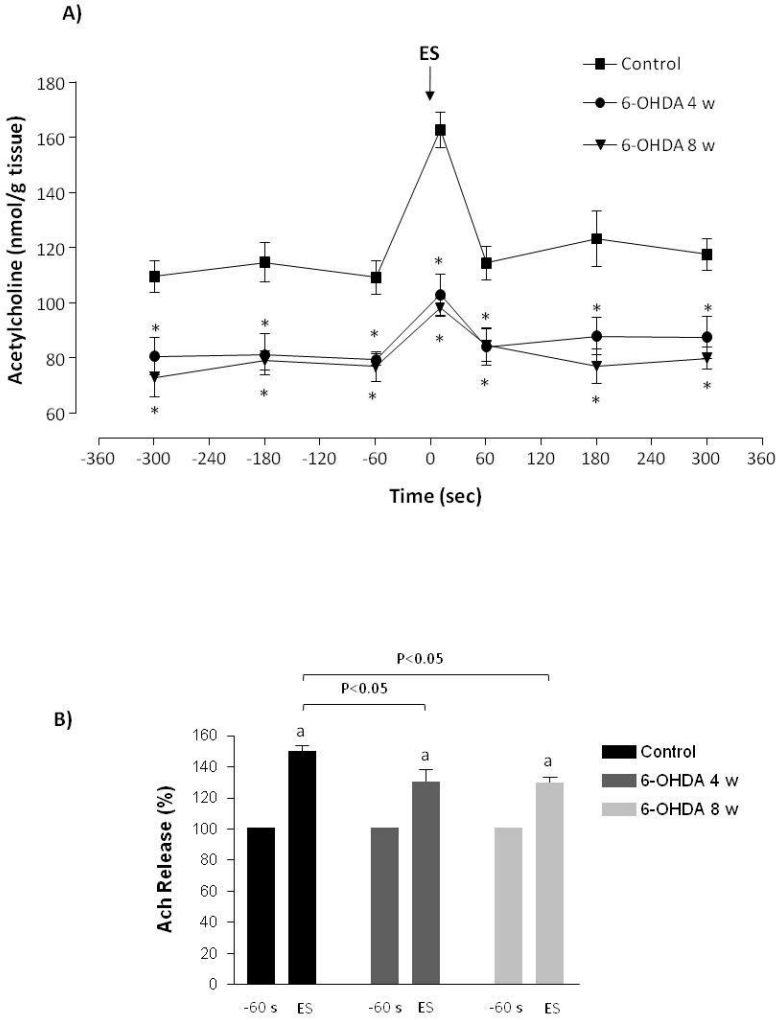


Figure 6. (A) Acetylcholine content in aliquots of Krebs solution incubating colonic longitudinal muscle preparations from control animals or rats after 4 and 8 weeks from nigrostriatal denervation with 6-OHDA. Aliquots were collected at -300, -180, -60, +60, +180 and +300 s with respect to the onset 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. *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. [a]P<0.05 vs the respective value at -60 s.

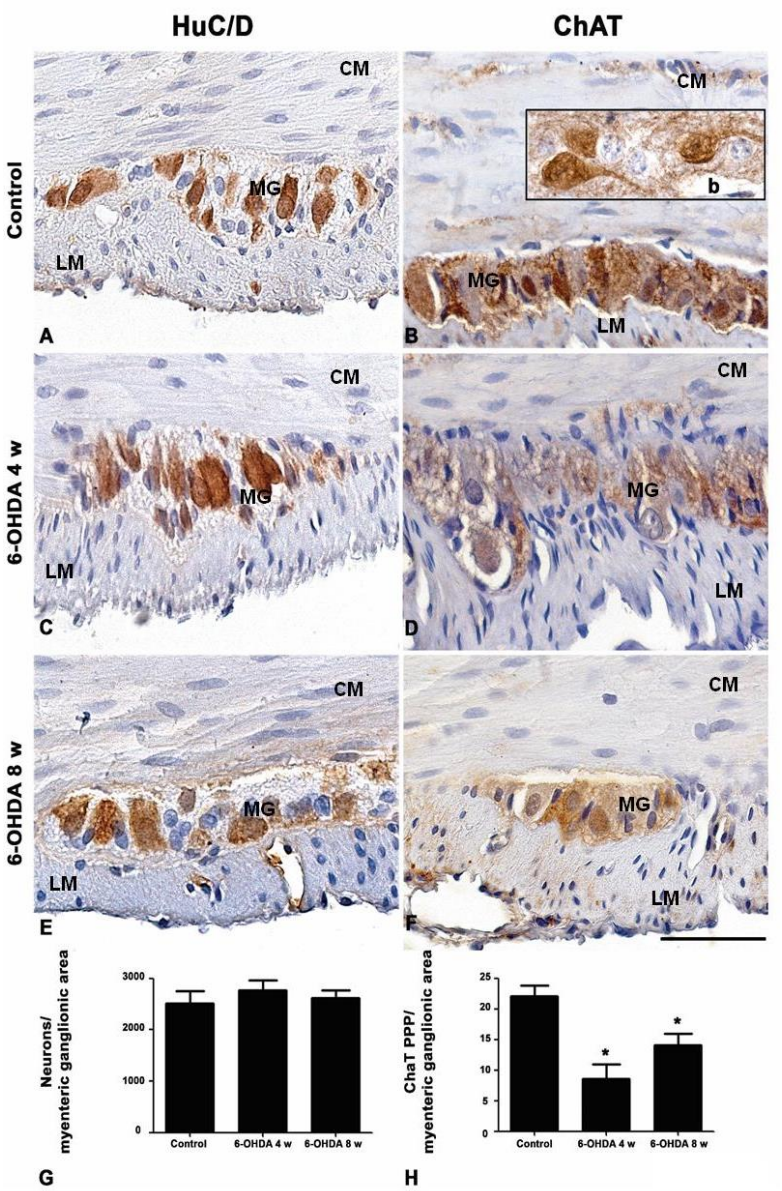


Figure 7. Representative pictures of HuC/D and ChAT immunostaining in rat colonic specimens. Myenteric ganglia (MG) from control and 6-OHDA rats show HuC/D immunoreactive neurons (A, C, E) without changes in neuron density (G). Myenteric neurons of control colon contain abundant amounts of ChAT staining, 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. ^{a}P<0.05 vs controls. Abbreviation: CM: circular muscle; LM: longitudinal muscle.

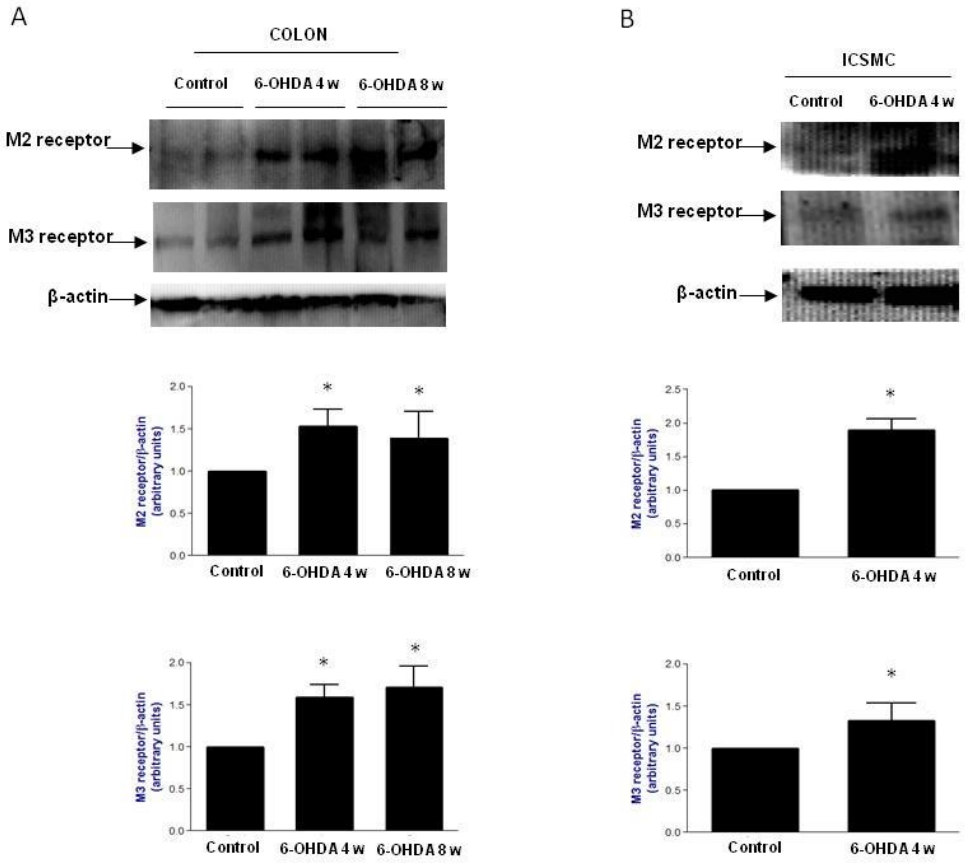


Figure 8. Western blot analysis of muscarinic M2 and M3 receptors in the colonic neuromuscular layer (A) and isolated colonic smooth muscle cells (ICSMCs) (B) from control rats and animals after 4 and 8 weeks from nigrostriatal denervation with 6-OHDA. Each column represents the mean±S.E.M value obtained from 5 to 6 animals. *P<0.05; significant difference vs control.

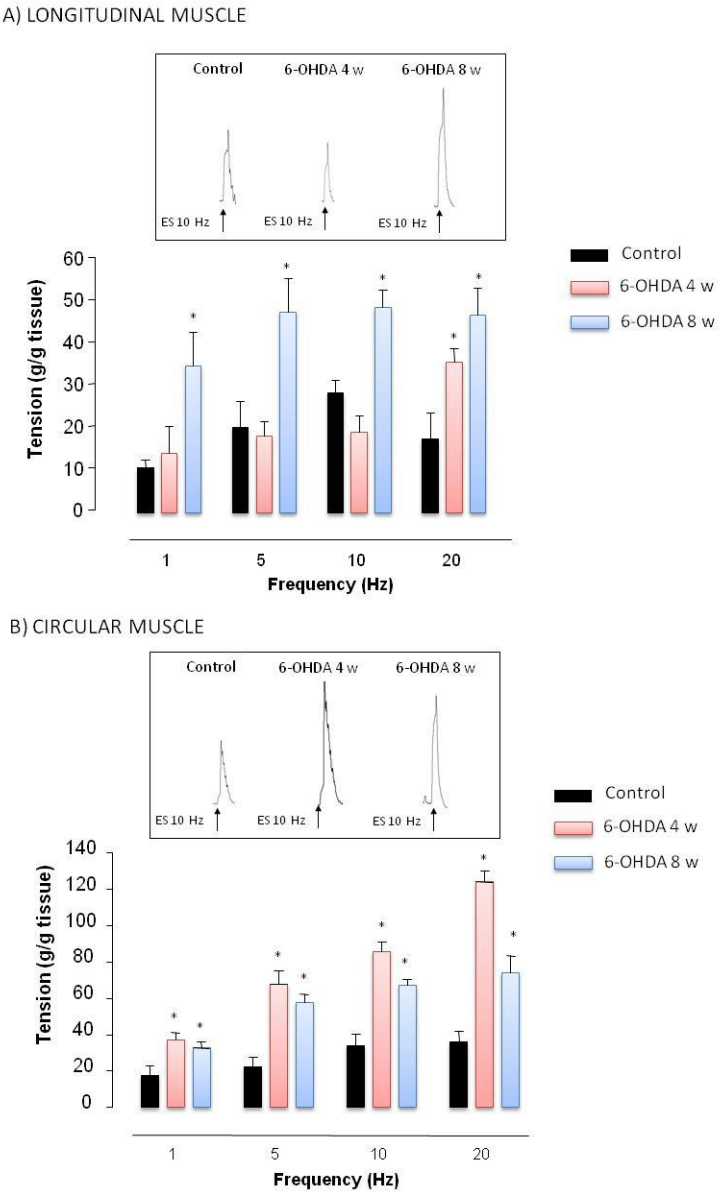


Figure 9. Longitudinal (A) and circular (B) smooth muscle preparations isolated from control animals or rats treated with 6-OHDA at 4 and 8 weeks and maintained in Krebs solution, containing L-NAME (100 μM), guanethidine (10 μM), atropine (1 μM). Contractile responses were induced by electrical stimulation (ES: 0.5 ms, 30 mA, 10 s, 1- 20 Hz). Each column represents the mean ± S.E.M value obtained from 8 experiments. *P < 0.05; significant difference vs control.

