Investigating intestinal smooth muscle dysfunction in the 6-OHDA rat model of Parkinson's disease

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Cover illustration:

Sagittal projections of the nigrostriatal pathway in the healthy brain of the rat, a tyrosine hydroxylase immunostaining.

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To my beloved Daniel

"Research is to see what everybody else has seen, and to think what nobody else has thought."

Albert Szent-Györgyi

Abstract

The neurodegenerative progression of the nigrostriatal pathway in Parkinson's disease (PD) entails the appearance of motor and nonmotor symptoms. Nonmotor symptoms are common, having a negative impact on the quality of life of patients living with PD. Among these symptoms, autonomic dysfunction is particularly bothersome affecting the intestinal function. This thesis explores the role of the smooth muscle in the impaired enteric neurotransmission seen in PD using the 6-hydroxydopamine (6-OHDA) rat model, a well-standardized and validated model of PD.

A priori experimental revision of the model demonstrated that a commonly used neuroprotective drug in 6-OHDA rat stereotaxic surgery, desipramine, interferes with the peripheral assessment of urinary bladder and intestinal motility. Therefore, it was omitted to minimize confounding factors when studying peripheral autonomic dysfunction.

Ileal and colonic dysfunction was assessed by electrical field-, cholinergic, and monoaminergic stimulation in smooth muscle strips and/or segments. Colonic enhancement of electrically-induced and cholinergic-evoked contractile responses was found, correlating with previously described upregulation of muscarinic receptors. Moreover, this cholinergic impairment shown to be related to altered inhibitory signaling. In addition, serotonergic neuromodulation displayed alterations in both colon and ileum following central dopamine lesion. Catecholaminergic-induced intestinal motility, however, showed no differences despite previous studies identifying a reduction of dopaminergic and noradrenergic neurotransmission. These findings increase the understanding of complex interactions between enteric neurotransmitters after central dopamine neurodegeneration affecting the intestinal function and may contribute in the future to discover novel therapeutic approaches based on these interactions.

Keywords:

Intestinal motility, smooth muscle, enteric nervous system, dopamine neurodegeneration

Sammanfattning på svenska

Den neurodegenerativa utvecklingen av den nigrostriatala banan hos Parkinsons sjukdom (PS) medför motoriska- och icke-motoriska symptom. Icke-motoriska symptom är vanliga och har en negativ inverkan på livskvalitén hos patienter som lever med PS. Bland dessa symptom är den autonoma dysfunktionen speciellt besvärlig då det påverkar tarmfunktionen. I denna avhandling undersöks hur den enteriska neurotransmissionen och vidare den glatta muskulaturens funktion påverkas hos PS genom att använda råttmodellen 6-OHDA, en väl standardiserad och validerad Parkinsonmodell.

A priori validering av modellen visade att ett vanligt använt neuroprotektivt läkemedel i den stereotaktiska kirurgin, desipramin, påverkar urinblåsans samt tarmens funktion och därmed de responser som studeras. Den har därför utelämnats för att minimera störfaktorer vid studien av perifer autonom dysfunktion.

Elektrisk, kolinerg och monoaminerg stimulering av de glatta muskelsegment (ileum och kolon) utfördes för att studera effekten av 6-OHDA-behandlingen. I kolonsegment sågs ökade kontraktila svar vid elektrisk och kolinerg stimulering, vilket stämmer väl överens med tidigare rapporter som beskriver uppreglering av muskarina receptorer och minskad endogen utsöndring av acetylkolin i 6-OHDA-modellen. Vidare visade det sig att försämringen i det kolinerga svaret var relaterat till förändrad inhibitionssignalering. Utöver detta hittades förändringar i serotonerg neurotransmission i både kolon och ileum efter centrala dopamincellförlust. Dock sågs inga skillnader i katekolaminerg-inducerad tarmmotilitet trots tidigare studier där man såg förändringar i dopaminerg och noradrenerg neurotransmission. Dessa fynd ökar förståelsen av de störningar som uppkommer i tarmens komplexa transmittorinteraktioner efter central degenering av dopaminerga neuron. Förståelsen kan bidra till framtida upptäckter av nya terapeutiska metoder där perifera biverkningar kan reduceras.

Nyckelord

Tarmmotilitet, glatt muskulatur, enteriska nervsystemet, dopaminerg neurodegeneration

Resumen

La progresión neurodegenerativa de la vía nigroestriatal en la enfermedad de Parkinson (EP) conlleva la aparición de síntomas motores y no motores. Los síntomas no motores son comunes y tienen un impacto negativo en la calidad de vida de los pacientes que viven con EP. Entre estos síntomas, la disfunción autonómica es particularmente molesta y afecta la función intestinal. Esta tesis explora el papel del músculo liso en la alteración de la neurotransmisión entérica que se observa en la EP utilizando el modelo de la 6-hidroxidopamina (6-OHDA) en ratas, un modelo bien estandarizado y validado de la EP.

Una revisión experimental *a priori* del modelo demostró que un fármaco neuro protector de uso común en la cirugía estereotáxica en ratas para la inyección intracerebral de 6-OHDA, la desipramina, interfiere con la evaluación periférica de la motilidad de la vejiga urinaria y los intestinos. Por lo tanto, se omitió para minimizar los factores de confusión al estudiar la disfunción autonómica periférica.

La disfunción ileal y colónica se evaluó mediante estimulación eléctrica, colinérgica y monoaminérgica en tiras y/o segmentos de músculo liso. Se encontró una respuesta contráctil aumentada en el colon al ser estimulado con agentes colinérgicos e inducida eléctricamente, lo que se correlaciona con el aumento de receptores muscarínicos postsinápticos. Además, se ha demostrado que este deterioro colinérgico está relacionado con la alteración de la inhibición de la motilidad. Asimismo, la neuromodulación serotoninérgica mostró alteraciones tanto en el colon como en el íleon. Sin embargo, la motilidad intestinal inducida por catecolaminérgicos no mostró diferencias a pesar de estudios previos que identificaron una reducción de la neurotransmisión dopaminérgica y noradrenérgica. Estos hallazgos proveen información acerca de las complejas interacciones entre neurotransmisores entéricos tras la deficiencia de dopamina central, lo que puede contribuir en el futuro a descubrir nuevos enfoques terapéuticos basados en estas interacciones.

Palabras clave

Motilidad intestinal, músculo liso, sistema nervioso entérico, neurodegeneración dopaminérgica

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Murillo, M.D.P.**, Aronsson, P., Winder, M., Carlsson, T. *Desipramine, commonly used as a noradrenergic neuroprotectant in 6-OHDA-lesions, leads to local functional changes in the urinary bladder and gastrointestinal tract in healthy rats*. **Heliyon.** 6: e05472.

II. **Murillo, M.D.P.**, Johansson, E., Bryntesson, V., Aronsson, P., Tobin, G., Winder, M., Carlsson, T. *Alterations in smooth muscle function of colonic segments in the 6-hydroxydopamine rat model of Parkinson's disease.* **Manuscript submitted**

III. **Murillo, M.D.P.**, Aronsson, P., Tobin, G., Winder, M, Carlsson, T. *Monoaminergic smooth muscle responses in colonic and ileal segments in the 6-hydroxydopamine rat model of Parkinson's disease.* **Manuscript**

Content

Abbreviations

5-HT	5-hydroxytriptamine (serotonin)
6-OHDA	6-hydroxydopamine
ACh	Acetylcholine
ANS	Autonomic Nervous System
AP	Anterior-posterior
ATP	Adenosine triphosphate
cAMP	cyclic Adenosine monophosphate
cGMP	cyclic Guanosine monophosphate
CNS	Central nervous system
DA	Dopamine
DAB	3,3'-diaminobenzidine
DMV	Dorsal motor nucleus of the vagus
DV	Dorsal-ventral
DVC	Dorsal vagal complex
EC	Enterochromaffin cells
EFS	Electrical field stimulation
ENS	Enteric Nervous System
GABA	γ-Aminobutyric acid
GI	Gastrointestinal
ICCs	Interstitial cells of Cajal
IP	Intraperitoneal
L-DOPA	L-3,4-dihydroxyphenylalanine
L-NAME	N^{γ}-Nitro-L-arginine methyl ester
mAChRs	Muscarinic acetylcholine receptors

MeCh	Methacholine
MFB	Medial forebrain bundle
ML	Medial-lateral
MLC	Myosin light chain
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NA	Noradrenaline
NANC	Non-adrenergic non-cholinergic
NET	Noradrenaline transporter
NK	Neurokinin
NHS	Normal horse serum
NMS	Non-motor symptoms
NO	Nitric oxide
NOS	Nitric oxide synthase
NTS	Nucleus of the solitary tract
PBS	Phosphate buffered saline
PD	Parkinson's Disease
PFA	Paraformaldehyde
PNS	Peripheral nervous system
SC	Subcutaneous
SM	Smooth muscle
SN	Substantia nigra
SP	Substance P
TH	Tyrosine hydroxylase
VIP	Vasoactive intestinal polypeptide
α-syn	α-synuclein

Introduction

"So slight and nearly imperceptible are the first inroads of this malady, and so extremely slow its progress, that it rarely happens, that the patient can form any recollection of the precise period of its commencement"

James Parkinson, Essay on the shaking palsy, 1817

This thesis focuses on the study of possible enteric adaptations to nigrostriatal neurodegeneration to understand the Parkinson's disease (PD)-related intestinal dysfunction. An altered intestinal function significantly affects patients living with PD, and it worsens over time along with the disease. In this introduction, concepts and physiological processes of the gastrointestinal (GI) system are first summarized to then discuss the pathological changes occurring in the intestine as a consequence of PD progression. In addition, animal models of PD are briefly described with special emphasis on the 6-hydroxydopamine (6-OHDA) rat model.

The gastrointestinal system

From the mouth (oral) to the anus (aboral), the main function of the GI system is to digest and absorb nutrients from the food. For this purpose, synchronized GI motility facilitates and maximizes absorption. With the ingestion of food there is a risk of the entry of potentially harmful substances or microorganisms; therefore, mucus production and immune responses act jointly with resident microbiota to protect the local microenvironment.

A structured organization is essential to guarantee an optimal function. On the intestine, epithelial invaginations facing the lumen increase the absorptive area due to a tightly polarized monolayer of epithelial cells that includes enterocytes and other multifunctional cells specialized on mucus production (goblet cells), immune protection (Paneth cells), paracrine signaling (enterochromaffin cells, ECs) and transcytosis (M cells). Epithelial proliferation is promoted by fibroblasts, located in the submucosa, supporting the extracellular matrix. In proximity, two neural plexuses are responsible for secretory and motility regulation in connection with two spatially aligned smooth muscle (SM) layers. The inner SM lays concentrically

around the circumference of the intestinal tube, known as circular, and the outer SM is displayed orthogonally in the oral-aboral direction, known as longitudinal.

The GI functional responses are carried out locally by the enteric nervous system (ENS) and by interactions between enteroendocrine cells (Latorre et al., 2016), closely regulated by the autonomous nervous system (ANS). Responses are well influenced by interactions between the intestinal mucosa and the resident microbiota.

Central control of intestinal function

The GI tract is tightly connected to the central nervous system (CNS) by parasympathetic and sympathetic branches of the ANS, relaying enteric sensory and motor signals, thereby regulating and modulating GI functions. The GI response to extrinsic neural control exercised by the ANS gradually becomes more independent towards the anus. Distally, the motility of the large intestine relies more on enteric reflexes than on central innervation [a detailed review can be found in (Browning and Travagli, 2014)].

Parasympathetic afferent and efferent projections are anatomically separated between small and large intestine. For the small intestine, afferent (sensory) and efferent (motor) signaling travel to and from the dorsal vagal complex (DVC). While for the large intestine, afferent and efferent signaling are carried out by splanchnic and sacral pelvic nerves to the lumbosacral dorsal root ganglia. Once in the spinal cord, they travel to and from the solitary tract nucleus (NTS) within the DVC. The DVC is composed mainly of the dorsal motor nucleus of the vagus (DMV), the NTS and the area postrema, located in the brainstem. This complex is an integrative center of autonomic regulation in close connection to the hypothalamus, Barrington's nucleus (also known as pontine micturition/defecation center) and periaqueductal grey, located in the midbrain. Higher integrative centers in the forebrain converge descending responses via midbrain and brainstem nuclei based on cognitive processes such as emotion, memory, and motivation.

Parasympathetic motor preganglionic nerves exert control of intestinal motility through inhibitory non-adrenergic non-cholinergic (NANC) and excitatory cholinergic pathways (Chang et al., 2003), modulated by the DMV and, to less extent, by the spinal cord. In addition, vago-vagal reflexes from the DMV are regulated by the NTS via GABAergic tonic inhibition or excitatory glutamatergic signaling (Davis et al., 2004). In contrast, the

sympathetic noradrenergic nerves regulate the GI motility by antagonizing the parasympathetic effect, through presynaptic inhibitory signaling. Hence, noradrenergic fibers are extrinsic in origin reaching myenteric and submucosal ganglia from the prevertebral sympathetic ganglia. In addition, non-adrenergic sympathetic neurotransmission occurs in the ENS (De Ponti et al., 1996). Sympathetic action also tonically inhibits secretion, and regulates blood flow by inducing vasoconstriction.

Enteric control of the intestinal motility

The intestine can function partially independently, via the ENS, without extrinsic control (Furness et al., 1995). This system, intrinsically, regulates intestinal motility, through the peristaltic reflex, to induce caudal propulsion of feces and promote defecation. Each peristaltic wave has an anteroposterior direction, a coordinated wave between an ascending contraction from the oral end and a descending relaxation from the aboral end (Wood, 2008). This coordination relies on multiple neuronal circuits connected to intrinsic primary afferent neurons, which are sensitive to chemical, thermic, mechanic, and osmotic stimuli, having a direct effect on enteric interneurons (Kunze and Furness, 1999). Upon stimulation, enteric interneurons influence excitatory and inhibitory motor neurons to regulate SM activity (Furness, 2000, Grider, 1989a).