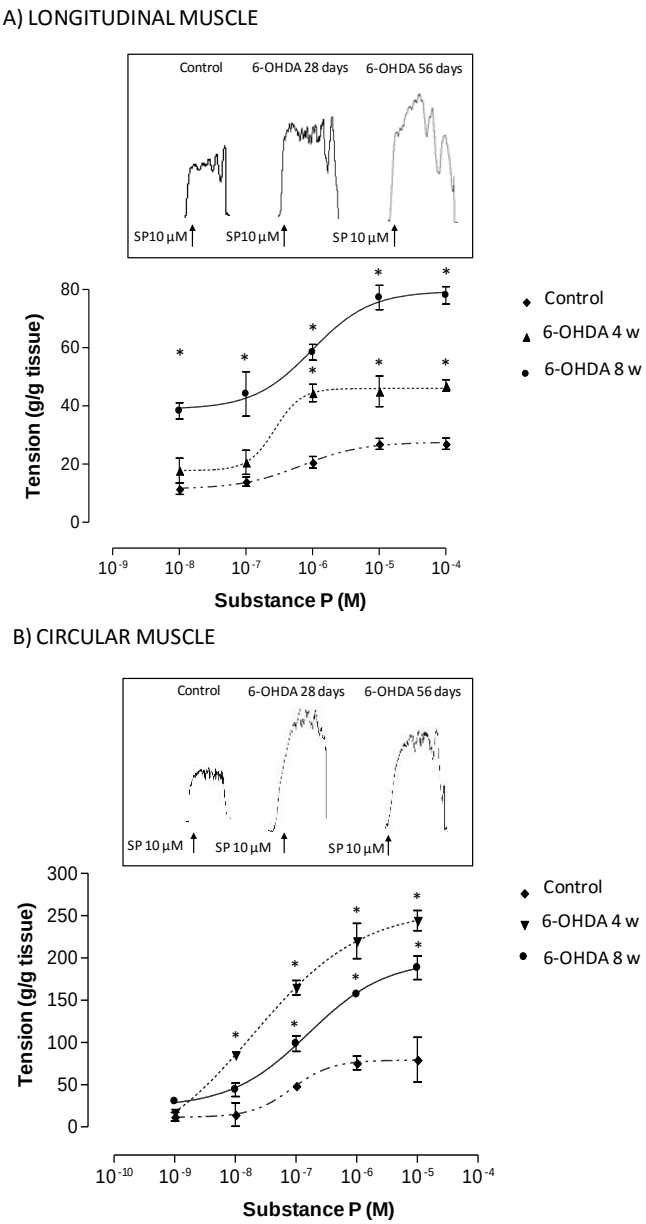


Figure 10. Colonic smooth muscle preparations isolated from controls or rats treated with 6-OHDA at 4 and 8 w and maintained in standard Krebs solution, containing tetrodotoxin (1 µM). Contractile responses of colonic longitudinal (A) and circular (B) smooth muscle preparations induced by increasing concentration of exogenous substance P (0.01-10 µM). Each points represents the mean ± S.E.M value obtained from 8 experiments. *P < 0.05; significant difference vs control.

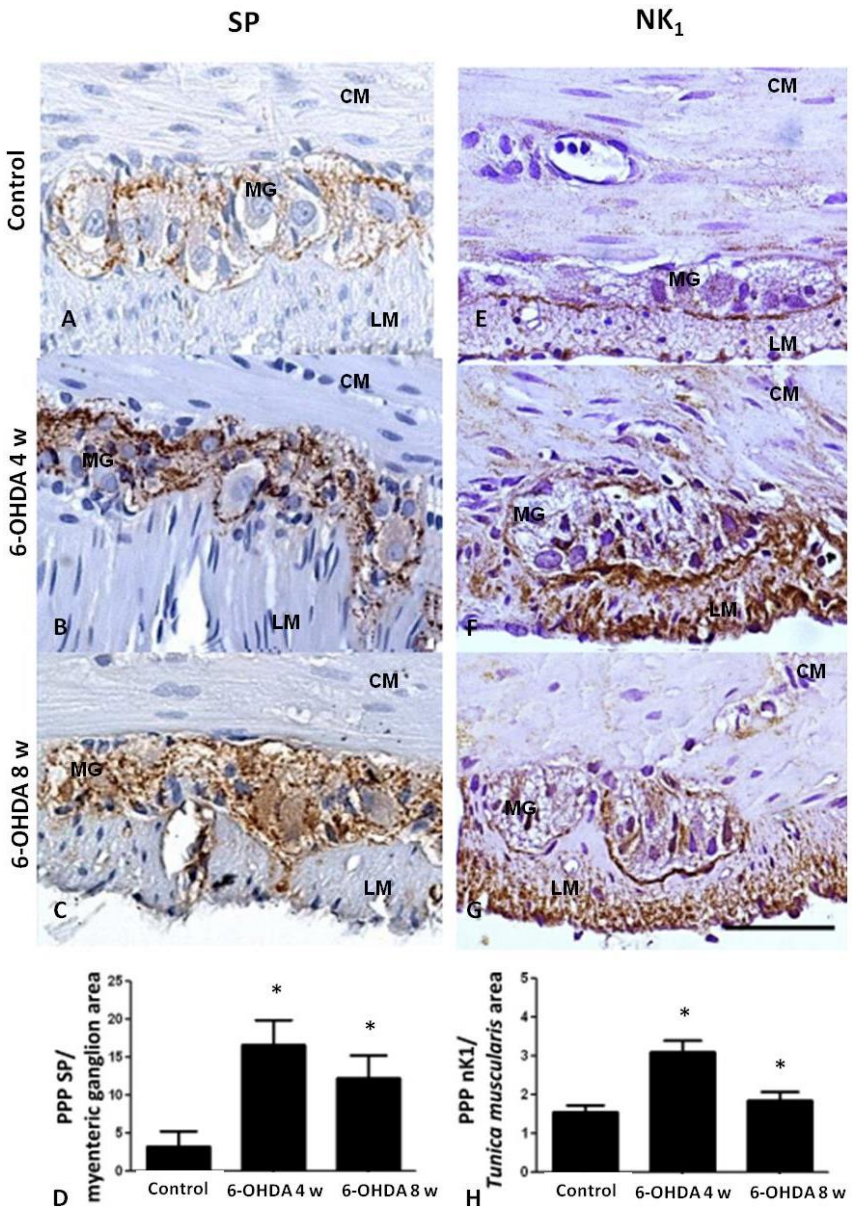


Figure 11. Representative pictures of SP and NK_1 receptor immunostaining in cross-sections from full-thickness rat colonic specimens (A-D, E-G, respectively) from controls (A and E) or animals with 6-OHDA-induced PD at weeks 4 (B and F) and 8 (C and G). By comparison with controls, both 6-OHDA animal groups show significant increments of NK_1 receptor and SP expression in myenteric ganglia (MG). Scale bar = 100 μm. Quantitative evaluation of NK_1 receptor and SP immunostaining in myenteric ganglion area (D and H, respectively): column graphs represent the mean values of PPP ± SEM obtained from 6 rats. $^{*}P \leq 0.05$ *versus* controls. Abbreviation: CM: circular muscle; LM: longitudinal muscle.

GFAP

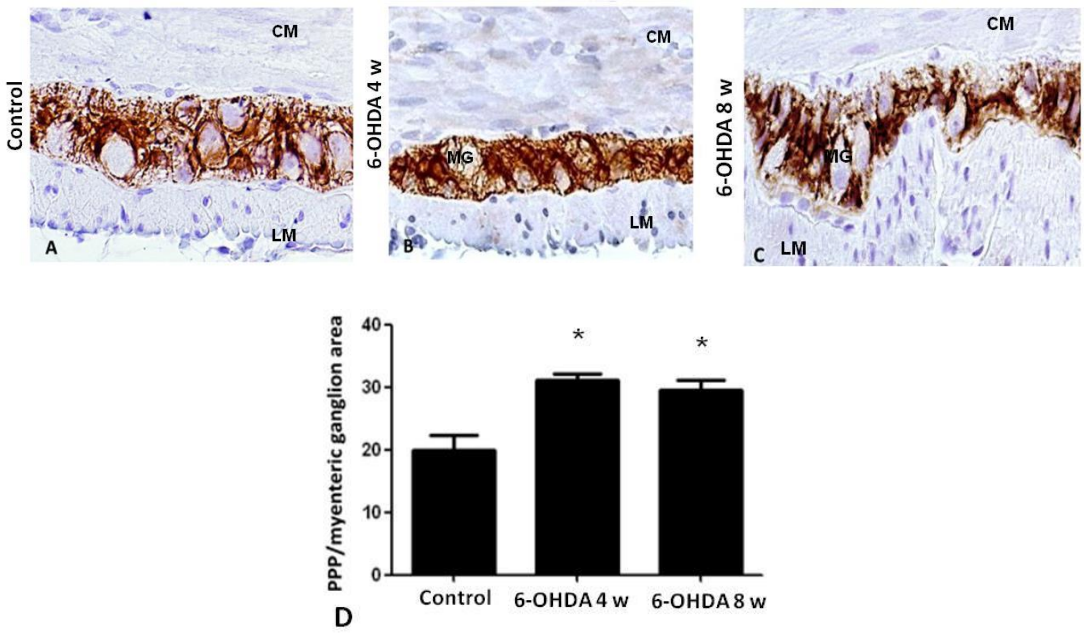


Figure 12. Representative pictures of GFAP immunostaining in cross-sections from full-thickness rat colonic specimens (A-C) from controls (A) or animals with 6-OHDA-induced PD on weeks 4 (B) and 8 (C). By comparison with controls, both 6-OHDA animal groups show significant increases in GFAP expression of myenteric ganglia (MG). Scale bar = 100 μm. Quantitative evaluation of GFAP immunostaining in myenteric ganglion area (D): the column graphs display mean values of PPP ± SEM obtained from 6 rats. $^{*}P \leq 0.05$ *versus* controls. Abbreviation: CM: circular muscle; LM: longitudinal muscle.

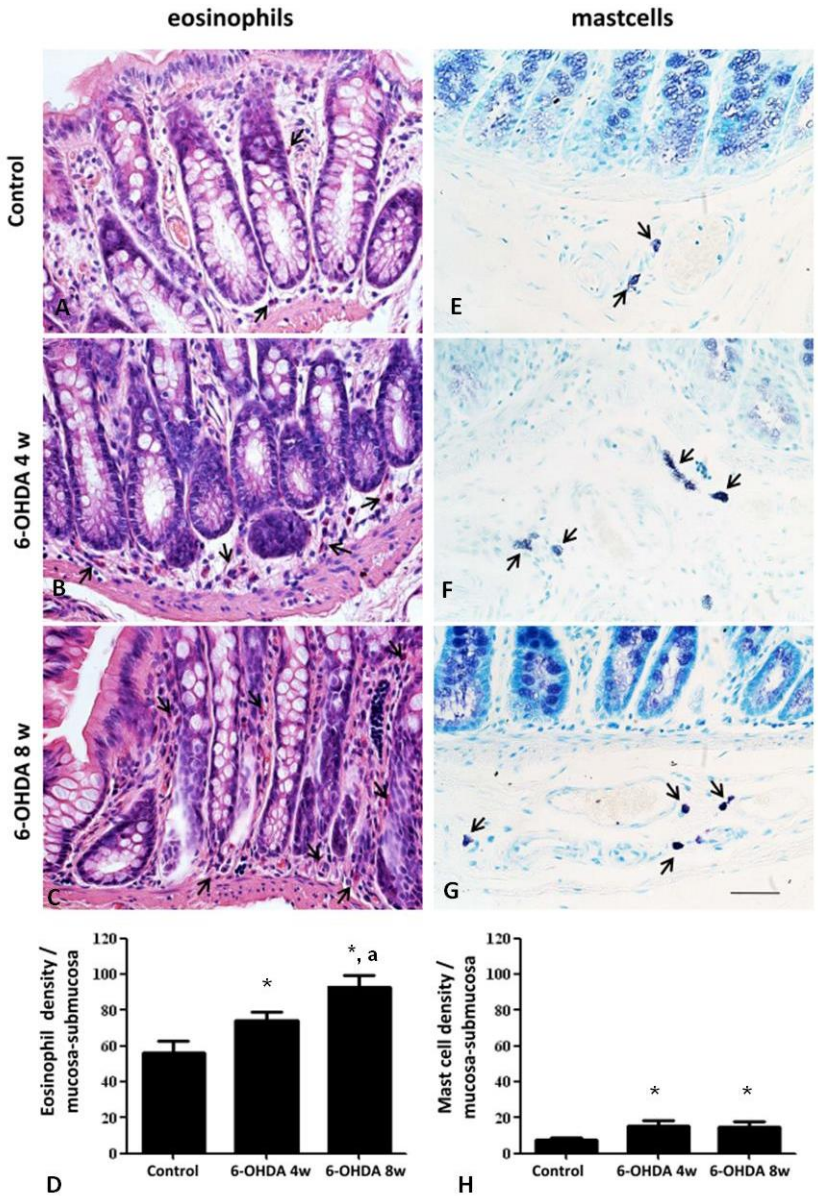


Figure 13. Representative pictures of rat colonic samples haematoxylin/eosin- and toluidine blue-stained from controls or animals with 6-OHDA-induced PD on weeks 4 and 8. Eosinophils (arrows), resident in the *tunica mucosa and submucosa*, are significantly increased in 6-OHDA animals at both time points. Mast cells (arrows), which are found occasionally in the colonic *tunica submucosa* in controls, are significantly increased in 6-OHDA animals. Scale bar = 50 μm. The column graphs display mean values of eosinophil or mast cell density per mm^2 of *tunica mucosa/submucosa* areas (cells/mm^2) ± SEM obtained from 8 rats. $^{*}P<0.05$ *versus* controls; $^{a}P<0.05$ *versus* 6-OHDA 4 week rats**.**

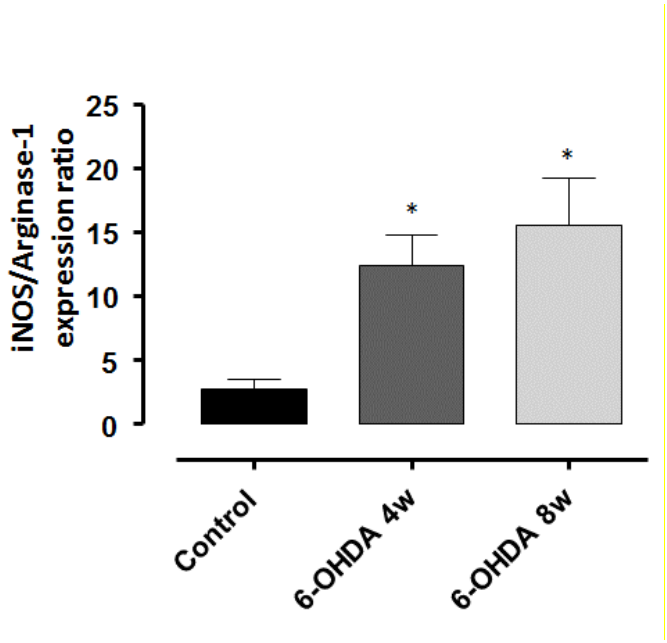


Figure 14. iNOS/Arginase-1 expression ratio in peritoneal macrophages from 6-OHDA and control rats. Data represent the mean ± S.E.M value obtained from 8 experiments. *P < 0.05; significant difference vs control.

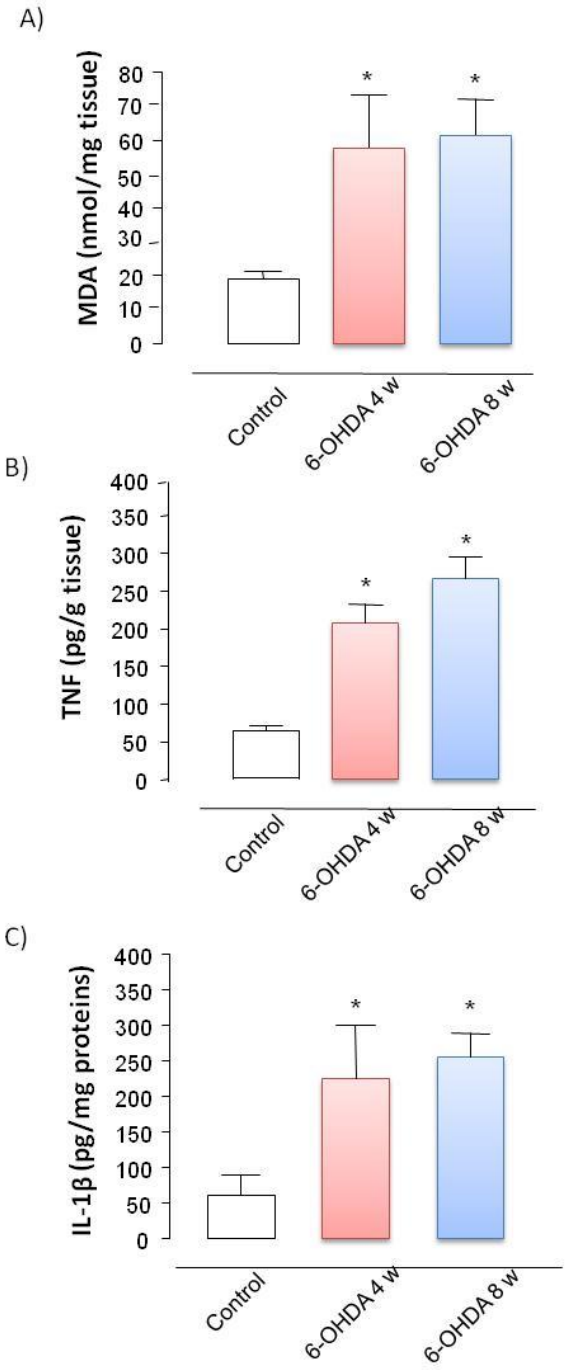


Figure 15. MDA (A), TNF (B) and IL-1β (C) levels in colonic tissues from control animals and rats after 4 and 8 weeks from nigrostriatal denervation with 6-OHDA. Each column represents the mean±S.E.M value obtained from 8 animals. *P<0.05; significant difference vs control.

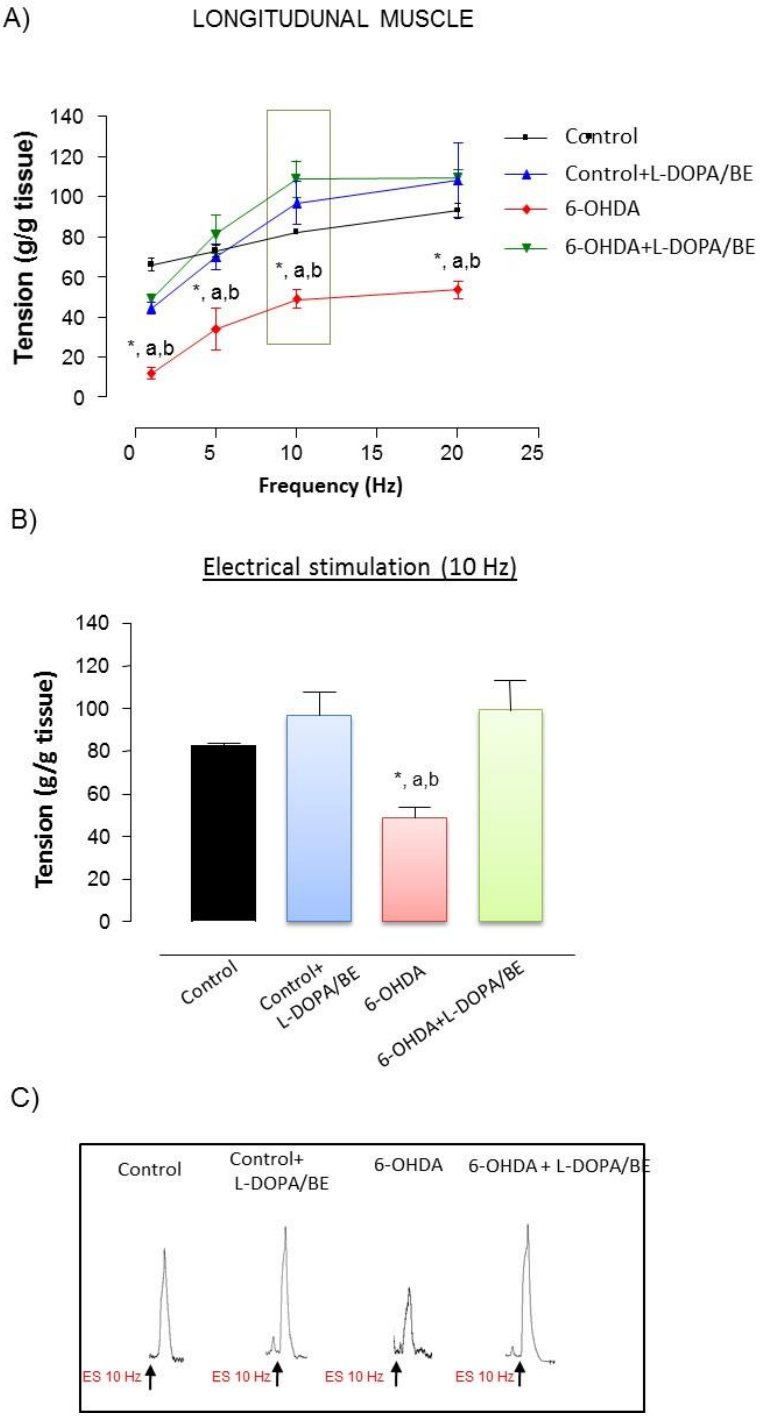


Figure 16. Effects of electrical stimulation (ES, 1-20 Hz) on the motor activity of colonic longitudinal (A) smooth muscle preparations isolated from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. (B) Column bars showing the effect of L-DOPA/BE treatment on contractile responses to ES recorded at the frequency of 10 Hz. (C) Tracings in the inset on the bottom 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. *P<0.05; significant difference vs control; [a]P<0.05 vs control+ L-DOPA/BE ; [b]P<0.05 vs 6-OHDA + L-DOPA/BE

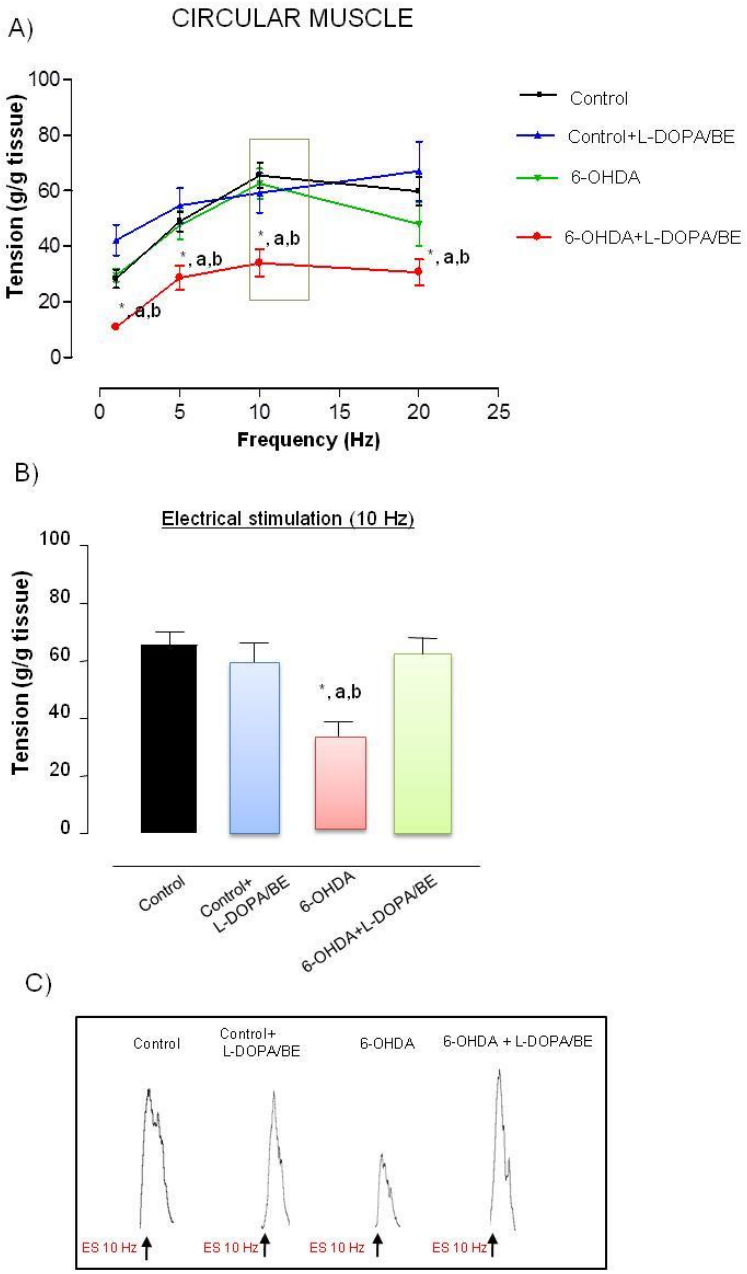


Figure 17. Effects of electrical stimulation (ES, 1-20 Hz) on the motor activity of colonic circular (A) smooth muscle preparations isolated from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. (B) Column bars showing the effect of L-DOPA/BE treatment on contractile responses to ES recorded at the frequency of 10 Hz. (C) Tracings in the inset on the bottom 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. *P<0.05; significant difference vs control; [a]P<0.05 vs control+ L-DOPA/BE ; [b]P<0.05 vs 6-OHDA + L-DOPA/BE

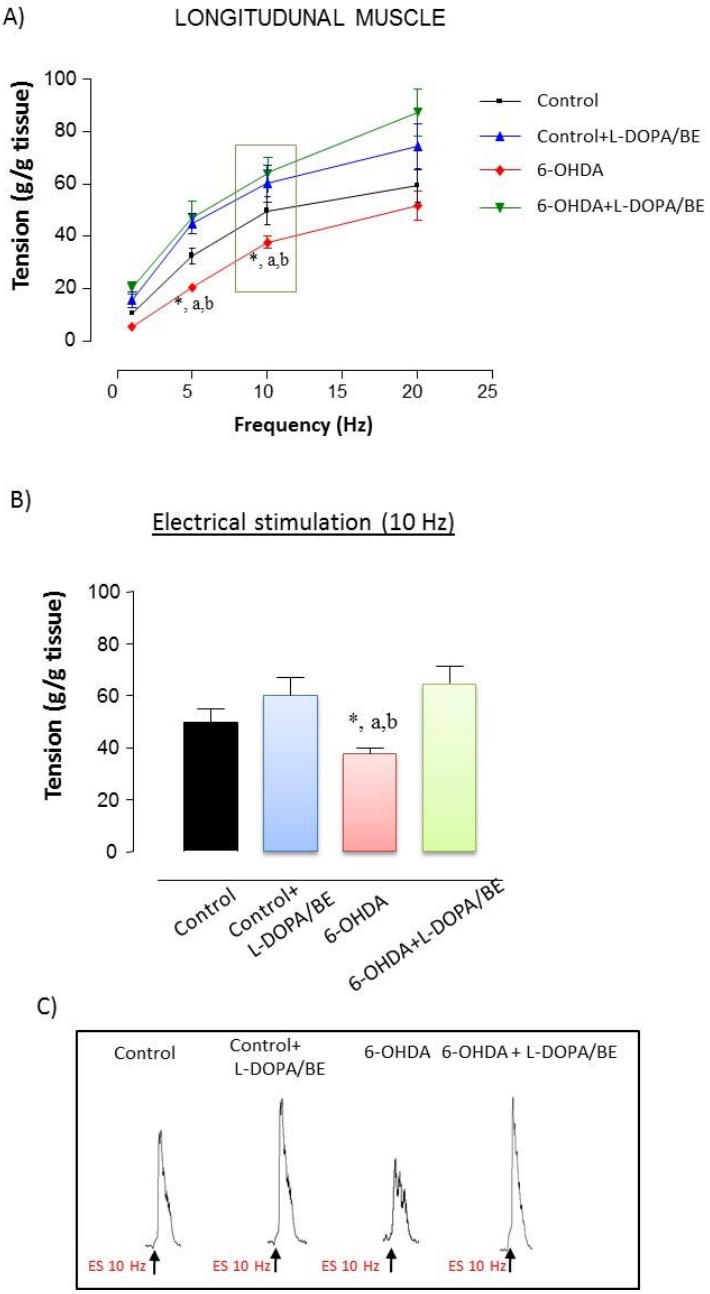


Figure 18. Effects of electrical stimulation (ES, 1-20 Hz) on the motor activity of colonic longitudinal (A) smooth muscle preparations isolated from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. Colonic preparations 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. (B) Column bars showing the effect of L-DOPA/BE treatment on contractile cholinergic responses to ES recorded at the frequency of 10 Hz. (C) Tracings in the inset on the bottom 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. *P<0.05; significant difference vs control; [a]P<0.05 vs control+ L-DOPA/BE ; [b]P<0.05 vs 6-OHDA + L-DOPA/BE.