The intrinsic innervation of the intestine relies on two plexuses located in the submucosa, known as Meissner, and in between circular and longitudinal SM layers, known as Auerbach or myenteric (Furness and Costa, 1980). Although the submucosal plexus focuses on the regulation of blood flow and mucus secretion and the myenteric plexus specializes in motility, both plexuses have a bidirectional communication due to ascending and descending pathways to render the enteric responses to stimuli (Costa et al., 2000).

The intrinsic dual action, excitatory and inhibitory, eliciting intestinal movement is due to highly specialized neurons from the myenteric plexus directly activating circular and longitudinal SMs. The excitatory component depends mainly on cholinergic transmission by acetylcholine (ACh) acting on muscarinic receptors (mAChRs) to induce contraction; however, other nerve-mediated substances also intervene such as substance P (SP) (Bartho and Holzer, 1985) and serotonin (5-HT). The sympathetic NANC inhibitory component releases vasoactive intestinal polypeptide (VIP), adenosine 5´-triphosphate (ATP) and nitric oxide (NO) to modulate relaxation.

SM contractile mechanisms

Intestinal SM layers are highly organized and functional units connected by gap junctions, ensuring a coordinated response to maintain GI motility. The SMs have a spatiotemporal relationship with neuronal networks and pacemaker cells, known as interstitial cells of Cajal (ICCs), that enables the propagation of a contractile wave to adjacent interconnected units. Therefore, to propel food contents forward the circular SM responds to chemical- or mechanical-induced distension by first contracting orally and then propagating aborally. In response to circular SM contraction, the longitudinal SM shortens during the contractile wave and relaxes once it has receded (Hennig et al., 1999).

ICCs maintain oscillatory membrane potentials by allowing the flow of Ca^{2+} into the cell, which then produces intermittent depolarizations known as slow waves (Langton et al., 1989). A resting potential characterizes the slow waves as they are autonomous and independent of external stimuli. Upon external stimulation, an upstroke potential induces a transient depolarization that once it reaches its peak causes a repolarization recovering to resting potential, followed by a refractory period. As ICCs are in close contact with SM cells, elicited slow waves alter the membrane conductance of SMs through gap junctions [for a detailed review see (Sanders, 2019)]. Therefore, neuronal and paracrine modulation of ICCs pacemaker activity influences SM motor responses.

For the SM to contract, the light chain of myosin must be phosphorylated to be able to interact with actin filaments. This phosphorylation occurs as a result of increasing intracellular Ca^{2+} concentrations, which then bind to cytosolic calmodulin activating the myosin light chain (MLC) kinase in the presence of ATP (Webb, 2003). To revert contraction, the MLC phosphatase dephosphorylates the light chain of myosin leading to SM relaxation. This process is regulated by Rho kinase phosphorylating the myosin-binding subunit of MLC phosphatase, therefore inhibiting its action (Fukata et al., 2001). In consequence, the interplay between SM contraction and relaxation depends on fluctuating inward intracellular Ca^{2+} concentrations and MLC phosphatase catalysis. Local changes in resting membrane potentials occur after the activation of L-type voltage-gated ion channels that allow extracellular Ca^{2+} to enter the SM depolarizing the cell membrane. The change in voltage evokes an action potential leading to a contractile wave (Berridge, 2008).

Although the SM contractile function is regulated by changes in membrane potentials, action potentials can also be triggered by receptor

activation. Neuronal release of ACh in the neuromuscular junction provokes SM contraction by triggering mAChRs. Direct action of ACh on the muscarinic M_3 receptor leads to phosphoinositide hydrolysis causing an increase of intracellular Ca^{2+}, while M_2 activation inhibits neuronal release of ACh and prevents cyclic adenosine monophosphate (cAMP) accumulation (Ehlert et al., 1997). In addition, M2 and M3 activation modulate membrane depolarization through Na^+ and K^+ channels, a Ca^{2+}-dependent mechanism that prevents excessive SM activation [for detailed review see (Tanahashi et al., 2021)]. It has been described that SP and ACh are co-released by interneurons and motoneurons to modulate intestinal motility. The neuropeptide SP, a tachykinin, acts as a non-cholinergic excitatory neurotransmitter in the intestine. It has been shown that SP, through neurokinin (NK) receptor 1 activation, has a direct prokinetic action mainly acting on ICCs and SM (Grider, 1989b), as well as an indirect inhibitory action stimulating nitrergic neurons, facilitating NO release (Lecci et al., 1999).

NO, VIP and ATP mediate the NANC-induced relaxation of the intestine through inhibitory motor neurons (Keef et al., 1994). The relaxatory effect of ATP and VIP/NO comprise a fast and prolonged hyperpolarization of the SM membrane, respectively (He and Goyal, 1993). In addition, NO activates cyclic guanosine monophosphate (cGMP)-dependent signaling pathways inhibiting phosphorylation of MLC by MLC phosphatase, and VIP activates adenylyl cyclase increasing cAMP concentrations blocking the MLC kinase enzymatic activity. Both, cAMP and cGMP mechanisms, leading to SM relaxation (Makhlouf and Murthy, 1997).

Other neurotransmitters act as modulators of the SM motility in the GI tract, such as dopamine (DA) and 5-HT. Epithelial non-neuronal release of 5-HT activates sensory neurons mediating excitatory and inhibitory motor responses (Foxx-Orenstein et al., 1996) in response to changes in intraluminal pressure or induced by microbiota-epithelium interactions. The 5-HT release induces ascending contraction by activating 5-HT_4 receptors, releasing SP, and facilitates descending relaxation by 5-HT_3 receptors, stimulating VIP production. DA release appears to facilitate relaxation through D_2 receptors, where its absence has prokinetic properties, as shown by D_2 knock-out mice (Li et al., 2006). The action of DA has also shown to be biphasic, inducing a fast contraction at lower concentrations and a slow relaxation at higher concentrations. The relaxatory concentration-dependent effect attributed to the activation of presynaptic α- and post-junctional β-receptors (Kirschstein et al., 2009).

Disturbances in epithelial permeability, changes in GI motility patterns and microbial dysbiosis can lead to suboptimal intestinal function. Acute trauma and chronic impairment shape physiological adaptations in enteric and central neuronal pathways. How these adaptations are linked to the autonomic dysfunction seen in PD preceding or ensuing central DA loss still needs to be fully identified.

Parkinson's Disease

PD is the most common movement disorder with a global prevalence estimated to 1−4%, increasing with age and displaying geographic variability (Pringsheim et al., 2014). The incidence rate is estimated around 17 cases per 100,000 individuals per year. It is more common in the mid-to-late adulthood, with earlier onset in men than in women (Twelves et al., 2003). Idiopathic PD accounts for most of the cases, while only 5-10% are related to familiar PD.

PD is a multifactorial disease caused by an interplay between genetic susceptibility and environmental exposure, lacking diagnostic tools for an early detection. Risk factors for developing PD include relatives diagnosed with PD, family history of tremor, constipation, depression or bipolar disorders, exposure to herbicides or insecticides, head injury, living in rural areas, and agricultural occupation, among others (Noyce et al., 2012). Genetic mutations associated to an increased risk of developing PD have been described to interfere with multiple biological processes (Billingsley et al., 2018). However, the etiology of the disease is still controversial.

The neurodegenerative progression in PD affects the nigrostriatal pathway, where substantia nigra (SN) *pars compacta* neurons are projecting to the putamen, causing DA loss in in the SN and DA fiber loss in the putamen (German et al., 1989). The neurodegeneration is partially attributed to abnormal aggregates of α-synuclein (α-syn) in the cytosol of the dopaminergic neurons (Spillantini et al., 1997). These intracellular inclusions, known as Lewy bodies and Lewy neurites, are considered the hallmark pathology of PD (Goedert et al., 2013).

The dopaminergic nigrostriatal pathway, as part of the basal ganglia, is a neuronal pathway that facilitates voluntary movement (Hauber, 1998, Lanciego et al., 2012). DA loss in this pathway leads to the characteristic motor symptoms, bradykinesia also known as slowness of movement, tremors at rest, and rigidity (Postuma et al., 2016). Post-mortem studies estimated that more than 56% of the dopaminergic neurons are lost in the SN

(Fearnley and Lees, 1991) and DA levels almost inexistent (less than 1%) in the putamen (Kish et al., 1988).

Non-motor symptoms

While PD has been traditionally considered a motor disorder, non-motor symptoms (NMS) are also recognized to progress along with the motor impairment (Postuma and Berg, 2017, Hawkes et al., 2010) such as autonomic dysfunction, olfactory loss, and cognitive impairment (Schapira et al., 2017, Katunina and Titova, 2017). While, NMS showed great variation between individuals and symptom severity, they significantly deteriorate the health-related quality of life of PD patients (Gallagher et al., 2010, Duncan et al., 2014). NMS are often unreported or undiagnosed and, therefore, left untreated (Chaudhuri et al., 2010).

The dysfunction of the ANS is a common NMS. It occurs due to generalized or local impairment of para- and sympathetic neurons innervating peripheral organs, which can be seen as urinary bladder dysfunction, bowel dysmotility and orthostatic hypotension (Gallagher et al., 2010, Chen et al., 2020). However, the mechanism of PD-related autonomic dysfunction has not yet been fully described. The nigrostriatal denervation alone is unlikely to explain its development; although, it might play an important physiological role in its regulation (Sakakibara et al., 2011, Coon et al., 2018). As in PD, α-syn aggregates are found in peripheral ANS neurons, where autonomic denervation has also occurred (Orimo et al., 2008, Phillips et al., 2008). In brainstem autonomic regulatory areas, these aggregates have shown to precede the significant DA loss seen in the nigrostriatal pathway (Kingsbury et al., 2010).

Initially, Braak and colleagues suggested that PD pathology first occurs peripherally via the glossopharyngeal, olfactory, and vagal nerves, and then progressively migrates into the CNS where it spreads (Braak et al., 2003). Recently, two PD phenotypes has been described depending on whether the pathological changes occur first, peripherally or centrally. In the *PNS-first* phenotype, the autonomic dysfunction precedes the central degeneration, while in the *CNS-first* phenotype the central and SN degeneration develop before the autonomic impairment (Borghammer and Van Den Berge, 2019).

Intestinal dysfunction in PD

One common PD-related autonomic symptom is GI dysmotility. Peristalsis alterations have been described such as dysphagia, delayed gastric emptying and slow colonic transit (Cersosimo and Benarroch, 2012), partially attributed to cholinergic denervation in the DMV (Braak et al., 2007, Benarroch et al., 2006). Peripheral parasympathetic denervation has been traced by positron emission tomography occurring among all PD stages confirming that autonomic dysfunction occurs in PD (Gjerloff et al., 2015).

Intestinal dysmotility is particularly bothersome. More than half of PD patients reported decreased stool frequency (less than three times per week) or difficulty to evacuate stools (Edwards et al., 1991, Cersosimo et al., 2013). Constipation can result from one or both of these conditions (Krogh et al., 2008). Fecal incontinence and diarrhea may result from inadequate anorectal stool storage or overflow secondary to constipation (Stocchi et al., 2000). Slow colonic motility is the general cause of PD-related constipation (Sakakibara et al., 2003, Knudsen et al., 2017). Such delay might occur due to peripheral degeneration of ANS nuclei and impaired central autonomic regulation (den and Bethlem, 1960, Braak et al., 2007). It has been suggested that the basal ganglia have a role in modulating the intestinal motility (Sakakibara et al., 2011, Cersosimo and Benarroch, 2008).

All this evidence strongly suggests an important connection between the ANS and the CNS where the basal ganglia and, in particular, the nigrostriatal pathway play a regulatory role on the intestinal SM contractility.

Available treatment for GI dysfunction in PD

The enteric regulation of the GI functions is complex and it is influenced by ANS/CNS feedback. This regulation depends on the interaction between peptides, hormones, and neurotransmitters. Therefore, treating GI dysfunction requires multiple approaches and currently there are limited treatment options available [for review see (Poirier et al., 2016). Available management only focuses on symptom relief to improve the quality of life of PD patients (Pedrosa Carrasco et al., 2018).

Non-pharmacological management includes lifestyle changes aiming to minimize upper and lower GI symptoms. Dietary recommendations include to consume meals low in fat content, increase fiber (20-30g/day) and water intake, and follow a fractionated diet. In addition, oral administration of

prescribed drugs before food intake is advised to optimize drug absorption, which also improves by doing moderate physical activity.

Pharmacological treatment of GI symptoms in PD is limited. Therefore, symptomatic treatment should consider comorbidities and the risk of drug interactions. Recently, little evidence about *Helicobacter pylori* screening in PD patients suggests that it may interfere with L-DOPA absorption. When treated, it may improve L-DOPA-induced motor fluctuations (Rees et al., 2011). In addition, treatment should focus on alleviating NMS. To facilitate defecation in chronically constipated patients, bulk-forming laxatives like methylcellulose and osmotic laxatives like lactulose are drugs of choice as they do not directly promote peristalsis. To relief pain, opioids should be avoided as they worsen gastroparesis and constipation. Aside from laxatives and pain management, only drugs acting on dopaminergic and serotonergic receptors have been available.

Previous efforts to develop prokinetic drugs were made with unsatisfactory results. Domperidone, D_2 antagonist, alleviates gastroparesis, increasing the risk of cardiac arrythmias. It is prescribed in specific cases after an individual benefit-risk assessment. Metoclopramide, D_2/5-HT_3 antagonist and 5-HT_4 agonist, used for dyspepsia and nausea, is no longer recommended as it worsens PD motor symptoms. Cisapride, 5-HT_4 agonist, improves motor fluctuations and L-3,4-dihydroxyphenylalanine (L-DOPA) absorption, however it increased the risk of having ventricular arrhythmias therefore it was removed from the market in 2000. Tegaserod, 5-HT_4 agonist, improved constipation, but then again it was removed from the market in 2007 due to increased risk of ischemic events. The only prokinetic drug currently deemed safe is prucalopride, 5-HT_4 agonist, which stimulates colonic transit by increasing the amplitude and frequency of slow waves in the colon, without effects on the cardiovascular system (Omer and Quigley, 2017).

The challenge to treat GI dysfunction in PD is due to limited knowledge about impaired regulatory mechanisms in the ENS and lack of treatment options. Clinical trials evaluating the efficacy and safety of prokinetic drugs in PD is also limited. Understanding exactly which neuronal populations are affected due to PD pathology and how they interact in health and disease is a priority to develop novel therapeutic targets.

Animal models in PD research

Animal models are critical in PD research as they significantly contribute to the understanding of the pathophysiology behind the multisystemic effects of the disease. In addition, gathered knowledge from reliable and validated animal models may help to develop adequate therapies in humans. Hence, it is essential to distinguish the main characteristics of current existing models available.

In the literature, non-mammalian and mammalian models are described. There are basically genetically-modified, pharmacological- or neurotoxin-induced models. In non-mammalian animals, genetically-modified models of PD focus on the identification of genes responsible for dopaminergic signaling that can be manipulated to identify new therapeutic targets or to study neuroprotective compounds. Neurotoxin-induced models were developed to assess locomotor impairment after dopaminergic neurodegeneration. They include *Drosophila melanogaster*, *Caenorhabditis elegans,* and *Danio rerio* [for review see (Lim, 2010, Vaz et al., 2018)]. The advantages of using these models comprise short life-span, easy to breed and easy to interpret. However, the main disadvantage is that they lack the pathological complexity of the disease occurring in humans.

Mammalian models of PD basically differ on how peripheral or central impairment is induced. Pathological changes can be induced by the administration of drugs, neurotoxins, or pesticides, or can be developed by genetic modifications linked to known human PD-associated genes or α-syn expression [for an up-to-date review see (Lama et al., 2021)]. These models have been developed in different strains of mice and rats, cats, dogs, pigs and non-human primates.

Pharmacologically-induced models study the effect that drug therapies have on the control of specific symptoms. For this, reserpine was commonly used. Reserpine inhibits the reuptake of monoamines by presynaptic neurons leading to a temporal and systemic sympatholytic effect recreating PD-related motor symptoms. Once induced, specific DA-replacement treatment has been tested to evaluate recovery of motor deficits (Colpaert, 1987).

Genetically-modified or transgenic models are created to mimic familiar forms of PD after recognition of specific genes linked to the disease. For instance, α-syn, leucine-rich repeat kinase and glucocerebrosidase. They provide valuable information about disease progression, making possible the study of disease-modifying therapies [for review see (Dawson et al., 2010)].

Neurotoxin-induced models are often used because they mimic pathological features of the disease. The most common neurotoxins that induce selective loss of DA neurons in the nigrostriatal pathway are 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The former is described in the next section. MPTP hinders the mitochondrial respiration, and halters Ca^{2+} flow leading to oxidative stress and neuronal death, selectively targeting dopaminergic neurons (Nicklas et al., 1985). Non-human primates are commonly used in this model, where cognitive impairment has been studied to test pharmacological treatments, including L-DOPA (Decamp and Schneider, 2009).

Animal models in PD employing environmental neurotoxins have also been established. Rotenone, a pesticide and piscicide, alters also mitochondrial respiration. This model has shown to produce nigrostriatal degeneration and α-syn inclusions after chronic exposure (Cannon et al., 2009). Rotenone toxic effects on dopaminergic neurons are highly variable with fluctuating behavioral and histological effects (Fleming et al., 2004), therefore limiting its use.

The 6-OHDA rat model of PD

In the absence of spontaneous occurrence of PD in animals, the 6-OHDA rat model of PD, is reliable, reproducible, and well-standardized. This model has contributed to understand the pathological mechanisms derived from the central DA loss as well as to investigate how the specific denervation of the nigrostriatal pathway leads to motor, neurological, and physiological deficits. It is a model that resembles PD in humans except from the presence of α-syn aggregates and the characteristic neurodegenerative age-related progression of the disease.

Animals bearing nigrostriatal lesions induced by 6-OHDA display fine motor and postural deficits as well as impaired sensorimotor coordination [for a detailed review see (Simola et al., 2007)]. Mostly, motor slowness (as parkinsonian-like akinesia), decreased forepaw usage (reduced fine motor control of arms and hands), lack of vibrissae sensing to reposition their body (sensory-motor deficits) and quivering jaw movements (as parkinsonian tremor), are part of the altered motor features seen.

These animals also exhibit intestinal dysmotility. The nigrostriatal denervation may slow down intestinal peristalsis through dopaminergic D_2 receptor downregulation (Blandini et al., 2009, Colucci et al., 2012) due to enhanced enteric DA levels (Zhang et al., 2015). Others stated that enteric

cholinergic neurotransmission is impaired, upregulating muscarinic M_2 and M_3 receptor expressions (Fornai et al., 2016). Likewise, slow colonic transit occurs due to reduced ACh release from myenteric nerves affecting cholinergic-evoked contractility. This effect on the cholinergic signaling could be reverted by oral administration of L-DOPA in combination with benserazide (Pellegrini et al., 2017). Mucosal permeability, activation of local adaptive immunity, and proinflammatory responses also accompany the dysfunctional intestinal neurotransmission (Feng et al., 2019).

Among neurotoxic models of PD, the 6-OHDA rat model of PD is still one of the most widely used models to study PD-related pathophysiology. By using this model, it is not only possible to study motor impairment and behavioral changes, but also non-motor features resembling PD in humans. This model is, therefore, suitable for the study of autonomic dysfunction. A comparison between human PD and the 6-OHDA rat model of PD is shown in figure 1.

Characteristics	Human PD	6-OHDA rat model of PD
Nigrostriatal degeneration	✓	✓
Peripheral neuronal dysfunction	✓	✓
α-syn cytosolic inclusions	✓	✗
PD progression	✓	✗
Sensorimotor alterations	✓	✓
Cognitive/behavioural changes	✓	✓
NMS impairment	✓	✓

Figure 1. *A comparison between pathophysiological characteristics of human PD and neurotoxic-induced deficits in the 6-OHDA rat model of PD*

Aims

This thesis attempted to provide new insights into how the 6-OHDA rat model of PD can be used to study autonomic dysfunction, by investigating the SM activity. The thesis consisted of two intertwined aims.

The first aim was to refine the animal model, making it more physiologically relevant. Briefly, it assessed the contractile response to desipramine injection (**study I**) and to different tissue preparations (**study II**).

The second aim was to examine the altered autonomic regulation of the SM contractility of the intestines in response to nigrostriatal DA denervation in the 6-OHDA rat model of PD. More specifically, to investigate the intestinal contractile response to neuronal and cholinergic stimulation (**study II**) and to ENS neuromodulators (**study III**).

Methodology

Experimental design

The thesis combined preclinical methods from neuroscience and pharmacology, allowed by ethical permits no. 145-15 and 1911/21 approved by the ethical committee at the University of Gothenburg.

To achieve the aims previously described, two phases were designed. Phase I included revision of experimental parameters possibly affecting the peripheral study of autonomic dysfunction in the 6-OHDA rat model of PD and phase II involved the *in vitro* experimentation for the study of intestinal SM function.

In phase I, unforeseen actions of desipramine hydrochloride, a neuroprotective substance against 6-OHDA-induced noradrenergic neurotoxicity, on SM were examined in study I. In addition, an assessment of different intestinal tissue preparations was performed in study II. In phase II, the intestinal SM contractility was assessed *in vitro* by neuronal and direct receptor stimulation, attained in studies II and III.

Detailed description of outcome variables and experimental groups for each study is as following:

Study I explored the effects of the drug administration (saline as placebo, desipramine) and the injection site (subcutaneous SC, intraperitoneal IP). Healthy animals were included in this study.

Study II measured the intestinal contractile responses evoked by electrical field stimulation (EFS) and a cholinergic muscarinic agonist, methacholine (MeCh), in the presence and absence of a non-selective cholinergic antagonist, atropine, and a nitric oxide synthase (NOS) inhibitor, N^{γ}-Nitro-L-arginine methyl ester (L-NAME). Responses were examined in different tissue preparations (circular/longitudinal strips, intestinal segments), where 6-OHDA-lesioned animals were compared to controls (saline injection in sham-operated, no intervention in healthy animals.

Study III quantified the intestinal contractile and relaxatory responses to monoamines, DA, 5-HT and NA, before and after cholinergic muscarinic stimulation and in the presence of non-selective cholinergic antagonist.

This study was carried out in intestinal segments from 6-OHDA-lesioned and sham-operated animals.

Male Sprague Dawley rats were used in all studies. The total time of intervention in all studies lasted maximum 30 days. Study I gathered additional physiological parameters by using metabolic cages, arranged one day prior to euthanasia of the animal, after which tissue samples were harvested to perform isolated tissue baths experiments. Studies II and III did not include metabolic cages. For these studies, tissue samples (intestinal segments, brain) were harvested on the day of euthanasia to perform isolated tissue baths the same day and subsequent cerebral immunohistochemical analysis.

The 6-OHDA rat model

Early development of the model showed that peripheral injection of 6-OHDA in rats caused chemical sympathectomy, resulting in a multisystemic autonomic dysfunction (Finch et al., 1973), without modifying the catecholaminergic neurons in the brain. Therefore, the neurotoxin needed to be delivered intracerebrally by means of stereotactic surgery, where it chemically induces a selective dopaminergic neurodegeneration on the lesion site in a dose-dependent fashion. Studies showed that this neurotoxin was unable to cross the blood-brain barrier when injected peripherally. In addition, as 6-OHDA was also internalized by noradrenergic nerve terminals, neuroprotection of sympathetic neurons was required when injecting 6-OHDA into the brain (Evetts and Iversen, 1970).

6-OHDA

6-OHDA is a structural analogue of DA and an isomer of noradrenaline (NA), resembling a catecholamine. It acts as a neurotoxin producing selective degeneration of catecholamine neurons (Ungerstedt, 1968). When dopaminergic and noradrenergic neurons are exposed to 6-OHDA the active catecholamine transporters, dopamine transporter (DAT) and NA transporter (NET) respectively, located on the cellular membrane, pumps this neurotoxin from the extracellular space into the cytosol. As it is internalized by the neurons, its cytoplasmatic concentration increases and accumulates multiple impairing cellular processes that lead to oxidative stress by production of hydrogen peroxide (H_2O_2) and other reactive oxygen species

(Blum et al., 2001). In addition, cytoplasmatic 6-OHDA inhibits the oxidative phosphorylation required for energy production by the mitochondria leading to neuronal death (Kupsch et al., 2014, Glinka and Youdim, 1995).

Preparation of the compound. 6-OHDA needs to be prepared immediately before the injection as it is chemically unstable and prompt to easily oxidize. The compound is dissolved in sterile saline solution (0.9%; B. Braun, Melsungen AG, Germany) containing 0.2% ascorbic acid, which stabilizes the solution's pH preventing the oxidation of the neurotoxin (Powell and Heacock, 1973). The ascorbic acid solution was prepared and stored in small aliquots at −20°C until required. In addition, the 6-OHDA solution is temperature and light sensitive, once prepared it should be kept in the fridge or on ice and retrieved just prior to injection. The solution must be used within 2−3 hours after preparation. If oxidation of the neurotoxin occurs, a brown/red color can be observed and the solution should be discarded.

Desipramine

Desipramine is a tricyclic antidepressant inhibiting the reuptake of NA and 5-HT by presynaptic neurons, exhibiting high affinity for NET (Deupree et al., 2007). It has antagonistic action on histaminergic H_1-receptors, mAChRs, α-adrenoceptors and 5-HT transporters.

Preparation of the compound. Desipramine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile saline solution. It is often required to heat the solution for 10−15 minutes at ~50°C to make it more soluble before injecting the animal (personal observation).

Intracerebral injection of 6-OHDA by stereotactic surgery

In rodents, the stereotactic surgery is a multipurpose approach that can be used to produce mechanical or chemical neurological lesions, to modify gene expression, or to implant cannulas aiming to investigate neurotransmitters' signaling pathways (Fornari et al., 2012). The intracerebral stereotactic surgery is a three-dimensional minimally invasive intervention performed to induce changes on specific areas of the brain by using a set of coordinates in relation to anatomical features on the skull (Ferry et al.,

2014). This technique assures that an accurate manipulation of the neuronal microenvironment can be achieved and its effects can be followed in time after the procedure is performed.

Surgical procedure

Under general anesthesia, the animal was mounted on a stereotactic frame that allows a fixed placement of the animal's skull by securing it with ear bars. The mouth was fixed with a tooth bar and nose clamp meanwhile ventilation and inhalation anesthetics (isoflurane in air; Forene Abbott, Wiesbaden, Germany) were maintained. Isoflurane 2.5−3.0% was used for induction and kept at 2.0−2.5% for maintenance. Before surgery, the animal's scalp was shaved and disinfected. At first, local anesthetics (Marcain 2.5mg/mL, AstraZeneca AB, Sweden) were intradermally injected in the forehead to block pain signaling. Thereafter, an approx. 1.5cm anterior-posterior incision with a scalpel was made to access the frontal muscle and the surface of the skull. Of importance, control of local bleeding and gentle separation of the muscle was required for an optimal visual inspection of the bones.

For guidance, a stereotactic atlas was used to determine the exact spatial location of the targeted area of the brain in a three-dimensional coordinate system consisting of three axes: anterior-posterior (AP), medial-lateral (ML), and dorsal-ventral (DV) (Paxinos and Watson, 2007). The most prominent feature of the skull should be visualized and used as reference: the bregma. The bregma is an intersectional point located in the middle line, created by the fusion of the frontal bones (known as coronal suture) and the parietal bones (known as sagittal suture).

Once the bregma was located, AP and ML coordinates were obtained by placing the tip of the drill over the bregma without exerting force. Next, the corresponding values were recorded by reading the respective micrometer Vernier scales. These values act as reference to calculate the precise coordinates to reach the targeted area. Once in place, the drill was used to carefully make a hole in the bone avoiding disruption of the meningeal tissue that envelops the brain. The drill was then removed from the microdrive screw and a NanoFil 10μl syringe coupling to a 33-gauge blunt needle (World Precision Instruments Inc, Florida, USA) was placed instead. The DV coordinate was obtained by placing the microneedle's tip in close contact with the first layer of the meninges, the dura mater, and calculation of the precise coordinates of the targeted area was made.