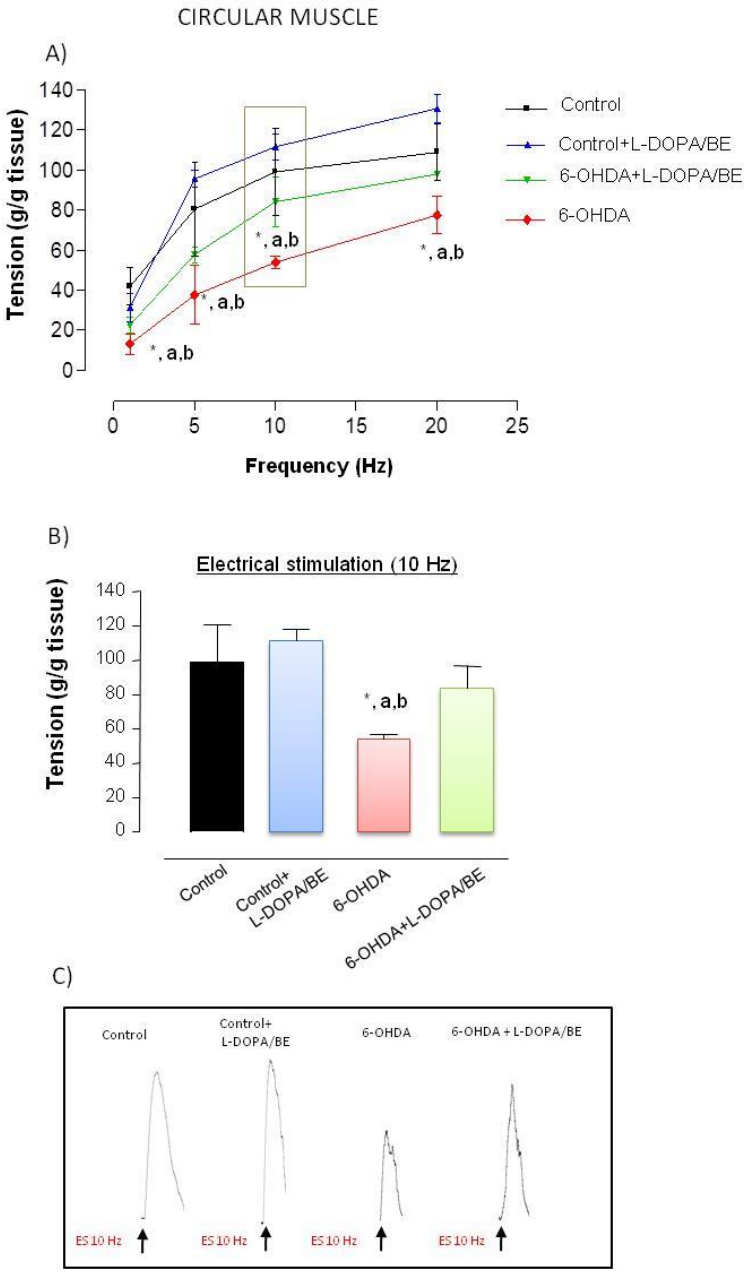


Figure 19. Effects of electrical stimulation (ES, 1-20 Hz) on the motor activity of colonic circular (A) smooth muscle preparations isolated from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. Colonic preparations 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. (B) Column bars showing the effect of L-DOPA/BE treatment on contractile cholinergic responses to ES recorded at the frequency of 10 Hz. (C) Tracings in the inset on the bottom 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. *P<0.05; significant difference vs control; ^{a}P<0.05 vs control+ L-DOPA/BE ; ^{b}P<0.05 vs 6-OHDA + L-DOPA/BE.

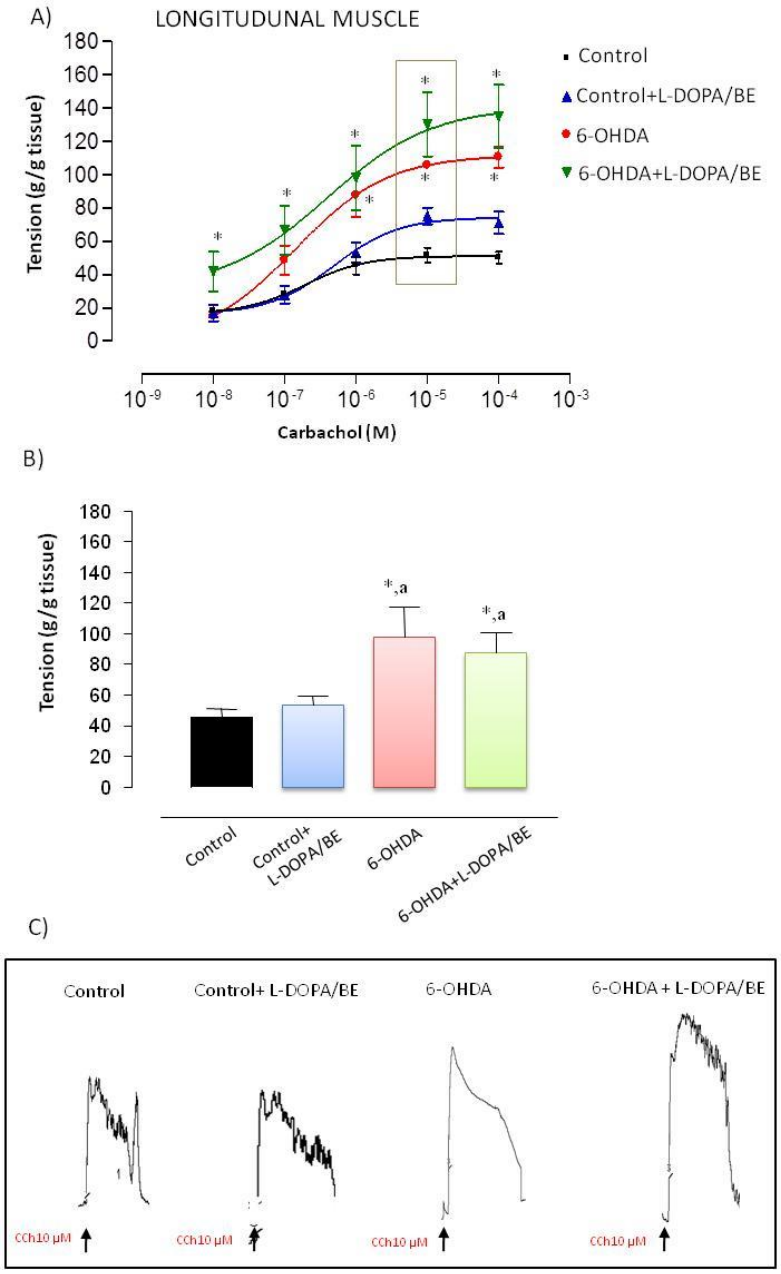


Figure 20. Effects of increasing concentrations of carbachol (Cch, 0.01-100 µM) on the contractile activity of colonic longitudinal (A) and smooth muscle preparations isolated from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. Colonic preparations were maintained in Krebs solution containing tetrodotoxin (1 µM). (B) Column bars showing the effect of L-DOPA/BE treatment on contractile cholinergic responses to Cch 10 µM. (C) Tracings in the inset on the bottom 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. *P<0.05; significant difference vs control; [a]P<0.05 vs control+ L-DOPA/BE.

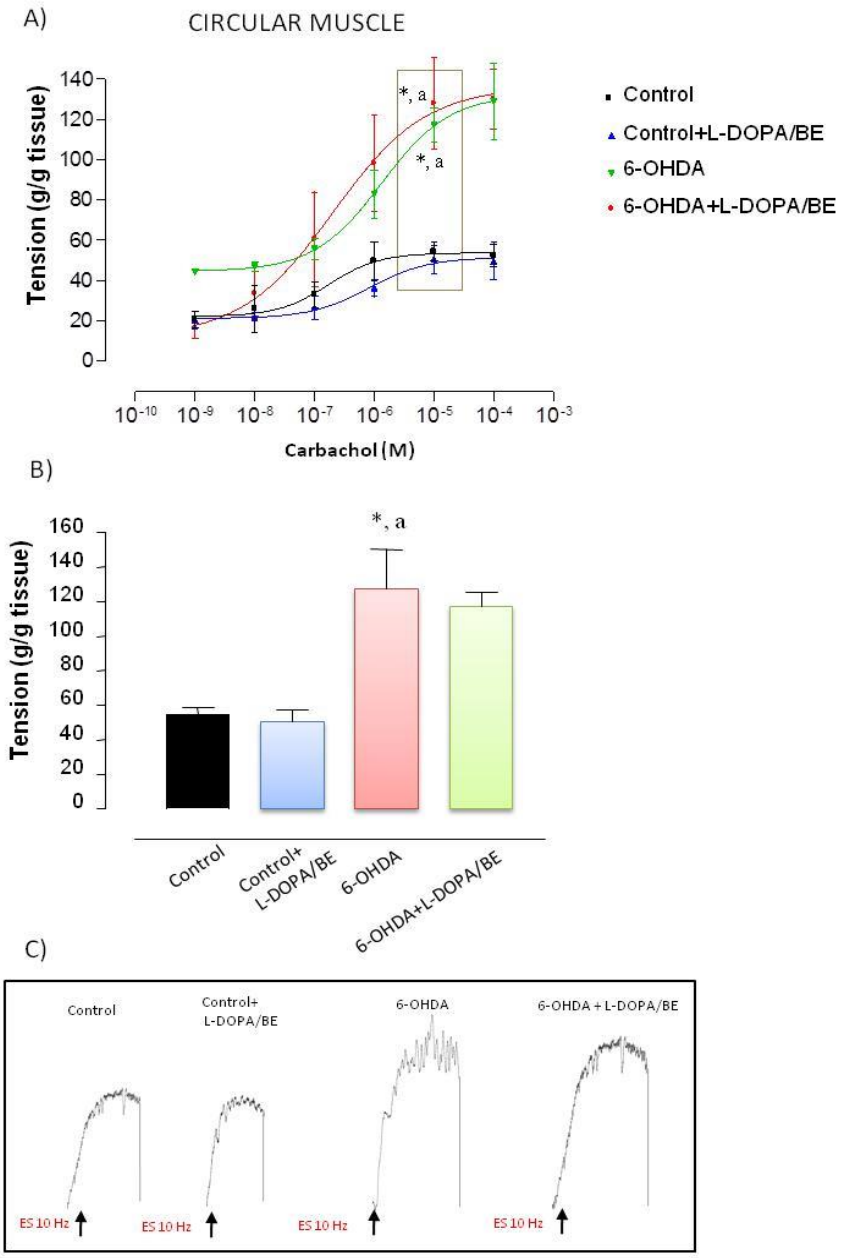


Figure 21. Effects of increasing concentrations of carbachol (Cch, 0.01-100 μM) on the contractile activity of colonic circular (A) and smooth muscle preparations isolated from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. Colonic preparations were maintained in Krebs solution containing tetrodotoxin (1 μM). (B) Column bars showing the effect of L-DOPA/BE treatment on contractile cholinergic responses to Cch 10 μM. (C) Tracings in the inset on the bottom 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. *P<0.05; significant difference vs control; [a]P<0.05 vs control+ L-DOPA/BE.