Thereafter, 6-OHDA or sterile saline solution was injected in the medial forebrain bundle (MFB) region at an injection speed of 1μl/min keeping the needle in place for 3 minutes before retracting it slowly. The surgical area was then cleaned and the incised wound was closed by pulling the edges together with suture clips. After surgery, the animals had time to recover in individual clean cages and pain management post-surgery was given subcutaneously (Romefen, 5mg/kg; VET, Merial, Lyon, France). Animals were then placed back into their home cages under standard laboratory conditions.

In particular, the stereotactic surgery is convenient to deliver the 6-OHDA into the nigrostriatal pathway since the neurotoxin is unable to cross the blood-brain barrier due to physical and enzymatic obstacles (Kostrzewa, 2007). The blood-brain barrier contains enzymes that metabolize peripheral catecholamines before they enter in contact with endothelial cells and pericytes, such as monoamine oxidase and catechol-*O*-methyltransferase. As it is injected, the neurotoxin is released in the vicinity of the site of injection and degeneration spreads along the neuronal axons once it is up taken by the neurons. The detailed coordinates to inject it into the MFB were in mm: AP -4.4, ML -1.2, and DV -7.8, with the tooth bar at -2.4. In this thesis, intracerebral administration of 6-OHDA (3.5mg/kg; Sigma-Aldrich, St Louis, MO, USA) was utilized to induce neurodegeneration. Sham-operated animals, acting as the control group, underwent the same surgical procedure receiving sterile saline as a substitute.

Figure 2 shows the nigrostriatal pathway in the rat brain, where dopaminergic cell bodies project from the SN into the striatum. A schematic representation of the MFB stereotactic injection is also shown. When the MFB is targeted, dopaminergic neuronal cell death occurs as the neurotoxin acts first on the axons and then on the cell bodies leading to an anterograde degeneration to the striatum and retrograde to the SN within days (Zuch et al., 2000).

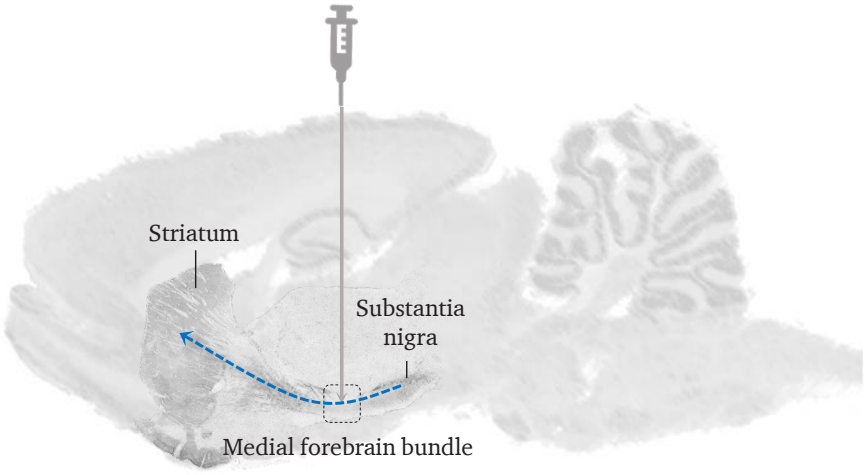


Figure 2. *Schematic representation of the stereotaxic lesion on the medial forebrain bundle in a sagittal section of the rat brain. Background image modified from (Paxinos and Watson, 2007).*

Immediate evaluation of the lesion can be performed by behavioral analysis or administrating dopamine agonists, e.g. apomorphine (0.25mg/kg) or more commonly amphetamine (5mg/kg), to verify the lesion-induced motor impairment (Yuan et al., 2005). Rotation of the animal to the contralateral side occurs spontaneously after surgery or can be evoked by apomorphine, meanwhile the animal rotates to the ipsilateral side after 30 minutes of a given dose of amphetamine, both suggesting that the lesion has been performed properly (Perese et al., 1989).

Experimental considerations. It is relevant to minimize the operational error when performing stereotactic surgery in rats. The possibility of animal variations due to sex, strain, and body weight was suggested previously (Paxinos et al., 1985). They found that variations in sex and strain do not affect the stereotactic accuracy; however, the animal's body weight can create greater variation. They suggested that the correct localization of the bregma must be guaranteed to avoid inaccurate intracerebral lesions in animals weighing more than 290g.

Moreover, the intended degree of lesion of the nigrostriatal pathway can vary depending on the structure targeted. For instance, the MFB lesion is the most commonly used; however, it produces a more extensive damage of the neurons in the SN and the ventral tegmental area in comparison to intrastriatal and SN lesions, which are more selective (Deumens et al.,

2002). Likewise, targeting the MFB will lead to partial loss of dopaminergic neurons projecting to the nucleus accumbens affecting the mesolimbic pathway (Perese et al., 1989). Bilateral lesions of the MFB region are not recommended due to an increased risk of disturbance in the feeding behavior causing high mortality among these animals (Ungerstedt, 1971).

Animal perfusion, brain fixation and sectioning

Decay of harvested tissue rapidly occurs by enzymatic proteolysis and by contamination with microorganisms. Hence, the purpose of tissue fixation is to preserve the cellular structures, while preventing its decomposition over time. Tissues are preserved for further histological analysis by immunohistochemistry (IHC) or microscopy.

One of the most extensively used methods to perfuse and fix animal tissues is chemical fixation by aldehyde compounds. To achieve chemical fixation, tissues can be exposed to the fixative by immersion or by perfusion. Fixation by immersion broadly consist on submersion of the tissue in fixative, which over time will diffuse through the tissue. This method can be used in small tissues or cellular samples. In contrast, fixation by perfusion involves the circulatory system to deliver the fixative to the targeted tissue (Gage et al., 2012) making transcardial perfusion the gold standard procedure for brain fixation.

Transcardial perfusion fixation procedure

Prior to the procedure, a flexible tube coupled to a peristaltic pump (MINIPULS 3, Gilson, Saint-Avé, France) was filled up with saline solution at room temperature, avoiding air bubbles within the system.

The animal was injected intraperitoneally with an overdose of pentobarbital sodium (100−150mg/kg; APL, Stockholm, Sweden) in the caudal left quadrant. In this way, there is a minimized risk of puncture of abdominal organs (Laferriere and Pang, 2020). Once anesthetic depth was reached, acknowledged by the absence of reflexes, the animal is placed in supine position on a polystyrene plate previously tilted 15 degrees to the left. The limbs were secured to the surface with 23-gauge needles (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Thereafter, a right thoracotomy was performed by doing an anterolateral incision to grant access to the pleural space. The left rib cage was lifted and secured with a 15-gauge

needle (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to the polystyrene plate next to the left clavicle. Connective tissue to the heart was carefully removed before lifting the rib cage.

Thereafter, visual inspection of the descending aorta was done and upon identification the vessel was clamped using a mosquito forceps to interrupt blood flow to the lower part of the body. A small incision was then made in the bottom of the left ventricle to insert the blunt perfusion needle into the heart. The needle was secured in placed by a mosquito forceps. At this point, the peristaltic pump was turned on to infuse saline solution to replace the animal's blood at an infusion rate of 20−25mL/min. Immediately after an incision was made on the right atrium to facilitate drainage. Once the drained liquid appeared clear, the pump was stopped and the saline solution was switched by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (Sigma-Aldrich, St. Louis, MO, USA) at 4˚C. This was followed by continuous infusion of 200−250mL of 4% PFA over 10 minutes. At the end of the procedure, the upper body of the animal was stiff.

Experimental considerations. The optimal degree of fixation depends on the perfusion time and the pH of the fixative solution. A slow fixation rate assures a homogenous distribution of the fixative throughout the brain tissue, while a physiological pH (7.2−7.6) minimizes the risk of cross-linking, loss of antigen immunoreactivity and is required to get an optimal perfusion (Berod et al., 1981).

Brain dissection and sectioning

A midline incision on the head using curve dissecting scissors was made from the dorsal neck to the forehead to access to the skull. For better visualization it is required to turn aside the muscle *frontalis* and the cranial portion of the muscle *levator auris longus* from the midline (Greene, 1935). As the base of the skull became visible, surgical sharp scissors were inserted into the foramen magnum to carefully fracture the occipital bone and the sagittal suture to divide the parietal bones, without damaging the brain. Similar cuts were performed on the temporal bone of each side. It was required to cut the meningeal layers before removing the fragmented bones with Friedman rongeurs. This maneuver needed to be repeated until the brain was completely exposed. The olfactory bulbs were detached before the brain was removed. Finally, the brain was placed in a plastic bottle containing 4% PFA and left overnight at 4˚C. The day after, the PFA was

replaced by 25% sucrose (Sigma-Aldrich, St. Louis, MO, USA) in 0.1M phosphate buffer.

Sectioning of the whole brain was performed by using a cryostat (Leica, CM1950; Leica Microsystems, Nussloch, Germany). Prior to sectioning, a coronal cut was made with an adult rat slicer through the hypothalamus to divide the brain in two halves. Each half of the brain was then placed on a disc and fixed using a frozen section compound (FSC 22 Blue; Leica Biosystems, Richmond, IL, USA) to quickly freeze it on the Peltier element for 15-20 minutes. After, each frozen half was placed in the object head with temperature set at -15°C. The temperature of the cryostat chamber was set to -20°C. A disposable microtome blade was used to slice the brain, collecting free-floating sections (35μm thick) by 1 in 5 series with a round brush. The sections were then placed and stored in cryoprotectant solution (25% ethylene glycol and 25% glycerol in 0.1M phosphate buffer) for further immunohistochemical analysis. Unstained series were kept at -20°C.

Experimental considerations. To optimize the quality of the histological sections, care should be taken to section the brain when it is completely frozen. Otherwise, the sections can either be broken, be incomplete or have marks from the microtome blade. In addition, if the brain is frozen below -20°C the sections can coil up making it difficult to stain them (personal observation).

Immunohistochemistry

Antigens are proteins within the tissue, able to be recognized by immune proteins (known as antibodies). Once they are attached to each other, they form an antigen-antibody complex. The antigen-antibody complex formation is the basis of IHC. Their detection and staining is a compelling method in the laboratory to identify and characterize cellular or tissular components (Kabiraj et al., 2015). It is a very sensitive and commonly used technique where the antigen-antibody complexes are labelled by fluorophores or produced by enzymatic reactions to visualize them.

Tyrosine hydroxylase as targeted antigen

Tyrosine hydroxylase (TH) is a cytosolic enzyme that converts L-DOPA to DA, in the presence of tetrahydrobiopterin and oxygen; L-DOPA is the

precursor of DA (Nagatsu et al., 1964). TH is present in all catecholaminergic neurons as DA is required for the sequential generation of NA and adrenaline. Therefore, this enzyme is the rate-limiting step of the catecholamine biosynthesis due to multiple regulatory mechanisms [described in detail in (Daubner et al., 2011)]. These mechanisms include enzyme inhibition by catecholamines, activation by phosphorylation of other cytosolic enzymes, and modulation by interactions with enzymes from the same pathway or from the tetrahydrobiopterin pathway.

Immunostaining procedure

Free-floating brain sections, 35μm thick in phosphate-buffered saline (PBS; Gibco, Thermo Fisher Scientific, MA, USA), were used for the detection of TH in dopaminergic neurons located in the SN and dopaminergic fibers in the striatum.

At first, quenching of endogenous peroxidases was performed by submerging the sections in 3% H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) in 10% methanol for 10 minutes. This was followed by a 1-hour preincubation with 5% normal horse serum (NHS; Vector Laboratories, Burlingame, CA, USA) and 0.25% triton-x (Sigma-Aldrich, St. Louis, MO, USA) in PBS, to block non-specific binding sites for the secondary antibody on the tissue. Thereafter, brain sections were incubated overnight at room temperature ($\sim$21°C) with unconjugated mouse anti-TH (1:1000-1:2000, multiple providers) mixed with 5% NHS and 0.25% triton-x in PBS to help the binding of this primary antibody to the TH in the neurons' cytosol.

After approx. 15$-$18 hours of incubation, brain sections were washed with PBS and incubated at room temperature for 1 hour with biotinylated horse anti-mouse antibody (1:250; BA2001, Vector Laboratories), mixed with 5% NHS and 0.25% triton-x in PBS to detect and bind to primary antibodies attached to TH. Thirty minutes before the incubation period finished, horseradish peroxidase avidin was mixed with biotin (ABC Elite Kit, Vector Laboratories) to facilitate the quick formation of avidin-biotin complexes, making the solution optimal and in equilibrium prior to usage. In this way, the already made avidin-biotin complexes (known as ABC solution) were further attached to biotinized secondary antibodies, which later enhanced the detection signal. Brain sections were then incubated at room temperature for 1 hour with the ABC solution immediately after they were washed with PBS.

Finally, 3,3'-diaminobenzidine (DAB; Vector Laboratories) was used as the chromogen substrate for the horseradish peroxidase in the presence of H_2O_2 to reveal the immunostaining. The enzyme catalyzes a redox reaction that appears brown to the human eye. TH-stained sections were mounted on adhesive microscope slides (HistoBond®+, Paul Marienfeld GmbH & Co.KG, Lauda-Königshofen, Germany) in rostral-caudal direction and left to dry at room temperature for a few days. Mounted sections were then dehydrated by using increasing concentrations of ethanol to xylene (Solveco, Rosenberg, Sweden), submerging the slides for two minutes in each step. Thereafter, dehydrated sections were covered with DePeX (Merck KGaA, Darmstadt, Germany) and a 24x60mm coverslip was mounted on the glass slide. Afterwards, they were left to dry in a fume hood for at least one week.

TH immunoreactivity is a widely-used, validated, and quantifiable measurement of dopaminergic neurons (Pickel et al., 1975). Its detection by IHC aids to reveal the degree of lesion of the nigrostriatal pathway. When 6-OHDA is injected unilaterally in one hemisphere, more than 95% of the dopaminergic neurons degenerate in comparison to the contralateral hemisphere, depending on the lesion site and dose. In contrast, when injecting sterile saline solution there is a complete preservation of dopaminergic neurons in both hemispheres, as it is shown in figure 3.

Experimental considerations. In some cases, the PFA fixation can mask the TH localized in the tissue, noticeable when the DAB staining is deficient, absent or not uniform. As described by Torres *et al*., problems with immunoreactivity are likely produced by PFA cross-linking to proteins or lipids present in the cell membranes creating a physical barrier that hampers the penetration of immunohistochemical solutions (Torres et al., 2006). This artificially produced physical barrier can be diminished by adding detergents, such as triton-x, to increase the permeability of the cell membranes during the IHC process.

To optimize the access to TH, two methods can be used: digestion with proteolytic enzymes and exposure to heat (Kabiraj et al., 2015). When using enzymatic digestion, the staining is less efficient, often increasing the background or creating false positive/negative staining. In contrast, heating methods improve the staining significantly as they may revert the fixation barrier. In this thesis, when TH staining was not optimal, heat-induced antigen retrieval was performed by heating the brain sections in a microwave for 5 seconds in the presence of citrate buffer 0.1M (sodium citrate and citric acid pH 5.0). Thereafter, TH re-staining significantly improved.

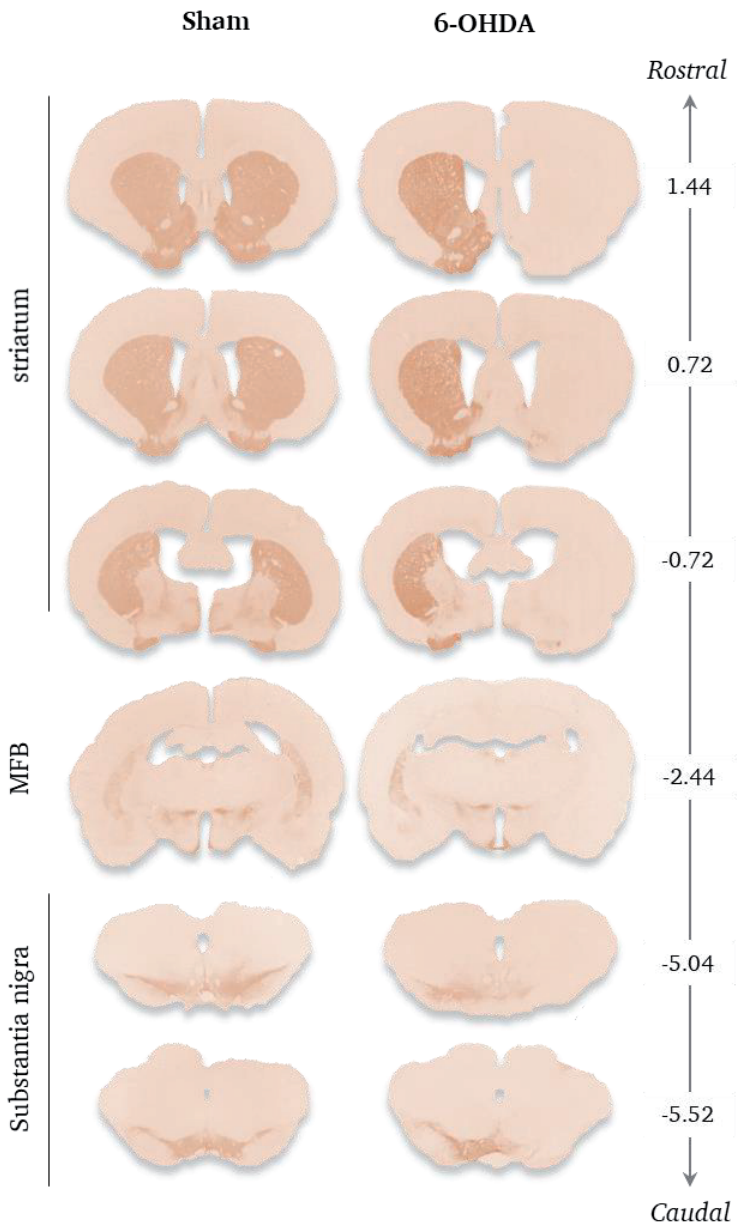


Figure 3. *Rostral-caudal sections of the intracerebral unilateral lesion in the right nigrostriatal pathway in sham-operated (left) and 6-OHDA-lesioned animals (right) according to bregma. Immunohistochemistry by Maria del Pilar Murillo Angarita and Timóteo Ladeira. Photos by Ka Chun Tsang and Lindsay Zentveld.*

Worth noticing is the risk of breaking the tissue if the heat is maintained for more than 8 seconds (personal observation).

Immunohistochemical image analysis by optical densitometry

The evaluation of the nigrostriatal denervation by optical densitometry is required to validate functional data (Xavier et al., 2005). In general, this technique semi-quantifies the intensity of the color in TH-stained areas in comparison to area(s) of the brain without staining.

Densitometric procedure

Pictures of TH-stained coronal sections of the striatum were taken by Dino-Lite digital microscope system (Dino-Lite Edge AM4515T8, AnMo Electronics Cooperation, Taipei, Taiwan) and digitalized by DinoCapture 2.0. Image analysis was performed by using ImageJ 1.53e with 64-bit Java v. 1.8.0 (downloaded from https://imagej.nih.gov/ij/).

Six striatal equally-spaced sections were analyzed: +2.16 +1.56, +0.96, +0.36, −0.36 and −0.96mm according to bregma (Paxinos and Watson, 2007). At first, the brain image was transformed to grey scale (8-bit), followed by inverting white and black colors. Prior to analysis, set measurements of area, mean gray value, min and max gray values, and display level were selected. Next, striatal areas of each hemisphere were manually highlighted and measurements were obtained, where the left hemisphere was labelled as *intact* and the right hemisphere as *lesioned*. Similarly, an area within the corpus callosum of each hemisphere was analyzed for correction of staining background. Immediately after, the optical densities of intact and lesioned areas were calculated by subtracting background densities to their side-matching stained densities. The percentage of TH-positive fibers was calculated as lesioned density divided by intact density, for each section. Finally, a total mean optical density for the striatum was obtained separately for each sham-operated and 6-OHDA-lesioned animal.

Experimental considerations. In very rare cases where the staining is very poor, the histogram may be adjusted to increase contrast. In addition, areas damaged during sectioning or immunostaining procedures should be excluded when manually highlighting stained areas for measurement.

Isolated tissue baths

Isolated tissue bath is an *in vitro* technique used to characterize receptors, to make inferences about receptor signal transduction, to study drug-tissue interactions, and to evaluate the effect of neurotransmitter release on tissue responses (Kenakin, 1984). It is based on the measurement of isotonic and isometric contractile responses, under controlled physiological conditions of temperature, pH, oxygen supply, and nutrients (Jespersen et al., 2015).

This technique was used in this thesis to evaluate the effects that direct stimulation has on the SM response, by electrically evoking neuronal release of neurotransmitters or by using compounds imitating the ANS action.

Tissue preparation and mounting

Tissue samples harvested from the animal were transported in reusable plastic bottles containing room-temperate Krebs solution [in mM: sodium chloride NaCl 118, potassium chloride KCl 4.6, Potassium dihydrogen phosphate monobasic KH_2PO_4 1.15, magnesium sulphate $MgSO_4$ 1.15, sodium bicarbonate $NaHCO_3$ 25, calcium chloride $CaCl_2$, 1.25, and glucose $C_6H_{12}O_6$ 5.5]. Upon arrival to the laboratory, tissues were transferred to beakers with clear Krebs solution. When urine was found inside the urinary bladder, the liquid was cleared during the preparation. For intestinal preparations, intestinal content was flushed out with clear Krebs solution very gentle to avoid mechanical damage to the tissue. All tissues were prepared according to standardized procedures depending on the study. To prepare urinary bladder strips, an incision was made from the urethral sphincter to the apex dividing it in half. Then, two urinary bladder strips (approx. 2−6mm long) were dissected. To prepare circular SM strips, a segment of approx. 5mm long was cut, rotated 90 degrees and dissected through the mesenteric line until approx. 10mm wide. To prepare longitudinal SM strips, a segment of approx. 10mm long was dissected through the mesenteric line and divided in half approx. 5mm wide. For intestinal segments, a 10mm long piece was dissected and for inverted intestinal segments, a similar piece was cut exposing the luminal side by carefully introducing blunt forceps to flip the tissue from the inside out. Table 1 shows the characteristics of intestinal preparations based on permeability, muscle contractile direction, layering, and mucosa exposure.

Table 1. *Characteristics of the intestinal tissue depending on tissue preparation.*

	Intestinal preparations			
Characteristics	*circular strip*	*longitudinal strip*	*segment*	*inverted segment*
response by muscle	circular	longitudinal	combined	combined
layering	full-thickness	full-thickness	full-thickness	full-thickness
direct exposure to stimuli	all layers	all layers	serosa	mucosa
mucosa exposure	yes	yes	no	yes

As shown in figure 4, the isolated tissue baths were calibrated, prefilled with Krebs solution, constantly aired with a mixture of 95% oxygen and 5% carbon dioxide, and maintained at 37˚C by a water coat. Next, tissue preparations were individually mounted in each bath hanging at both ends by silk threads, one attached to a fixed hook and the other one attached to a force transducer (TSD125C or SS63L, BIOPAC Systems Inc., Goleta, CA, USA) and real-time recordings were gathered by a MP100WSW data acquisition system.

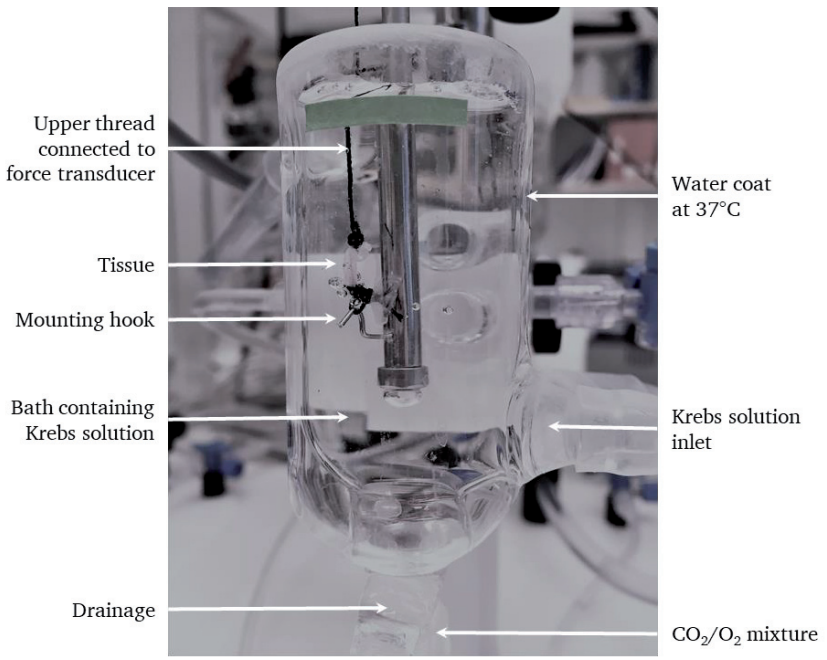


Figure 4. *Components of an isolated tissue bath setup used for in vitro experiments.*

In vitro protocol

The basal tension for the preparations varied according to the tissue mounted: for urinary bladder between 5−8mN and for intestine between 10−15mN. After the preparations were stretched to their basal tensions they were left to equilibrate for 45 minutes. Krebs solution was then replaced by isotonic high K^+ Krebs, osmolar substitution of NaCl for KCl 124mM, to yield a contractile response, testing the viability of the tissue preparations and stimulating them. For urinary bladder strips, it was done only one time, while for intestinal preparations, it was repeated three times. For urinary bladder strips, a tension threshold of 8mN after high K^+ was specified to exclude non-responsive preparations. For intestinal tissues, non-responsive preparations to either EFS, MeCh or high K^+ were excluded. At the end of the protocol, a new high K^+ Krebs test was performed to verify the survival of the tissue along the experiment.

Electrical field stimulation (EFS) was applied at the following increasing frequencies: 1, 2, 5, 10, 20 and 40Hz, at supramaximal voltage (50V) with pulse duration of 0.8 sec and width of 0.8 msec. Tissues were stimulated continuously until the maximal response was obtained for each frequency with a resting period in between stimulations (MultiStim system D330, Digitimer Ltd, Letchworth Garden City, Hertfordshire, UK or Grass S44 stimulator, Grass medical instruments, Quincy, Mass., USA). Direct receptor stimulation was performed by adding cumulative concentrations of each respective agonist to the isolated tissue bath to try to reach maximal response, and repeated in the presence of an antagonist depending on the study protocol. Table 2 shows the range of concentrations for each substance tested in isolated tissue bath experiments.

Table 2. *Concentrations for agonists and antagonists tested in isolated tissue bath experiments.*

Substance	**Range of concentration (M)**
Agonists	
MeCh	10^{-8} – 10^{-3}
DA	10^{-11} – 10^{-5}
NA	10^{-8} – 10^{-3}
5-HT	10^{-8} – 10^{-4}
Antagonists	
Atropine	10^{-6}
L-NAME	10^{-6}

Washout and resting periods of 10 minutes were included after the tissue preparations were challenged with high K^+ Krebs, EFS or agonists. An incubation period of 20 minutes was required for the antagonists to facilitate competitive binding or enzymatic inhibition.

Quantitative data was extracted from the recordings with Acknowledge Software v4.3 or BSL Student Lab v4.1 (BIOPAC systems Inc., Goleta, CA, USA).

Experimental considerations. Three experimental variables are important to optimize the data collection: flow of air, pH, and temperature.

Intestinal tissues are sensitive to mechanical stimulus. A constant airflow is recommended to avoid generating spontaneous peristaltic waves. Worth to notice, big bubbles tend to induce this phenomenon (personal observation).

A physiological pH of the Krebs and high K^+ Krebs solutions is required to obtain optimal recordings. Changes in pH can be seen when the solution turns whitish. If this occurs it is may be due to residual $CaCl_2$ left in the glassware.

If changes in temperature occur during the experiment or the temperature is below 37°C, it is often seen by intestinal slow waves becoming low in frequency and high in amplitude. In addition, the contractile response to stimuli is slow and suboptimal (personal observation).