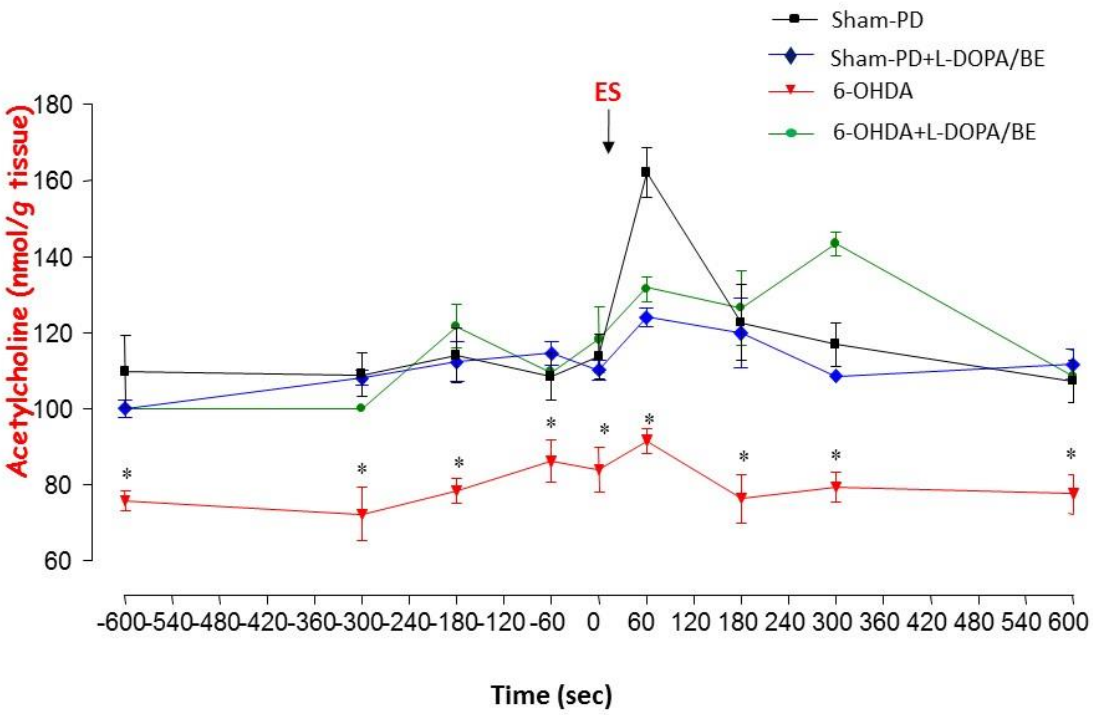


Figure 22. Acetylcholine content in aliquots of Krebs solution incubating colonic longitudinal muscle preparations from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. Aliquots were collected at -300, -180, -60, +60, +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. *P<0.05, significant difference vs control values, control+L-DOPA/BE and 6-OHDA+L-DOPA/BE.

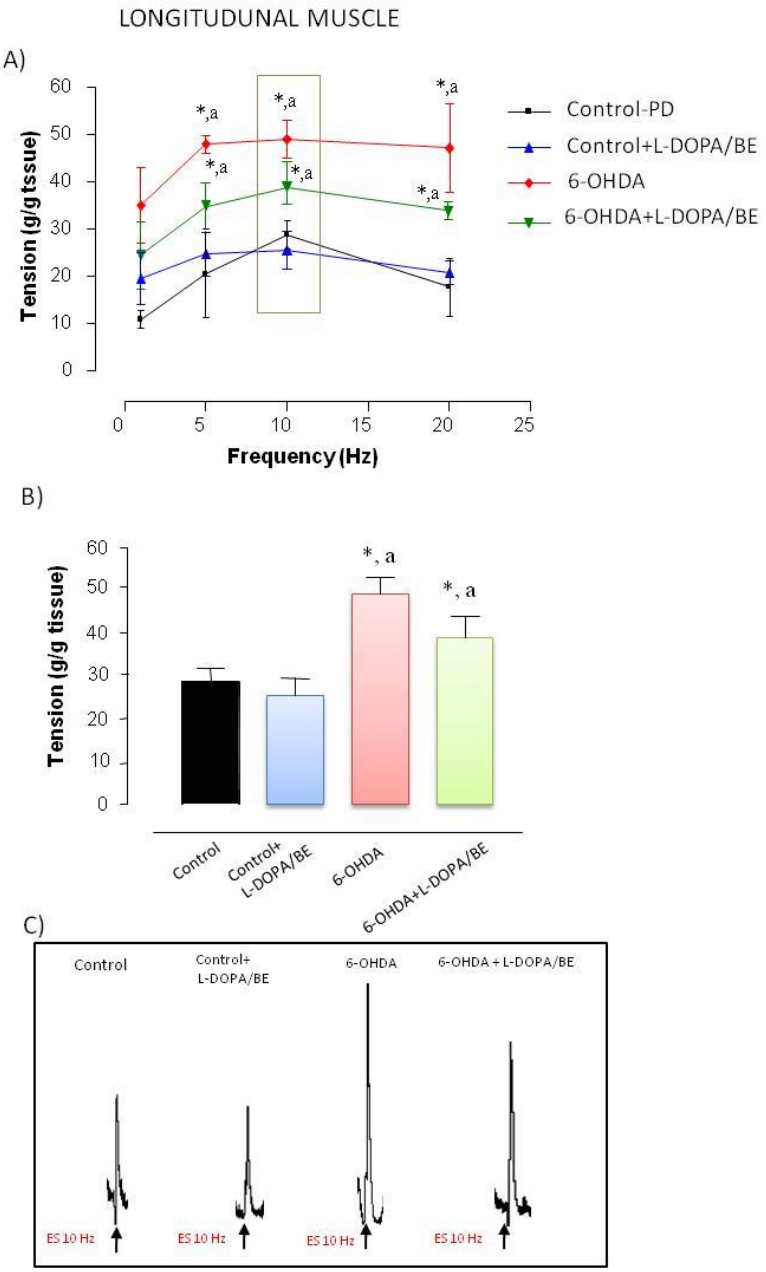


Figure 23. Effects of electrical stimulation (ES, 1-20 Hz) on the motor activity of colonic longitudinal (A) smooth muscle preparations isolated from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. Colonic preparations were maintained in Krebs solution containing L-NAME (100 μM), guanethidine (10 μM), and atropine (1 μM) to record cholinergic contractions. (B) Column bars showing the effect of L-DOPA/BE treatment on contractile tachykininergic responses to ES recorded at the frequency of 10 Hz. (C) Tracings in the inset on the bottom 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. *P<0.05; significant difference vs control; [a]P<0.05 vs control+ L-DOPA/BE.

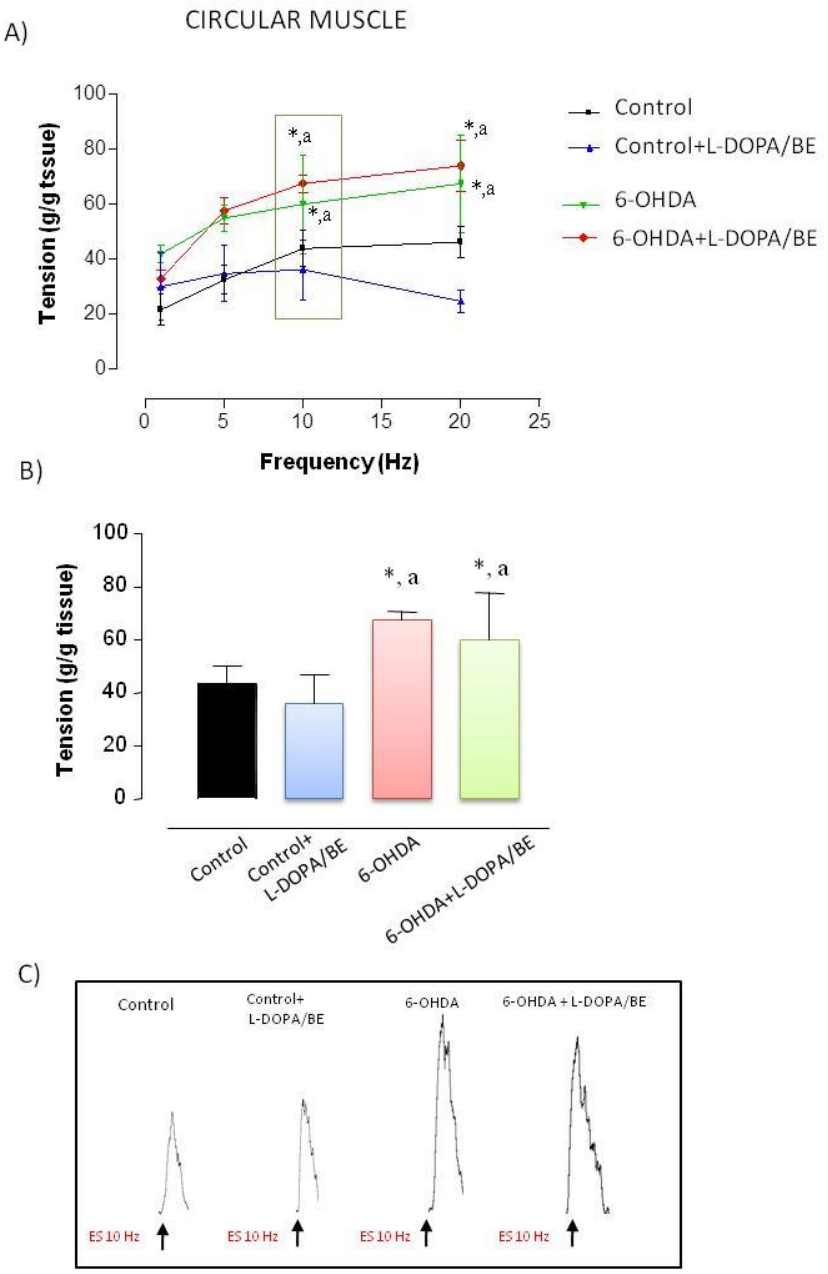


Figure 24. Effects of electrical stimulation (ES, 1-20 Hz) on the motor activity of colonic circular (A) smooth muscle preparations isolated from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. Colonic preparations were maintained in Krebs solution containing L-NAME (100 μM), guanethidine (10 μM), and atropine (1 μM) to record cholinergic contractions. (B) Column bars showing the effect of L-DOPA/BE treatment on contractile tachykininergic responses to ES recorded at the frequency of 10 Hz. (C) Tracings in the inset on the bottom 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. *P<0.05; significant difference vs control; [a]P<0.05 vs control+ L-DOPA/BE.

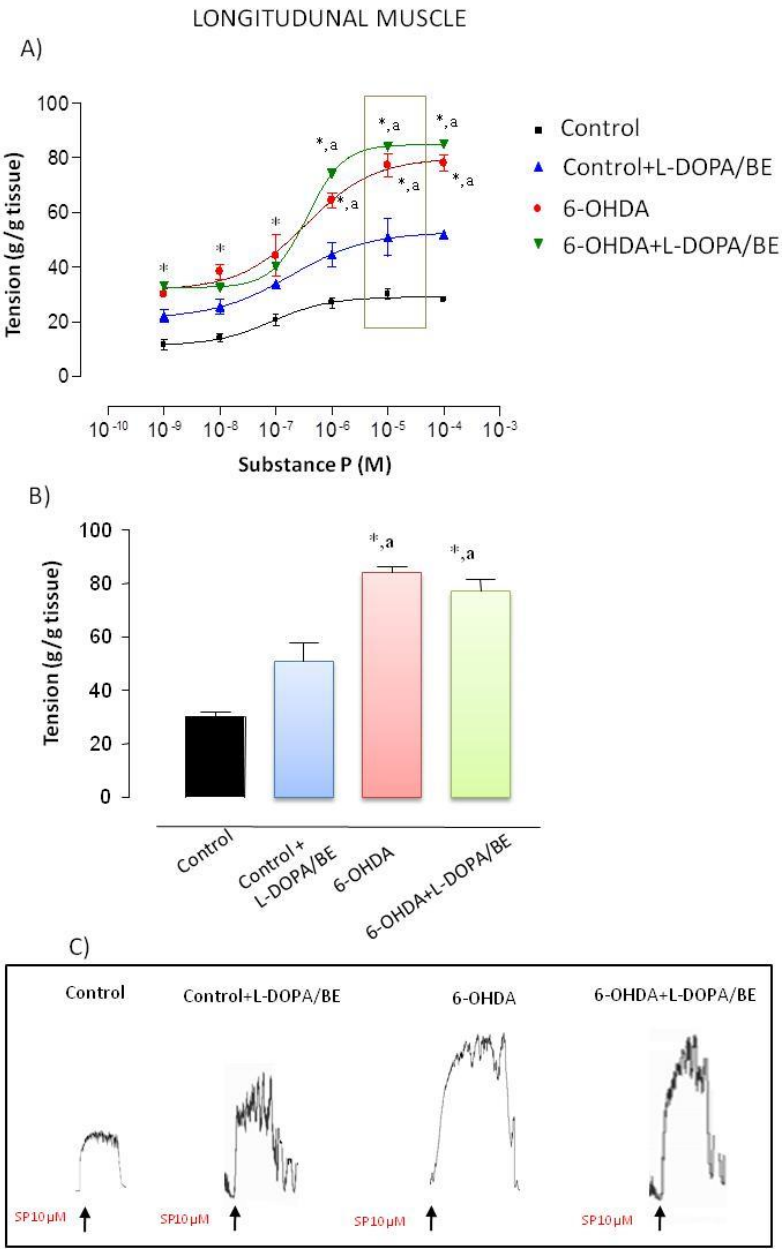


Figure 25. Effects of increasing concentrations of exogenous substance P (SP, 0.01-100 µM) on the contractile activity of colonic longitudinal (A) and smooth muscle preparations isolated from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. Colonic preparations were maintained in Krebs solution containing tetrodotoxin (1 µM). (B) Column bars showing the effect of L-DOPA/BE treatment on contractile cholinergic responses to SP 10 µM. (C) Tracings in the inset on the bottom of each panel display contractile responses to SP at the concentration of 10 µM. Each point represents the mean±S.E.M value obtained from 8 animals. *P<0.05; significant difference vs control; [a]P<0.05 vs control+ L-DOPA/BE.