Mechanism of action of agonists and antagonists

Acetyl-β-methylcholine (Methacholine)

MeCh is a non-selective cholinergic agonist acting on mAChRs. MeCh exerts an effect on all mAChRs subtypes (M1 to M5). Of clinical interest are inhibitory G_i-coupled M2 and excitatory G_q-coupled M3 receptors (Ehlert et al., 1997). It induces a physiological response comparable to the one exerted by ACh, although it has a prolonged action due to a slower degradation rate by acetylcholinesterases (Koppanyi et al., 1953).

Dopamine, noradrenaline and serotonin

DA, NA and 5-HT are known as monoamine neurotransmitters. DA is a non-selective agonist acting on dopaminergic receptors, exerting its action on excitatory G_s-coupled D_1-like receptors and inhibitory G_i-coupled D_2-like receptors (Sibley and Monsma, 1992). NA is a non-selective adrenergic agonist binding to α- and β- adrenoceptors. The activation of NA on G_i/G_0-coupled α_2-, G_q-coupled α_1- and G_s-coupled β-adrenoceptors produces an inhibitory response in the intestine (Jenkinson and Morton, 1967). 5-HT is a non-selective agonist acting on 7 families of serotonergic receptors (5-HT_1 to 5-HT_7). Only the 5-HT_3 receptor is a ligand-gated ion channel, while all the others are coupled to G-proteins [for review see (Saxena, 1995)].

Atropine

Atropine is a competitive and reversible non-selective muscarinic antagonist with high affinity for mAChRs. Atropine actively competes for the binding site blocking the action of cholinergic muscarinic agonists including ACh (Pauling and Petcher, 1970).

N^{γ}-Nitro-L-arginine methyl ester (L-NAME)

L-NAME is a N^G derivate of L-arginine acting as a non-selective NOS inhibitor. It hinders the enzymatic conversion of L-arginine to L-citrulline and NO by the NOS in the presence of NADPH and tetrahydrobiopterin. Upon non-enzymatic hydrolysis, L-NAME leads to the formation of N^{γ}-nitro-L-arginine, which then reversibly inactivates the enzyme (Pfeiffer et al., 1996).

Preparation of the compounds. All compounds were prepared as stock solutions in milli-Q water, kept frozen at –20˚C, and thawed at room temperature before use. Serial dilutions were aliquoted in 1.5−2.0mL vials and unused diluted solutions were frozen to preserve their stability amid trials.

Metabolic cages

Individual housing of a small rodent provides information about micturition and defecation patterns in real time meanwhile the animal has access to water intake and free movement. Independent collection of feces and

urine is possible by using this metabolic cage (Pfeiffer and Gass, 1963). Usually, it is for a fixed period of time according to the ethical permit.

Individual housing and data collection

In study I, the animal was placed on the support grid inside the cage chamber for a total time of 8 hours (21:00 to 05:00). The rat had access only to water. Feces and urine were collected separately, where urine drops were registered by a doppler sensor (SICK, Stockholm, Sweden) and recorded real-time by MP100WSW data acquisition system. Number and weight of fecal pellets, 8-hour urine volume, and total water intake was registered at the end of the enclosure.

Further analysis of the recordings provided information about the total number of micturitions, inter-micturition intervals and number of drops per micturition. Quantitative data was extracted with Acknowledge Software v4.3 (BIOPAC systems Inc., Goleta, USA).

Experimental considerations. Although the use of metabolic cages is a well-established method to evaluate physiological parameters in rodents, its utilization may reduce their welfare. Particularly, rats are more affected due to their need of social interactions, group thermoregulation and environment enrichment. It has been shown that placing the rat in a physically limited space without a solid floor, in the absence of bedding or cover, and without social contact negatively impacts the rat's response to stress (Sahin et al., 2019). This physiological response can be controlled by minimizing the isolation time of the animal. In this research project, the animal placement on a metabolic cage did not exceed 8 hours.

Statistical analysis

Hypotheses testing is necessary to make statistical inferences about the quantitative data collected in this thesis. In this section, the statistical methods are briefly described. All statistical analyses were performed and all figures were generated with GraphPad Prism version 9.3.1 for Mac OS X (GraphPad Software Inc, San Diego, CA, USA). A statistical significance of $p<0.05$ was set for all analyses.

Unpaired Student's t test was used to examine differences in rat weight, tissue weight and high K^+-evoked contractions between saline and

desipramine (IP or SC - **paper I**), healthy, sham-operated and 6-OHDA-lesioned animals (**paper II**), and sham-operated and 6-OHDA-lesioned animals (**paper III**). Student's t test was also used to examine differences in micturition parameters between saline and desipramine (IP only - **paper I**).

Two-way ANOVAs were used to estimate how the contractile responses varied according to a given stimulus, either to different frequencies or increasing concentrations of agonist, between saline and desipramine (IP or SC - **paper I**), between healthy, sham-operated and 6-OHDA-lesioned animals (**paper II**) or between sham-operated and 6-OHDA-lesioned animals (**paper III**). The Šidák test was used as *post hoc* ANOVA analysis for mean comparisons (**paper I**).

The non-parametric Mann-Whitney test was used to compare median rhythmic contractions for each tissue preparation between sham-operated and 6-OHDA-lesioned animals (**paper II**).

The Grubb's test was performed to detect if a given value was deviating significantly from the sample's distribution, with a significance level of 0.05 (**paper I**).

Ethical considerations

Studying SM physiology clinically requires an excisional full-thickness (transmural) biopsy of the organ. It is technically challenging to obtain due to high risk of complications such as hemorrhage, infection, perforation, peritonitis, need of multiple surgical interventions, and death (Shales et al., 2005, Collado et al., 2000). Additionally, only tissue samples that include the submucosal plexus are obtained in routine-endoscopic biopsies (Woitalla and Goetze, 2011). Therefore, a preclinical approach is more feasible and ethically accepted for understanding the SM pathophysiology in PD. It can help to generate potential novel pharmacological targets specific for PD treatment.

Choosing a preclinical approach should include revision of the 3R's principles (refinement, reduction and replacement) to ensure conducting appropriate animal research (Russell and Burch, 1959). Although many aspects of animal welfare are important, two ethical considerations are discussed in this thesis: the inclusion of females for sex-related comparisons and the use of sham-operated rats as controls, both aiming to refine the experimental design.

Inclusion of females in future experimental designs

There is a sex-related selection bias towards the use of male animals in preclinical studies, especially in pharmacology and neuroscience. Mostly, the general assumptions are that findings from males can be applicable to both sexes, that collected data is sensitive to physiological variations commonly linked only to estrous cycles, or that including females will increase the number of animals used in research (Beery and Zucker, 2011). In this thesis, only male Sprague-Dawley rats were used partially due to the reasons listed above. Although this thesis reflects the *status quo* of these research fields, recognizing that this sex bias exists is the first step in designing experiments that consider biological diversity as an advantage to make meaningful comparisons in animal research.

Sex differences not related to hormone actions do exist in the body and are not negligible. For instance, the embryonic development of monoaminergic neurons is not only influenced by the exposure to sex steroids but also to growth factors and adrenal hormones (Rohde et al., 1989). These additional points of exposure have the potential to alter the brain differentiation into adulthood. Likewise, the variation in number and size of dopaminergic neurons between the male and female rats appear to be independent of hormonal stimulation. Female mesencephalic dopaminergic neurons are higher in number and have an increased TH activity, while diencephalic neurons are smaller in size when compared to males (Reisert and Pilgrim, 1991). Therefore, experimental designs in the future could be refined if both sexes are equally represented. Studying sex differences in the 6-OHDA rat model of PD could yield new insights about how intestinal dysfunction, or autonomic dysfunction, occurs after central DA loss. If female animals are missing, such differences, which are likely to exist, will most certainly be overlooked.

Sham-operated animals' welfare

Control groups are often used in animal research to compare the effect of an intervention or treatment, aiming to minimize the impact of potential confounding variables (Johnson and Besselsen, 2002). In consequence, sham-operated animals are often used as controls of surgical interventions to adjust for variables derived from experimental procedures (e.g. anesthesia, surgery, drug administration). In fact, the experimental design should consider the purpose and type of control needed before the study begins. One important consideration is the animals' welfare, to avoid unnecessary

harm. Therefore, sham-operated animals require similar care as provided for treated animals. A possible alternative to replace sham controls is the use of healthy (nonmanipulated) animals, which not only balances benefits over risks of the intervention but also it reduces the costs in research.

In this thesis, sham-operated animals were subjected to stereotaxic surgery mimicking the same experimental conditions as the 6-OHDA-lesioned animals, except for the injection of 6-OHDA which was replaced by saline. Sham-operated animals served to adjust for the potential mechanical damage induced by the brain surgery and its related physiological adaptations post-surgery. In this way, a comparison between these two groups is made solely under the assumption that differences occurred due to the action of the neurotoxin, which has a clear predictable endpoint: central DA loss. However, can sham-operated animals be replaced by healthy controls? Findings from paper II showed differences in colonic contractile responses induced by high K^+ Krebs, mostly in intestinal segments, between healthy and sham-operated animals. Differences that could be explained by stereotaxic surgery and related procedures, bearing in mind that the animals could be exposed to stressful conditions despite measures to minimize them (e.g. pain management, habituation to human contact). Healthy animals were not exposed at all to the same stressful conditions. This could possibly indicate that the use of sham-operated animals as control group is a better alternative as their physiological responses to the stereotactic surgery may influence the outcome of the study. If possible in the future, it is relevant to evaluate physiological and behavioral parameters between these two control groups to provide a better understanding about their relevance in PD animal research.

Results

This thesis is divided into two separate experimental aspects to study autonomic dysfunction in the 6-OHDA rat model of PD. The first aspect included optimization of experimental conditions such as desipramine administration (**paper I**) and tissue preparation (**paper II**). Once these conditions were optimized, the second aspect involved the investigation of peripheral effects of central DA denervation. In particular, the study of these effects was focused on the SM response to different stimuli such as EFS and direct cholinergic activation (**paper II**), and monoamines acting as ENS neuromodulators (**paper III**). Papers II and III focused on the comparison between sham-operated and 6-OHDA-lesioned animals.

Optimization of experimental conditions

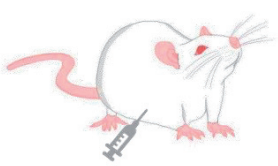
Desipramine modifies contractile responses

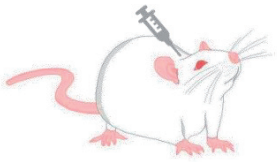
Paper I examined the effects of a single injection of desipramine on the urinary bladder and intestinal function. Figure 5 summarizes the main findings from this study.

Our results showed that desipramine produces significant local changes on the SM contractility when injected intraperitoneally. In urinary bladder strips, a significantly higher contraction was observed after electrical field- and cholinergic stimulation, while proximal colon segments showed a significantly lower response only after cholinergic stimulation.

SC administration of desipramine also showed changes in bladder and proximal colon contractility. In bladder, a significant reduction of the contractile response was detected after electrical field- and cholinergic stimulation, and in proximal colon segments a higher contraction was displayed.

No contractility changes occurred in distal ileum segments except for an increased contractile response after high K^+-induced depolarization in animals who received subcutaneous desipramine. The *in vitro* findings did not translate into alterations of micturition and defecation patterns.





INTRAPERITONEAL			Smooth muscle contraction evoked by	SUBCUTANEOUS		
Urinary bladder	Proximal colon	Distal ileum		Distal ileum	Proximal colon	Urinary bladder
↑	=	=	depolarization (high K+ Krebs)	↑	=	=
↑	=	=	neuronal release (EFS)	=	=	↓
↑	↓	=	direct mAChR activation (MeCh)	=	↑	↓

Figure 5. *Alterations in intestinal and urinary bladder smooth muscle contractile responses to different stimuli after intraperitoneal (left) and subcutaneous (right) injection of desipramine compared to animals injected with saline. Stimuli to evoke smooth muscle contraction included induced depolarization by high K^+ Krebs, neuronal release by EFS, and direct mAChR activation by MeCh. This summary only shows findings normalized by tissue weight from paper I.*

Contractile responses vary according to tissue preparation

Paper II compared the SM contractile responses in three different tissue preparations: circular SM strip, longitudinal SM strip, and intestinal segment from proximal and distal colon. The main findings regarding strips compared to intestinal segment are compiled in figure 6.

Our results showed that the contractile responses are influenced by the tissue preparation. Under the same physiological conditions, significantly higher contractile responses in intestinal segments from distal colon, as well as a tendency of increased responses in circular strips from proximal colon, were observed after EFS and MeCh. The frequency of slow waves in proximal colon was also higher, as shown in figure 6F in paper II.