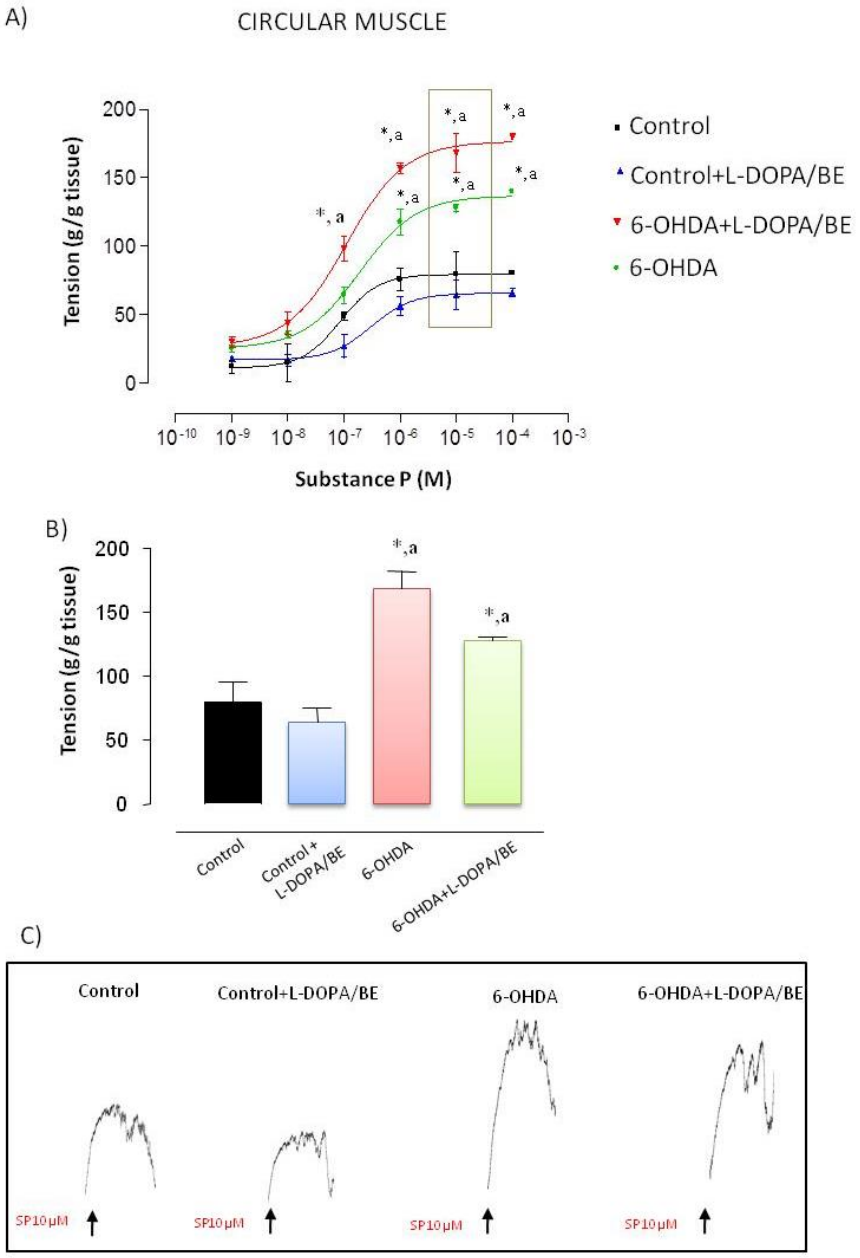


Figure 26. Effects of increasing concentrations of exogenous substance P (SP, 0.01-100 µM) on the contractile activity of colonic circular (A) and smooth muscle preparations isolated from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. Colonic preparations were maintained in Krebs solution containing tetrodotoxin (1 µM). (B) Column bars showing the effect of L-DOPA/BE treatment on contractile cholinergic responses to SP 10 µM. (C) Tracings in the inset on the bottom of each panel display contractile responses to SP at the concentration of 10 µM. Each point represents the mean±S.E.M value obtained from 8 animals. *P<0.05; significant difference vs control; [a]P<0.05 vs control+ L-DOPA/BE.

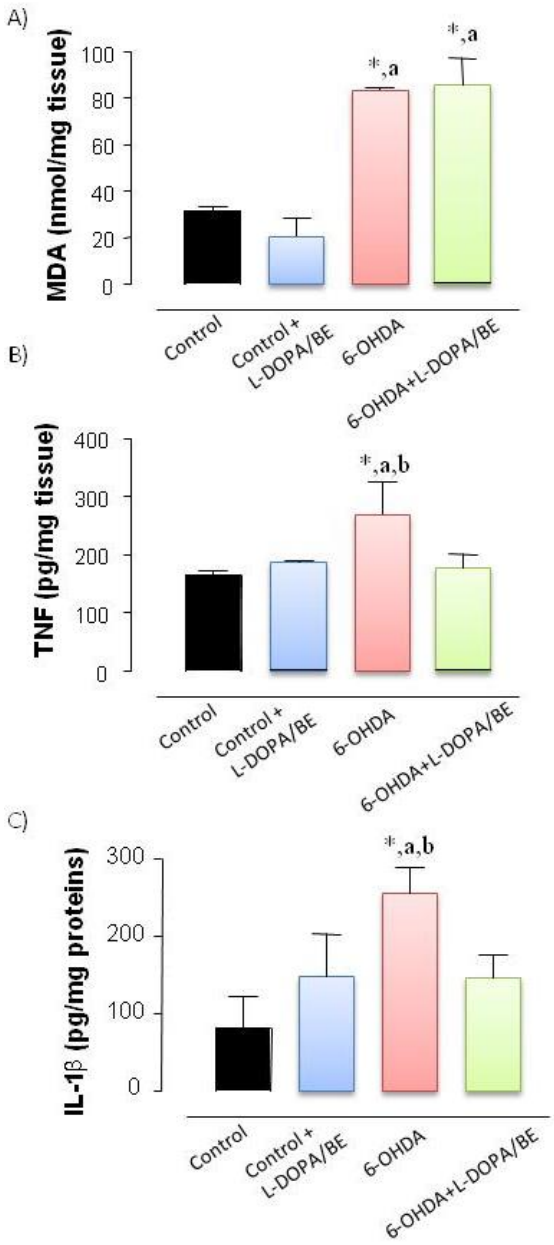


Figure 27. MDA (A), TNF (B) and IL-1β (C) levels in colonic tissues from control animals or treated with L-DOPA/BE, and rats after 8 weeks from nigrostriatal denervation with 6-OHDA or treated with L-DOPA/BE. Each column represents the mean±S.E.M value obtained from 8 animals. *P<0.05; significant difference vs control; [a]P<0.05 vs control+ L-DOPA/BE, [b]P<0.05 vs 6-OHDA+ L-DOPA/BE.

Discussion

Alterations of colonic excitatory cholinergic neurotransmission in the presence of central dopaminergic neurodegeneration

PD is commonly associated with alterations of gut motor functions, such as dysphagia, constipation, defecatory disorders and decreased frequency of bowel movements (Cloud and Greene 2011, Pfeiffer 2011). Consistently with these clinical disorders, abnormalities in the ENS have been observed in PD patients and experimental models of PD (Tian, Chen et al. 2008, Colucci, Cervio et al. 2012, Zhu, Zhao et al. 2012, Devos, Lebouvier et al. 2013, Sharrad, de Vries et al. 2013), even though the neurochemical bases of such alterations and their functional implications remain largely unclear and highly debated. A crucial issue is whether PD-related GI abnormalities result from α-syn pathology affecting the ENS neurons or depend on central dopaminergic denervation following nigrostriatal degeneration (Petrovitch, Abbott et al. 2009) showed that constipation in PD patients is associated with low SNc neuron density, independently of the presence of α-synuclein-positive Lewy bodies. Therefore, our purpose was to characterize the impact of nigrostriatal dopaminergic denervation on the patterns of colonic motility and related excitatory cholinergic control. In this context, the present study provides evidence that the induction of nigrostriatal denervation, which reflects one of the 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 of the patterns by which the contrast medium filled the caecum and progressed towards the rectum documented the development of a delayed colonic transit, as a consequence of the central dopaminergic denervation. These

findings suggest that the model of 6-OHDA-induced nigrostriatal denervation is suitable for the assessment of bowel dysmotility associated with PD. Consistent with our observation, several lines of evidence point out the notion that a decreased rate of bowel movements and severe constipation represent the most widely prevalent clinical signs of enteric dysfunction in PD patients (Pfeiffer 2011).

In order to verify whether the changes of *in vivo* propulsive colonic motility might depend on underlying alterations of enteric neurotransmission, I examined the patterns of *in vitro* excitatory cholinergic motor activity. The results show that the electrically evoked cholinergic contractions of both colonic longitudinal and circular 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, Zhao et al. 2012).

The 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, Cervio et al. 2012). In order to assess whether these motor abnormalities could depend on changes in the density of myenteric nerves, immunohistochemical assays, where myenteric ganglia were labeled with the neuronal marker HuC/D, and with ChAT, a specific marker of cholinergic neurons, were been carried out in the present study. 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. Similar findings were obtained in a recent study by (Toti and Travagli 2014), who described a reduced density of gastric and duodenal ChAT+ myenteric neurons, without a concomitant variation of total neuronal density, in the same experimental model of nigrostriatal denervation. 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, Arshad et al. 2012).

In an attempt of substantiating the hypothesis that the decrease in colonic ChAT would translate into hampered enteric cholinergic neurotransmission, the levels of acetylcholine released from *in vitro* colonic preparations into their incubation medium were assessed. 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 enteric 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 obtained in the same model, where nigrostriatal denervation was found to be associated with a reduced acetylcholine content in the gastric muscularis externa and a significant delay in gastric emptying (Zheng, Wang et al. 2011).

Besides the impaired neurogenic cholinergic motor activity in the colon of 6-OHDA rats, I observed an enhancement of colonic myogenic responses elicited by a direct activation of muscarinic receptors with carbachol. Based on the present results,

supporting a decrease in the production and release of acetylcholine from myenteric cholinergic neurons in 6-OHDA animals, this finding was hypothesized to result from an up-regulation of muscular muscarinic receptors occurring as a compensatory response to the impairment of cholinergic neurotransmission. To address this issue, I 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, Burleigh et al. 2003). In conclusion, based on my *in vitro* functional and molecular findings, it is conceivable that lowering of colonic transit in 6-OHDA rats depends, at least in part, on the impairment of cholinergic enteric neurotransmission.

Alterations of colonic excitatory tachykininergic neurotransmission

Besides the alterations of excitatory cholinergic neurotransmission, the present study provides evidence that the induction of experimental PD by 6-OHDA is characterized by alterations of the excitatory tachykininergic pathway, both at neuronal and muscular level.

The results show that electrically evoked tachykininergic contractions of both colonic longitudinal and circular muscle from 6-OHDA rats were increased, indicating an altered excitatory control of colonic motility. These findings provide the

demonstration that central nigrostriatal denervation is associated with a significant enhancement of excitatory tachykininergic motility in the large bowel.

In order to assess whether these motor abnormalities could depend on changes in the expression of SP, I carried out immunohistochemical assays, where myenteric ganglia were labeled with anti-SP antibody. In these experiments, colonic tissues from 6-OHDA rats displayed a significant increase in immunopositivity for SP, while the overall density of HuC/D+ myenteric neurons did not vary, suggesting that nigrostriatal denervation is associated with alterations in the chemical coding of enteric tachykininergic neurons, characterized by an increased expression of SP in myenteric tachykininergic neurons, which likely results in an enhancement of colonic tachykininergic neurotransmission. In line with this picture, evidence of increased SP expression was recently obtained in the brain tissues from rats with 6-OHDA-induced central dopaminergic denervation, and such increase remained for 21 days after toxin injection, suggesting that SP is pivotally involved in rearrangement in the chemical coding of central and enteric neurons in the presence of PD Thornton and Vink, 2012).

Besides the increased electrically-evoked tachykininergic motor activity in the distal colon of 6-OHDA rats, an enhancement of colonic myogenic responses elicited by a direct activation of tachykininergic NK_1 receptors with exogenous SP were detected in my experiments. In order to substantiate these results, I examined the expression of tachykininergic NK_1 receptors in specimens of colonic neuromuscular layer by western blot assays, and found that these tachykininergic receptor subtypes are up-regulated in the colon of 6-OHDA rats. Of note, increments of SP levels, as well as of tachykininergic NK_1 receptor density, have been previously described in the striatum of patients with PD, where the binding of SP to tachykininergic NK_1 receptors was found to be significantly increased in comparison with healthy people (Rioux, 1993).

In conclusion, based on the present findings, it is conceivable that an enhancement tachykininergic enteric neurotransmission contributes to colonic motor abnormalities in 6-OHDA rats.