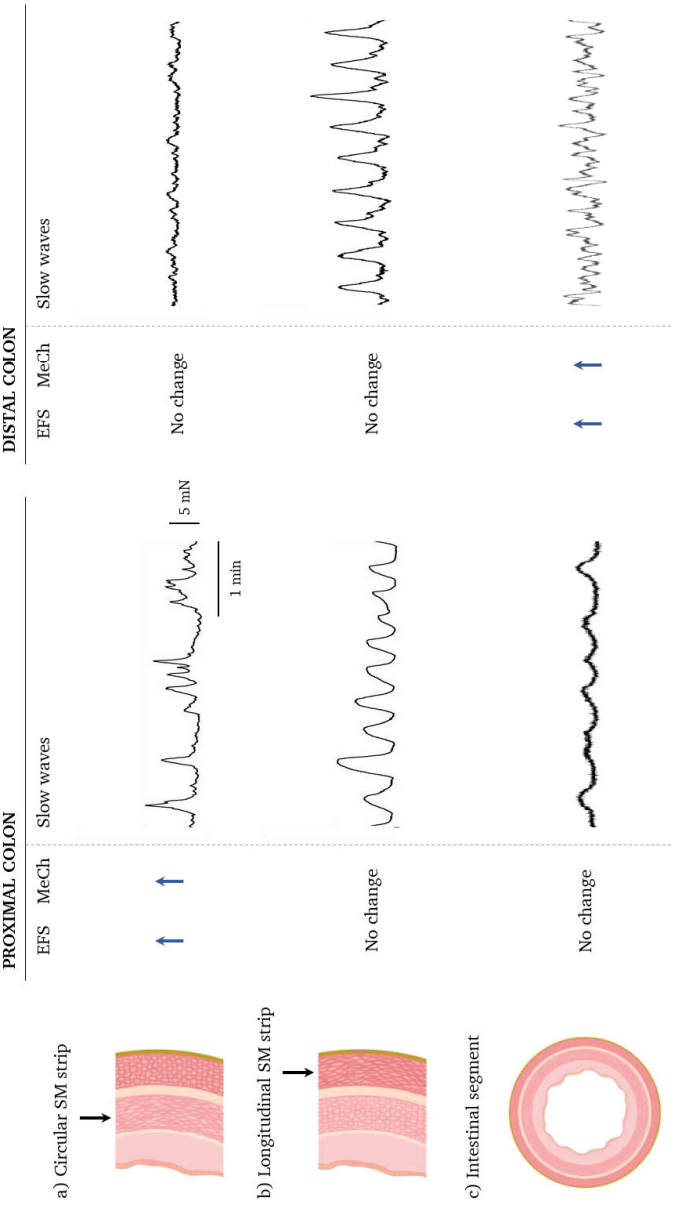


Figure 6. *Contractile responses to EFS and MeCh according to tissue preparation: a) Circular SM strip, b) longitudinal SM strip, and c) intestinal segment of 6-OHDA-lesioned animals compared to sham. The arrows show the SM type exerting the force on the strip preparation during the experiments. An example of slow waves in different tissue preparations, from the same animal, is presented. Findings from paper II.*

Distal ileum segments showed no differences in contractile responses, as shown in figure 5 in paper II.

Peripheral effects on SM after stimulation

The previous findings established the experimental conditions to carry out further experiments when investigating the intestinal response to major neurotransmitter systems. Modifications to the experimental design such as the omission of desipramine pre-treatment and the selection of intestinal segments were then implemented.

SM response to electrical and cholinergic activation

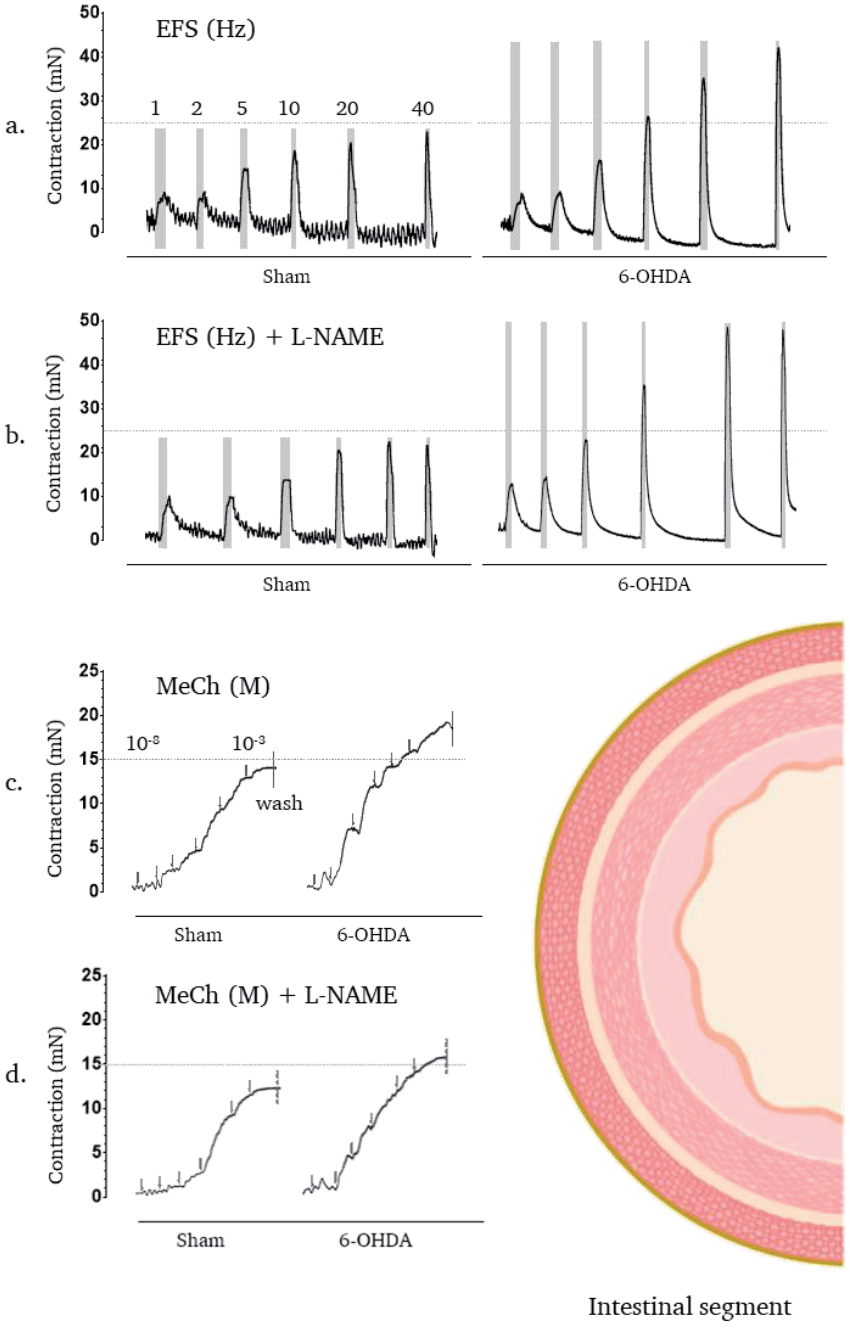
Paper II examined the intestinal contractile response to EFS and MeCh alone and in the presence of L-NAME in segments and inverted segments from distal colon. Figure 7 illustrates the main findings in this study from distal colon.

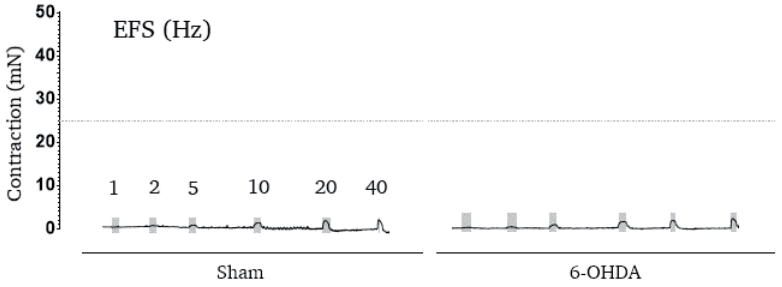
Distal colon results showed that L-NAME significantly reduced the contractile response to EFS in segments. A similar pattern was observed in inverted segments except for the sham-operated animals, where an increased response was observed instead. Contractile responses to a cumulative addition of MeCh showed a significant decrease in the presence of L-NAME only in 6-OHDA-lesioned animals. In inverted segments, an increased response was observed, only significant for the sham-operated animals. There was more than 60% inhibition of the contractile response after atropine (data not shown).

Paper II also examined the intestinal contractile response to EFS and MeCh alone and in the presence of atropine in segments from distal ileum, proximal and distal colon.

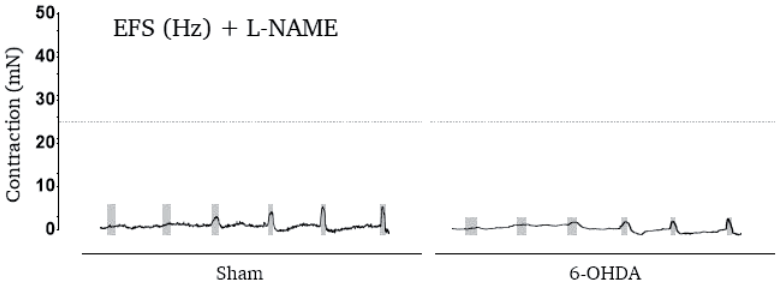
Proximal colon segments showed increased SM contractions evoked by high K^+ in 6-OHDA-lesioned animals (data not shown) compared to sham-operated animals; while SM evoked contractions in circular and longitudinal SM strips did not differ. Electrically-evoked contractile responses in circular SM strips showed a non-significant increase, as summarized in figure 6. In segments, a slight reduction was seen at higher frequencies, as shown in figure 4E in paper II. MeCh-induced contractions were similar between groups and tissue preparations.

Distal ileum segments showed no differences in contractile responses between sham-operated and 6-OHDA-lesioned animals. Moreover, EFS-induced and MeCh-evoked responses were abolished after atropine for all groups and tissue preparations (data not shown).

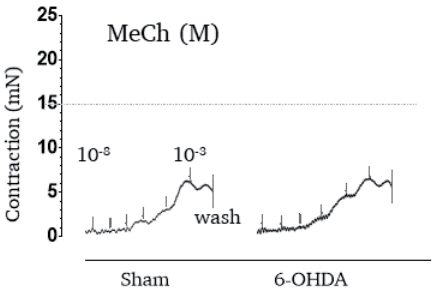

a.
EFS (Hz)
1
2
5
10
20
40
Contraction (mN)
50
40
30
20
10
0
Sham
6-OHDA
b.
EFS (Hz) + L-NAME
Contraction (mN)
Sham
6-OHDA
c.
MeCh (M)
10-6
10-3
wash
Contraction (mN)
25
20
15
10
5
0
Sham
6-OHDA
d.
MeCh (M) + L-NAME
Contraction (mN)
Sham
6-OHDA
Intestinal segment



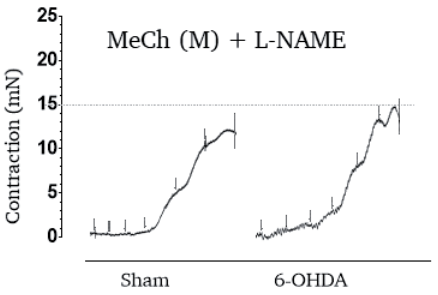
e.



f.



g.



h.

Inverted intestinal segment

← ***Figure 7.*** *Comparison of contractile responses evoked by EFS (a,b,e,f) and MeCh (c,d,g,h) between intestinal segments (left) and inverted intestinal segments (right) of the distal colon in the absence (a,c,e,g) and presence of L-NAME (b,f,d,h). Findings from paper II.*

SM response to monoaminergic stimulation

Paper III investigated the role of monoamines (DA, NA and 5-HT) on SM contraction in colonic and ileal intestinal segments. The main findings are shown in figure 8.

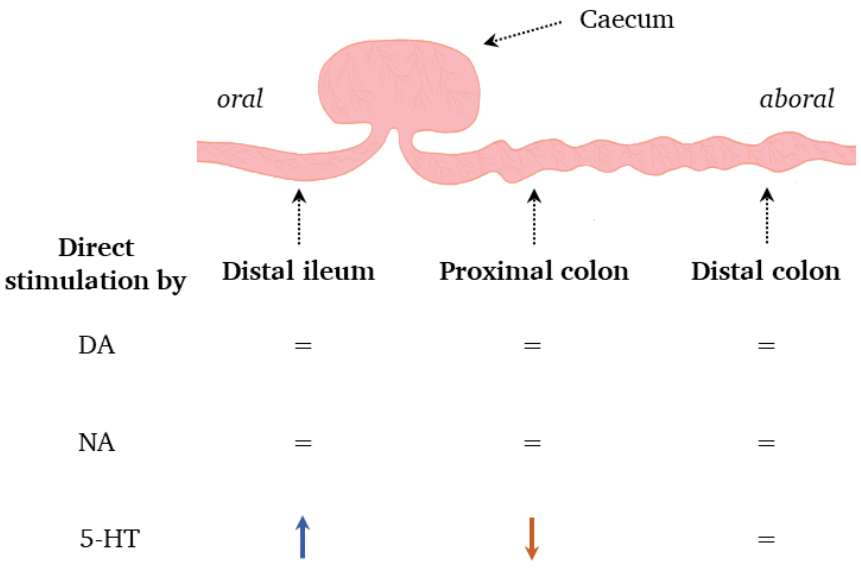


Figure 8. *Contractile responses of distal ileal, and proximal and distal colonic segments to monoamine stimulation by dopamine (DA), noradrenaline (NA) and serotonin (5-HT) in 6-OHDA-lesioned animals compared to sham. Findings from paper III.*

These results showed significant changes in 5-HT-induced contractile responses in distal ileum (increased) and proximal colon (decreased) after intestinal segments were exposed to cholinergic activation. Distal colon showed no differences. DA- and NA-induced responses were similar between the groups in all the tissues tested. After muscarinic inhibition by atropine, responses to DA were abolished, as shown in figure 2D-F in paper III, while responses to 5-HT they were atropine-resistant, as shown in figure 3D-F.

Discussion

Non-motor symptoms (NMS), specifically in the GI system, have been recognized in recent years as part of the broad multisystemic dysfunction accompanying the characteristic motor impairment in PD. NMS progressively and significantly have negative effects on the quality of life of people living with PD (Barone et al., 2009). PD as a multisystemic and neurodegenerative disease not only disturbs voluntary movement, controlled at the central level, but also hinders the ability of the SM, at the peripheral level, to function properly.

It is widely accepted that the autonomic impairment seen in PD, can occur both before and after nigrostriatal degeneration, is an important feature accompanying the disease progression (De Pablo-Fernandez et al., 2017). This autonomic impairment has direct effect on the SM performance due to the dysregulation of multiple excitatory and inhibitory signaling pathways within the ENS. Therefore, many studies in animals have focused on understanding the functional changes leading to GI dysmotility. The results found in this thesis confirm the enteric dysregulation affecting multiple enteric neurotransmitters and proposes to further study possible interactions of cholinergic, nitrergic, and serotonergic pathways.