Colonic neuroinflammation in the presence of central dopaminergic neurodegeneration

The changes in colonic cholinergic and tachykininergic neurotransmissions, as highlighted by the present findings, 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, Rub 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, Rub et al. 2003, Braak, Ghebremedhin et al. 2004, Zheng and Travagli 2007, Zheng, Wang et al. 2011).

The DMV-vagus nerve axis 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 and Boeckxstaens 2013). Indeed, there is evidence that the vagus nerve exerts tonic anti-inflammatory actions and contributes to the maintenance of intestinal homeostasis (Matteoli and Boeckxstaens 2013), while vagotomy confers an increased susceptibility to the development of inflammatory bowel diseases (Ghia, Blennerhassett et al. 2006). In addition, extrinsic enteric sensory neurons, containing also substance P, are located in the nodose ganglia and dorsal root

ganglia, and through the DMV they are involved in the regulation of sensory transmission between CNS and ENS (Costa, 1986). In particular, SP, released by capsaicin-sensitive primary sensory neurons, is a crucial mediator of neuroinflammation, and through its NK_1 receptors it takes part in a process known as neurogenic inflammation in several tissues, including the gut (Harrison and Geppetti, 2001).

Based on this knowledge, I hypothesized that 6-OHDA-induced nigrostriatal denervation might impair the DMV-vagal anti-inflammatory pathway, as well as increase the activity of extrinsic enteric sensory neurons, containing SP, resulting in a condition of mild chronic bowel neuroinflammation, that would in turn lead to persistent dysfunctions in the enteric neuromuscular compartment. To test this hypothesis, I assayed TNF, IL-1β and MDA levels in colonic tissues, and found that these parameters were increased in specimens from 6-OHDA rats, thus suggesting that experimental nigrostriatal denervation is associated with inflammatory activity and related oxidative stress in the colonic wall. In addition, an increase in eosinophil and mast cell density within the colonic *tunica mucosa* and *submucosa*, as well as glial cell activation in colonic myenteric ganglia were observed in 6-OHDA rats, thus confirming the occurrence of colonic neuroinflammation following central nigrostriatal denervation.

Of interest, the present 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, Lebouvier et al. 2013). In addition, the pro-inflammatory cytokine profile, as well as the recruitment of eosinophils, mast cells and glial cells observed in 6-OHDA rats represent conditions strikingly reflecting those observed in patients affected by inflammatory bowel

diseases (IBDs), thus suggesting that nigrostriatal denervation is associated with a process of gut inflammation that displays cytokine and cellular correlations with IBDs, even though less intensive in magnitude (Matsuda et al., 2009). In order to confirm the occurrence of colonic inflammation following central neurodegeneration, I examined also changes in the peritoneal macrophage, which are known to polarize towards the M1 pro-inflammatory phenotype (iNOS), or M2 "wound healing" (arginase-1) (Steinbach, 2014). In this series of experiments, I observed that 4 and 8 weeks after 6-OHDA injection peritoneal macrophages were polarized towards the pro-inflammatory M1 phenotype, with iNOS/Argenase-1 ratio increased in comparison with control rats, suggesting the presence of gut inflammation following intranigral neurodegeneration.
In conclusion, central dopaminergic denervation is associated with the occurrence of gut neuroinflammation, likely resulting from an impairment of DMV-vagal axis, which might contribute to colonic motor alterations.

Effects of L-DOPA/Benserazide co-treatment on the patterns of colonic neuromuscular excitatory cholinergic and tachykininergic pathways in the presence of experimental Parkinson's disease

At present, the oral administration of L-DOPA, in combination with a peripheral inhibitor of DOPA-decarboxylase, represents the first-line therapy for the management of PD (Cotzias, Papavasiliou et al. 1969). However, long-term L-DOPA treatment is associated with a variety of adverse effects, such as "on-off" fluctuations, freezing episodes, lack of responsiveness and dyskinesia. These complications are thought to be associated with fluctuations of L-DOPA plasma concentrations, which can be

exacerbated by alterations of gastric emptying, often observed in PD patients (Obeso, Olanow et al. 2000, Nyholm and Lennernas 2008).

Moreover, the treatment with L-DOPA can worsen further gastric emptying and through such a dysfunction L-DOPA is thought to contribute to its own absorption patterns, with subsequent changes in the therapeutic response (Pellegrini et al., 2015).

However, whether L-DOPA actually contributes to the severity of gastric dysmotility in PD patients, remains presently unclear and, most important, there is a lack of data regarding the effects of L-DOPA on motor dysfunctions of the lower digestive tract in PD patients.

On this basis, a part of my research program was dedicated to characterize the effects of L-DOPA/Benserazide treatment on the excitatory pathways regulating colonic motility in the presence of nigrostriatal dopaminergic denervation.

In this context, my results support the view that treatment with L-DOPA/BE was associated with a normalization of colonic motor activity, which was significantly impaired following 6-OHDA-induced nigrostriatal denervation. These findings suggest that L-DOPA/BE administration to animals with 6-OHDA-induced nigrostriatal denervation can restore normal pattern of colonic motility, likely through an increase in CNS dopamine levels. Consistently with these findings, a previous study by Tateno et al., (2011) showed that treatment with L-DOPA/carbidopa enhanced rectal contraction in PD patients, thus ameliorating anorectal constipation (Tateno et al., 2011).

In order to characterize the enteric neuronal pathways involved in the restoration of colonic motility by L-DOPA/BE, I went on to examine the patterns of *in vitro* evoked excitatory cholinergic and tachykininergic motor activities. The results showed that the evoked cholinergic contractions of both colonic longitudinal and circular muscle

from 6-OHDA rats were normalized following treatment with L-DOPA/BE, suggesting that an increase in central dopamine levels can influence the colonic cholinergic neurotransmission. This observation was supported by a study showing that the administration of domperidone, which does not cross the blood brain barrier, did not antagonize the effect of L-DOPA (Tateno et al., 2011). Therefore, these findings provide the first demonstration that, in the presence of central nigrostriatal denervation, the restoration of colonic motor activity by L-DOPA/BE, is associated with an improvement of excitatory cholinergic control in the large bowel, likely through a neuronal brain-gut link regulated by central dopaminergic systems. Of note, these results are in keeping with previous data, showing that, under stress conditions, the intracerebroventricular administration of dopamine stimulated colonic spike bursts, presumably via the hypothalamus, thus corroborating the hypothesis that L-DOPA might control the lower digestive tract functions via central neuronal circuity (Bueno et al., 1992).

As a further step, I examined the effects of L-DOPA treatment on electrically evoked tachykininergic contractions of colon, since 6-OHDA rats have shown an increase in colonic tachykininergic neurotransmission. In this setting, L-DOPA/BE administration did not modify the enhanced SP-mediated colonic contractions, suggesting that the restoration of colonic motility following the antiparkinson treatment did not extend to the enteric tachykininergic pathway.

Besides the impact of L-DOPA/BE treatment on neurogenic cholinergic and tachykininergic motor activities in the colon of 6-OHDA rats, I examined also the effects of this drug combination on the colonic myogenic responses elicited by direct activation of muscarinic and tachykininergic receptors, with carbachol and exogenous substance P, respectively.

In a previous series of experiments, I had observed an up-regulation of muscular muscarinic and tachykininergic NK_1 receptors in the colonic tissues of 6-OHDA animals. With regard for the muscarinic receptor up-regulation, it appeared to result from a compensatory response to the impairment of cholinergic neurotransmission following central nigrostriatal denervation. Conversely, the enhancement of tachykininergic colonic control was associated with an increase in both NK_1 receptor and SP expression.

In this context, the administration of L-DOPA/BE to 6-OHDA animals did not affect myogenic colonic contractile responses mediated by direct pharmacological stimulation of muscarinic and tachykininergic receptors, thus suggesting that the increment of dopamine levels in the CNS can contribute to restore colonic neuromotility at the neuronal pre-junctional level, while it doesn't appear to act on the alterations of muscarinic and tachykininergic motor responses at the post-junctional myogenic level.

In order to corroborate the above results and their interpretation, I am currently performing a set of morphological and molecular experiments on the neuronal markers (ChAT and SP), as well as M_2, M_3 muscarinic and NK_1 tachykininergic muscular receptors.

In the second part of the present study, I had observed that central nigrostriatal denervation is associated with the occurrence of colonic inflammation characterized by increased levels of TNF, IL-1β, and oxidative stress. Therefore, I went on to evaluate the impact of L-DOPA/BE treatment on the increase in oxidative stress and pro-inflammatory cytokine levels in colonic tissue from 6-OHDA animals.

The results showed that the administration of L-DOPA/BE to 6-OHDA rats normalized significantly the pro-inflammatory cytokine levels, without affecting the increased level of oxidative stress.

These findings represent a point of significant novelty, since it appears that the increment of dopamine levels in the central dopaminergic circuity can exert anti-inflammatory effects. However, there is a lack of knowledge concerning the effects of L-DOPA/BE on the gut inflammation in PD either in humans or experimental models. Therefore, further investigations are needed to better appreciate and disclose the mechanisms through which L-DOPA can modulate peripheral inflammation associated with PD.

In conclusion, based on the present findings, it is conceivable that the treatment with L-DOPA/BE following central nigrostriatal denervation can restore colonic motility, likely through a normalization of the cholinergic enteric neurotransmission, and that it can also improve the colonic inflammation associated with central dopaminergic denervation.

Overall conclusions

Taken together, the results obtained in the research program indicate that central nigrostriatal dopaminergic denervation is associated with an impairment of local cholinergic neurotransmission and an enhanced tachykininergic control, which are likely to contribute to the alteration of colonic transit rate. The occurrence of a condition of gut inflammation following central dopaminergic denervation might contribute to these colonic neuromotor dysfunctions.

Colonic changes in PD might results from an impairment of the vagal anti-inflammatory pathway, as well as from an enhanced activity of tachykininergic primary sensory neurons. However, it remains unclear whether the colonic motor alterations occurring upon central dopaminergic denervation result from gut neuroinflammation or a rearrangement of enteric neuronal coding or both. In addition, further investigations aimed at evaluating the alterations of extrinsic neuronal pathways linking the CNS to ENS in the presence of central dopaminergic neurodegeneration are needed.

Treatment with L-DOPA/BE in animal with central nigrostriatal denervation is associated with a recovery of the colonic motor activity, characterized by a normalization of enteric cholinergic neurotransmission. In addition, the increment of dopamine levels in the CNS of 6-OHDA rats seems to exert, at least in part, anti-inflammatory effects against the gut neuroinflammation associated with central dopaminergic neurodegeneration. However, the mechanisms thought which L-DOPA acts on enteric neuronal pathways as well as on colonic inflammation remain unclear and scarcely investigated.

In conclusion, the present study has shed some light on the pathophysiological mechanisms underlying colonic motor dysfunctions associated with central dopaminergic denervation (a condition which reflects one of the pathological hallmarks

of PD). These findings can provide a basis for better understanding the mechanisms underlying GI motor abnormalities in PD, and thereby paving the way to the development of suitable pharmacological treatments for the management GI dysfunctions associated with PD.

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Curriculum vitae et Studiorum

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EDUCATION AND TITLE

Degree: Degree in Chemistry and Pharmaceutical Technologies at the University of Pisa on the 16th of November 2011, 109/110

Fellowship/Scholarship

June-December 2014: Visiting PhD students at Manchester Collaborative Centre for Inflammation Research (MCCIR), University of Manchester

May 2012: titolare di borsa di studio del Centro Interdipartimentale di Ricerche di Farmacologia Clinica e Terapia Sperimentale di durata annuale per lo svolgimento di uno studio dal seguente titolo: *"Monitoraggio delle reazioni avverse ai farmaci impiegati off label nel paziente pediatrico: valutazione dei dati di segnalazione spontanea in Toscana e in Italia".*

January 2012: post-degree fellow in Laboratory of Gastrointestinal Pharmacology in the Department of Clinical and Experimental Medicine, University of Pisa.

LANGUAGES

English: Good

CONOSCENZE INFORMATICHE, COMPUTER SKILLS

- Office programs (word, power point, excel, access), Curve Expert; BIOPAC system, Inc (Registered to ISO 9001:2008); Graph Pad Prism; ChemWindow3.

ACTUALLY

PhD fellowship in Experimental Pharmacology and Toxicology

RESEARCH ACTIVITIES

Laboratory of Gastrointestinal Pharmacology

Role of adenosine system in the pathophysiology of inflammatory bowel diseases

- Involvement of adenosine receptors in neuromuscular gut dysfunctions associated with intestinal inflammation; .

Evaluation of non-steroidal anti-inflammatory drugs long term administration in the onset of bowel damage in rat.

- Characterization of injury induced by several chemical compounds: non and COX-2 selective drugs.

Role of purinergic P2X7 receptor in the pathophysiology of inflammatory bowel diseases

- Involvement of P2X7 receptor in neuromuscular gut dysfunctions associated with intestinal inflammation

Molecular and pharmacologic characterization of systems regulating neuromuscular functions of distal intestinal tract in Parkinson experimental disease.

Evaluation of L-DOPA + carbidopa administration on the gut neuro-muscolar activity in Parkinson experimental model.

Evaluation of rearrangement processes of myenteric ganglia cellular framework, as a consequence of experimental Parkinson induction and/or L-DOPA + carbidopa administration

Role of deubiquitinases in the activation of inflammasome induced by the parasite *Toxoplasma gondii*

Skills:

- ✓ Functional analysis of in vitro motility on smooth muscle

specimens: characterization of neuromotor systems.