Intestinal adaptations to central DA loss

Peristalsis is mediated by ascending excitatory and descending inhibitory pathways, influenced and regulated by autonomic connections to the brain, where neurotransmitter interactions are essential to maintain proper motility. Electrically-evoked responses from SM can be studied when the neurons are stimulated with increasing frequencies. Upon optimal stimulation, the release of neurotransmitters within the neuromuscular junction yields the maximal isometric tension possible for a given frequency as all neurons are simultaneously activated. In this way, neurogenic SM motor responses can be studied. Results from paper II showed an enhanced neuronally-evoked contractile response in distal colonic segments of 6-OHDA-lesioned rats, which was sensitive to atropine. This finding suggests that the absolute response is mainly excitatory and, to be expected, cholinergic

in origin. Experiments using circular and longitudinal full-thickness strips did not always show the same trend. In fact, there were no differences between sham-operated and 6-OHDA-lesioned animals.

The DMV is an intermediate brainstem synaptic center regulating the GI motility via preganglionic cholinergic neurons, relaying motor information between the ENS and CNS. It has been proposed that the characteristic α-syn pathology, seen in PD, spreads from the intestine to the brain through the DMV impairing its regulatory function over time (Braak et al., 2003). This Braak staging model has been suggested as a plausible way to explain the occurrence of peripheral damage preceding nigrostriatal involvement, model recently compared to PNS-first phenotype of PD (Borghammer and Van Den Berge, 2019). Previous studies showed decreased amounts of cholinergic neurons and increased TH^+ neurons in the DMV after central DA degeneration by 6-OHDA, suggesting that intestinal dysmotility occurs if the nigrostriatal pathway connects to the DMV through the hypothalamus (Zheng et al., 2011), which supports the CNS-first phenotype of PD. Regardless, this indicates that the impairment of enteric excitatory signaling develops due to abnormal cholinergic signaling from the DMV.

Pacemakers, ICCs, maintain spontaneous SM tone through oscillatory membrane potentials regulated by cholinergic, nitrergic, and serotonergic neurons, among others [for review see (Iino and Horiguchi, 2006)]. Although changes in peristaltic patterns cannot be directly linked to central DA loss, an increased frequency of slow waves without fluctuations in amplitude was found in proximal colon segments of 6-OHDA-lesioned animals (as shown in paper II). Alterations in slow waves can be attributed to unbalance within parasympathetic input from the DMV, sympathetic inhibition, enteric neuronal networks and/or neuronally-induced ICCs activity. This could possible indicate that the abnormal frequency of the slow waves is due to a reduction of ICCs populations in myenteric and submucosal plexuses, as seen in patients with severe slow colonic transit (Lyford et al., 2002) and PD-related chronic constipation (Nhou, 2018). A decrease of myenteric ICCs and their cellular extensions to the circular SM were also observed in 6-OHDA-lesioned animals (Pellegrini et al., 2020). However, from our results, there is not enough evidence to formulate a solid conclusion about this relationship. The complex neurotransmission mediating intestinal slow waves and ICCs activity remains elusive, in particular, for PD. The contribution of ICCs to slow colonic transit in PD is yet to be fully explored.

Cholinergic altered transmission

The cholinergic transmission, critical excitatory component within the parasympathetic system modulating GI motility, has been presumed to be responsible for the PD-related autonomic impairment. *In vitro* experiments presented in paper II showed an enhanced MeCh-induced contractile response in colonic segments. This was similar to what was previously described by Fornai et al. in 6-OHDA-lesioned rats, where they found an enhanced carbachol-induced contraction in dissected colonic circular and longitudinal SM after tetrodotoxin (Fornai et al., 2016). This could possibly be explained by upregulation of M2 and M3 receptors in response to a chronic reduction of ACh release from regulatory centers in the spinal cord (Zhang et al., 2021). However, an altered cholinergic transmission does not always correlate with changes in the amount of enteric cholinergic neurons (Colucci et al., 2012, Zhu et al., 2012), and a reduced number of cholinergic neurons did not modify the carbachol-induced contraction in colonic longitudinal SM from MPTP-treated marmosets (Coletto et al., 2021).

A previous study tracking the isolated neurogenic effect of ACh release on SM showed reduced circular and longitudinal SM contractile responses after electrical stimulation in 6-OHDA-lesioned animals when blocking the action of other endogenous excitatory and inhibitory substances, such as SP, NA and NO, respectively (Fornai et al., 2016). If, as suggested, there is a reduced endogenous release of ACh and mAChRs upregulation in PD, why is there an enhanced electrically-evoked contractile response? A possible explanation involves SP; since it has been shown that electrically-evoked release of SP leads to an enhanced contractile response in longitudinal SM of 6-OHDA-lesioned animals, correlating with an increased NK_1 receptor expression (Pellegrini et al., 2016). To date, studies evaluating the role that enteric SP plays in the pathophysiology of intestinal dysfunction in human PD are lacking [for review see (Tirassa et al., 2021)].

Catecholamines as modulators

In PD patients, the loss of dopaminergic neurons in the myenteric plexus and, consequently, the release of reduced amounts of DA has been suggested as contributing factors to PD-related constipation (Singaram et al., 1995). DA modulates peristalsis by indirectly interacting with myenteric inhibitory neurons through activation of D_1 receptors, facilitating NO release, in turn regulating cholinergic signaling (Nakamori et al., 2021). In

contrast, absence of D_2 receptors in knock-out mice has prokinetic effects seen as accelerated colonic transit, which alters the defecation pattern and hinders water absorption (Li et al., 2006). These studies showed that DA modulates descending inhibitory pathways, despite conflicting evidence about the type of receptor involved. In animal models of PD, MPTP-treated mice showed impaired relaxation after significant reduction of TH^+ neurons in the myenteric plexus (Anderson et al., 2007). However, as results from paper III showed, the absolute response to cumulative concentrations of DA in small and large intestinal segments was contractile, showing no effect in DA-induced responses in 6-OHDA-treated animals. A decrease in the DA-induced inhibitory response has been associated with the loss of D_2 receptors due to an increase in enteric DA synthesis possibly occurring after central DA loss and related autonomic dysfunction (Levandis et al., 2015). To what extent a reduced DA-induced inhibition and higher levels of DA production have functional effects on intestinal motility requires further study. Despite previously described alterations in dopaminergic receptor populations, the findings in this thesis do not support the idea that DA has functional implications in PD-related intestinal dysmotility.

The sympathetic system mainly acts through endogenous NA, inhibiting SM contraction via presynaptic modulation of neurotransmitters release. Exogenous NA mediates intestinal motility by activating: a) presynaptic α-receptors, inhibiting NA release or modulating cholinergic and NANC signaling, and b) post-junctional and postsynaptic β-receptors in SM, where NA directly mediates motor inhibition (De Ponti et al., 1996). In paper III, NA was the only neurotransmitter tested able to elicit an absolute inhibitory response in intestinal segments, leading to a concentration-dependent SM relaxation. No changes were found in the NA-induced relaxatory responses, despite previous studies demonstrating upregulation of β_1 and β_3-receptors in 6-OHDA-lesioned rats (Zhang et al., 2015, Song et al., 2014).

Serotonergic regulation

5-HT is a neurotransmitter known to influence the peristaltic reflex. In paper III divergent results after serotonergic stimulation were found in 6-OHDA-lesioned animals. In proximal colon, 5-HT-induced contractions were lower, while in distal ileum, they were higher than controls. The contractile responses to 5-HT were also atropine-resistant. These findings suggest that 5-HT produces an absolute excitatory response along the GI tract, unaltered by muscarinic inhibition. 5-HT has been shown to be an

important modulator of the cholinergic and nitrergic signaling in intestinal motility (Elswood et al., 1991, Briejer et al., 1992). Enteric 5-HT release, either epithelial by ECs or neuronal by serotonergic neurons located in the myenteric plexus, has also been shown to be regulated by ACh and NO (Kojima and Ikeda, 1998). Absolute responses after inhibiting NOS or nicotinic receptors were, unfortunately, not tested. Based on the experimental design, it is not possible to exclude that activation of nicotinic or nitrergic signaling occurred, in consequence masking the real action of 5-HT.

On the other hand, the opposite direction of change in response between intestinal segments can possibly be attributed to regional differences in the distribution of 5-HT receptors modulating intestinal motility. Even though a reduced protein expression of 5-HT_4 was described in 6-OHDA-lesioned colon their effects on colonic motility were not tested (Zhang et al., 2015). Additional *in vitro* testing with selective 5-HT agonists and antagonists is recommended to further elucidate the serotonergic involvement in the pathophysiology of PD, which based on the current available literature is limited. Despite therapeutic efforts to relieve bothersome GI symptoms in PD prescribing 5-HT_4 agonists and 5-HT_3 antagonists, it is still uncertain if serotonergic signaling is impaired or if this pharmacological treatment only has indirect prokinetic action by stimulating cholinergic signaling.

The role of nitric oxide

The activation of guanylyl cyclase by NO and subsequent accumulation of cytosolic cGMP pools enhance the enzymatic activity of protein kinase G and MLC phosphatase, leading to SM relaxation [for a detailed review see (Francis et al., 2010)]. Theoretically, NOS inhibition by L-NAME should therefore enhance the contractile responses induced by electrical stimulation, as there is no activation of NO-cGMP-dependent signaling pathways. However, *in vitro* results in paper II showed a significantly decreased electrically-evoked contraction in the distal colon of sham-operated and 6-OHDA-lesioned animals in the presence of L-NAME. Therefore, this reduction cannot be explained solely by a direct effect of NO on the SM relaxation. NOS inhibition has been shown to stimulate neuronal ACh release while L-arginine reduces it (Hryhorenko et al., 1994). It is plausible that the accumulation of L-arginine, due to the NOS inhibition, may hamper ACh release from nerve terminals. In contrast to the presented findings, inhibition of NOS in MPTP-treated marmosets showed no effect in neither

ileal electrically-evoked contractile nor relaxatory phases, with unchanged NOS-immunoreactive neurons (Coletto et al., 2019).

A decreased descending inhibitory modulation due to nitrergic neuronal loss in ileal and colonic myenteric plexus has been suggested as a possible cause of GI dysmotility (Blandini et al., 2009). In this thesis, a reduced cholinergic-induced contraction was found only in 6-OHDA-lesioned animals when NOS was inhibited by L-NAME. The absence of effect in sham-operated controls suggests that nitrergic signaling is impaired in the distal colon after central DA loss. It has been proposed that NO inhibits neuronal ACh release by a cGMP-independent pathway, which may indirectly provoke release of other modulatory neurotransmitters (Hebeiss and Kilbinger, 1996). If true, it is possible that the contribution of this indirect effect is amplified under pathological circumstances. Neuronal production of NO and release of VIP by inhibitory neurons has been demonstrated (Grider, 1993), it is possible that the inhibition of NO production by L-NAME partially decreases VIP release. This could explain the decreased MeCh-induced contractile response observed in 6-OHDA-lesioned animals. The actual mechanism by which the interplay NO/VIP modulates cholinergic transmission in PD requires further investigation.

Insights into the 6-OHDA rat model of PD

Interactions between the wide variety of enteric neurotransmitters, in response to CNS influence or changes in the intestinal microenvironment, are complex. Therefore, experimental findings from animal models should be interpreted with caution. In particular, the induced central DA neurodegeneration in the 6-OHDA rat model of PD is a shared pathological endpoint with human PD; however, the model lacks other pathognomonic features such as α-syn aggregates or the progressive nature of neurodegeneration. Therefore, gathering evidence from other animal models is crucial to develop a comprehensive explanation that applies to the human condition.

Revising the 6-OHDA rat model of PD is also relevant. Current results showed that desipramine influences SM contractility, where the urinary bladder appeared to be more susceptible. Desipramine has shown high affinity for NET over other monoamine transporters, making it a highly selective NA reuptake inhibitor (Tatsumi et al., 1997). However, desipramine has also shown a wide pharmacological profile binding to α_1-adrenoceptors, histamine H_1-receptors, and mAChRs with varying affinity (Deupree et al.,

2007). This could possibly explain the adverse effects seen on urinary bladder and intestinal contractility, as described in paper I. Therefore, data from studies using desipramine should be carefully interpreted. Likewise, the 6-OHDA rat model of PD focuses on the central DA loss as a *CNS-first* approach. If it is desired to develop a *PNS-first* model using 6-OHDA then prophylaxis with selective NA inhibitors, such as reboxetine or viloxazine, to protect peripheral noradrenergic neurons, may be an option.

Concluding remarks

PD-related intestinal symptoms are the portrayal of the GI dysfunction driven by the ANS. The mechanism of such peripheral autonomic dysregulation after nigrostriatal denervation remains unclear. To date, no comprehensive clinical management guidelines or effective solution for PD-related autonomic symptoms have been developed (Natale et al., 2008, Perez-Lloret et al., 2013). Currently available pharmacological treatments often interferes with the ANS function, which may worsen the autonomic symptoms (Palma and Kaufmann, 2018). Understanding how nigrostriatal denervation affects the ANS-regulated SM function in PD is needed to develop more targeted and selective pharmacological options.

Future directions

This thesis confirms that neurogenic and myogenic alterations occur in the intestine as a consequence of central DA loss. These alterations change the enteric modulatory mechanisms in many ways by: a) enhancing excitatory pathways, b) impairing inhibitory regulation, and/or c) weakening intrinsic regulatory mechanisms.

Knowing this, the aim for future research may include detailed investigation of possible pathological interactions between cholinergic, nitrergic, and serotonergic signaling pathways. These interactions may explain how the autonomic dysfunction occurs, leading to intestinal dysmotility, and will open the opportunity to test more targeted treatment options.

To move forward from only exploring cholinergic signaling, which does not appear to be the only PD-related impaired neurotransmitter system in the intestine, future experiments should include *in vitro* characterization of nitrergic and serotonergic receptor populations dysregulated by altered autonomic function. Once specific receptor populations are characterized novel experimental compounds should be tested *in vivo* to further prove their effects under physiological conditions. In addition, the involvement of VIP and SP may be of interest as their contribution to the altered intestinal motility has been poorly explored.

Last, but not least, despite the 6-OHDA rat model of PD being an adequate model for the study of the effects that the loss of DA in the brain has at the peripheral level, the use of other experimental models of PD that allow direct manipulation of the periphery should also be considered.

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