- ✓ Evaluation of inflammatory and oxidative stress parameters, with ELISA or colorimetric techniques, in bowel inflammation models
- ✓ RT-PCR
- ✓ Western blot analysis on cells and tissues
- ✓ Flow analysis
- ✓ Immunohistochemistry

AWARDS AND GRANTS

Travel Grant from Boehringer Ingelheim Fonds (May 2014)

Research fellowship from Italian Society of Pharmacology (SIF) (July 2014)

Research fellowship from Italian Society of Pharmacology- Merk Sharp & Dohme Corporation (SIF) (October 2015)

PUBBLICATIONS

1) Cristina Segnani, Chiara Ippolito, Luca Antonioli, **Carolina Pellegrini**, Corrado Blandizzi, Amelio Dolfi1, Nunzia Bernardini. Histochemical detection of collagen fibers by Sirius Red/Fast Green is more sensitive than van Gieson or Sirius Red alone in normal and inflamed rat colon. *PLoS One*. 2015 Dec 16;10(12):e0144630.
2) Matteo Fornai[1], **Carolina Pellegrini[1]**, Luca Antonioli, Cristina Segnani, Chiara Ippolito, Elisabetta Barocelli, Vigilio Ballabeni, Gaia Vegezzi, Zainab al Harraq, Fabio Blandini, Giovanna Levandis, Silvia Cerri, Corrado Blandizzi, Nunzia Bernardini, and Rocchina Colucci. Enteric dysfunctions in experimental Parkinson's disease: alterations of excitatory cholinergic neurotransmission regulating colonic motility in rats. *JPET Submitted.[1]The authors contributed equally to the manuscript*
3) **Carolina Pellegrini**, Luca Antonioli, Rocchina Colucci, Vigilio Ballabeni, Elisabetta Barocelli, Nunzia Bernardini, Corrado Blandizzi, Matteo Fornai. Gastric motor dysfunctions in Parkinson's disease: current pre-clinical evidence. *Parkinsonism and related Disorders, 2015(1-8) in press.*
4) Antonioli L, Colucci R, **Pellegrini C**, Giustarini G, Sacco D, Tirotta E, Caputi V,

Marsilio I, Giron MC, Németh ZH, Blandizzi C, Fornai M. The AMPK enzyme-complex: from the regulation of cellular energy homeostasis to a possible new molecular target in the management of chronic inflammatory disorders. *Expert Opin Ther Targets*. 2015 Sep 28:1-13.

5) Antonioli L, Giron MC, Colucci R, **Pellegrini C**, Sacco D, Caputi V, Orso G,Tuccori M, Scarpignato C, Blandizzi C, Fornai M. Involvement of the P2X7 Purinergic Receptor in Colonic Motor Dysfunction Associated with Bowel Inflammation in Rats. *PLoS One. 2014 Dec 30;9(12):e116253. doi:10.1371/journal.pone.0116253.*

6) Antonioli L, Fornai M, Awwad O, Giustarini G, **Pellegrini C**, Tuccori M, Caputi V, Qesari M, Castagliuolo I, Brun P, Giron MC, Scarpignato C, Blandizzi C, Colucci R. Role of the A2B receptor-adenosine deaminase complex in colonic dysmotility associated with bowel inflammation in rats. *Br J Pharmacol. 2014* Mar;171(5):1314-29. doi: 10.1111/bph.12539.

7) Fornai M, Antonioli L, Colucci R, **Pellegrini C**, Giustarini G, Testai L, Martelli A, Matarangasi A, Natale G, Calderone V, Tuccori M, Scarpignato C, Blandizzi C. NSAID-induced enteropathy: are the currently available selective COX-2 inhibitors all the same? *J Pharmacol Exp Ther*. 2014 Jan;348(1):86-95. doi: 10.1124/jpet.113.207118.

8) Antonioli L, Colucci R, **Pellegrini C**, Giustarini G, Tuccori M, Blandizzi C,Fornai M. The role of purinergic pathways in the pathophysiology of gut diseases: pharmacological modulation and potential therapeutic applications. *Pharmacol Ther*. 2013 Aug;139(2):157-88. doi: 10.1016/j.pharmthera.2013.04.002.

ABSTRACT

6th european congress of pharmacology. 17-20 july 2012, Granada, Spain.	V. Calderone, A. Martelli, L. Testai, A. Marino, **C. Pellegrini**, I. Pugliesi, S. Taliani, G. Nesi, S. Rapposelli, F. Da Settimo, MC Breschi. H_2S releasing properties of new thioamide, iminothioether and isothiocyanate derivatives.
Joint International Neurogastroenterology and Motility Meeting. 6-8 september 2012 Bologna, Italy	L. Antonioli, M. Fornai, R. Colucci, O. Awwad, G. Giustarini, **C. Pellegrini**, M. Tuccori, M. Qesari, I. Castagliuolo, P. Brun, M.C Giron, C. Scarpignato, C. Blandizzi. Role of A2B receptors in the control of colonic cholinergic motility in the presence of bowel inflammation.

Joint International Neurogastroenterology and Motility Meeting. 6-8 september 2012 Bologna, Italy	Matteo Fornai, **Carolina Pellegrini**, Luca Antonioli, Giovanna Levandis, Rocchina Colucci, Giulio Giustarini, Fabio Blandini, Corrado Blandizzi. Alterations of colonic cholinergic and tachykininergic motility in a rat model of Parkinson's disease.
Poster of distinction. Digestive Disease Week 18-21 may 2013 Orlando, Florida.	M. Fornai, **C. Pellegrini**, L. Antonioli, G. Levandis, R. Colucci, G. Giustarini, F. Blandini, C. Blandizzi. Characterization of excitatory and inhibitory neuromuscolar pathways regulating colonic motility in a rat model of Parkinson's disease.
Poster of distinction. Digestive Disease Week 18-21 may 2013 Orlando, Florida.	Luca Antonioli, Rocchina Colucci, Marco Tuccori, Giulio Giustarini, **Carolina Pellegrini**, Maria Grazia Zizzo, Rosa Serio, Ignazio Castagliuolo, Maria Cecilia Giron, Corrado Blandizzi, Matteo Fornai. Involvement of the P2X7 purinergic receptor in colonic motor dysfunction associated with bowel inflammation in rats**.**
35° CONGRESSO NAZIONALE DELLA SOCIETA' ITALIANA DI ISTOCHIMICA 12-14 June Santa Margherita di Pula (CA), Italy	Segnani C, Ippolito C, Stocchi S, Fornai M, **Pellegrini C**, Blandizzi C, Dini S, Dolfi A, Bernardini N. Morpho-functional alterations of colonic neuromuscular compartment in experimental Parkinson's disease.
67° CONGRESSO della Società Italiana di Anatomia e Istologia 20-22 September 2013 Brescia (BS)	Segnani C, Ippolito C, Stocchi S, Fornai M, **Pellegrini C**, Blandizzi C, Dini S, Dolfi A, Bernardini N. Morpho-functional alterations of colonic neuromuscular compartment in experimental Parkinson's disease.
36° Congresso Nazionale della SIF 23-26 October 2013 Torino, Lingotto, Italy	**C. Pellegrini**, M. Fornai, L. Antonioli, G. Levandis, S. Cerri, R. Colucci, G. Giustarini, F. Blandini, C. Blandizzi. Gastrointestinal dysfunctions in experimental Parkinson's disease: alterations of excitatory and inhibitory neuromuscular pathways regulating colonic motility
36° Congresso Nazionale della SIF 23-26 October	Luca Antonioli, Rocchina Colucci, **Carolina Pellegrini**, Deborah Sacco, Maria Grazia Zizzo, Rosa Serio, Ignazio Castagliuolo,Maria Cecilia Giron, Carmelo Scarpignato,

2013 Torino, Lingotto, Italy	Corrado Blandizzi, Matteo Fornai. Control of enteric neuromuscular functions by purinergic p2x7 receptors in normal rat distal colon and experimental bowel inflammation.
9th Congress of ECCO - Inflammatory Bowel Diseases 20-22 February 2014, Copenhagen, Denmark	L. Antonioli, M. Fornai, R. Colucci, **C. Pellegrini**, D. Sacco, V. Caputi, G. Orso, M.C. Giron, C. Scarpignato, C. Blandizzi. Role of P2X7 purinergic receptor in the control of enteric neuromuscular functions in normal rat distal colon and experimental bowel inflammation.
Digestive Disease Week 3-6 May 2014 CHICAGO// Illinois.	**C. Pellegrini**, M. Fornai, L. Antonioli, Colucci, D. Sacco, C. Ippolito, C. Segnani, G. Levandis, S. Cerri, R. F. Blandini, N. Bernardini, C. Blandizzi. Alterations of colonic neuromuscular excitatory cholinergic pathway in a rat model of 6-hydroxydopamine-induced Parkinson's disease
Digestive Disease Week 3-6 May 2014 CHICAGO// Illinois.	M.Fornai, **C. Pellegrini**, Colucci, L. Antonioli, D. Sacco, G. Levandis, S. Cerri, R. F. Blandini, C. Blandizzi. Effects of L-DOPA/Benserazide co-treatment on the patterns of colonic neuromuscular excitatory cholinergic and tachykininergic pathways in the presence of experimental Parkinson's disease.
Digestive Disease Week 3-6 May 2014 CHICAGO// Illinois.	L. Antonioli, M. Fornai, **C. Pellegrini**, D. Sacco, B. Csóka, C. Segnani, N. Bernardini, V. Caputi, M.C. Giron, G. Haskó, C. Blandizzi, R. Colucci. Modulatory role of adenosine A_{2B} receptors on pro-fibrotic signalling in experimental colitis.
Purines 23-27 July 2014 Bonn// Germany.	L. Antonioli, M. Fornai, **C. Pellegrini**, D. Sacco B. Csóka, C. Segnani, C. Ippolito, G. Haskó, C. Blandizzi, R. Colucci**.** Modulatory role of adenosine A_{2B} receptors in fibrotic gut wall remodeling associated with experimental colitis.
Society for Immunology Annual Congress 1-4 December 2014 Brighton// UK.	M. Lyall, **C. Pellegrini**, T. Yung, G. Lopez-Castejon. New insights into NLRP3 inflammasome regulation by ubiquitin.
Digestive Disease Week 16-	L. Antonioli, D.Sacco, M. Fornai, **C. Pellegrini**, E.

19 May 2015 Washington DC.	Tirotta, V. Caputi, M.C. Giron, G Orso, G. Haskó, C. Scarpignato, C. Blandizzi, R. Colucci. Colonic dysmotility associated with high fat diet-induced obesity: involvement of endogenous adenosine via A_{2B} receptors.
UEG week 24-28 October 2015 Barcellona, Spain.	R. Colucci, E. Ghelardi, E. Tirotta, E. Piccoli, D. Sacco, L. Antonioli, M. Fornai, C. Renzulli, **C. Pellegrini**, C. Blandizzi, C. Scarpignato. Rifaximin prevents enteric bacteria alterations and inflammation in a rat model of diclofenac-induced enteropathy.
37° Congresso Nazionale della SIF 26-30 October 2015 Napoli, Italy.	E. Tirotta, R. Colucci[1], E. Ghelardi, E. Piccoli, D. Sacco, L. Antonioli, M. Fornai, C. Renzulli, **C. Pellegrini**, C. Blandizzi, C. Scarpignato. Enteric bacteria alterations and inflammation associated with diclofenac-induced enteropathy: preventive effects of rifaximin.
Simposium 37° Congresso Nazionale della SIF 26-30 October 2015 Napoli, Italy	L. Antonioli, M. Fornai , R. Colucci, **C. Pellegrini**, E. Tirotta, M.C. Giron, V. Caputi, I. Marsilio, S. Sartini, V. Coviello, C. La Motta, F. Da Settimo, C. Blandizzi. Adenosine regulating agents as novel strategies for the pharmacological treatment of inflammatory bowel diseases.
Congresso Nazionale della SIF 26-30 October 2015 Napoli, Italy.	Fornai M., Antonioli L., **Pellegrini C**., Colucci R., Sacco D., Tirotta E., Natale G., Bartalucci A., Flaibani M., Renzulli C., Blandizzi C., Scarpignato C. **Protective effects of rifaximin against experimental enteropathy induced by indomethacin in rat.** *37°*
ORAL COMUNICATIONS *Congresso Nazionale della SIF 26-30 October 2015 Napoli, Italy.*	**C. Pellegrini**, M. Fornai, D. Sacco, E. Tirotta, V. Caputi, M.C. Giron, G. Orso, I. Marsilio, C. Scarpignato, C. Blandizzi, R. Colucci, L. Antonioli. Involvement of endogenous adenosine via A_{2B} receptors in colonic dysmotility associated with high fat diet-induced obesity.

CONGRESS AND MEETING

- **Medical training meeting "Osteoarthrosis: from diagnosis to specific**

therapeutic treatments", Junes 15-16th 2012, Pisa, Italy

- **Medical training meeting "Novel proposal for pharmacologic therapy of melanoma", May 30th 2012, Pisa, Italy**
- **Annual Meeting "Purine Club" and "Tuscany Adenosine Network", September 13-14th, Pisa, Italy**

Ai sensi della DL 196/2003 (tutela delle persone e di altri soggetti rispetto al trattamento dei dati personali), autorizzo il trattamento dei dati personali contenuti nel presente *curriculum vitae studiorum*