Ana Lúcia Oliveira da Silva

Premotor enteric nervous system impairment in an experimental Parkinson model

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Sónia Silva Santos (Faculdade de Farmácia da Universidade de Coimbra) e do Professor Doutor Ângelo Tomé (Faculdade de Ciências e Tecnologia da Universidade de Coimbra)

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This work was developed under a Project coordinated by Doctor Frederico Pereira in collaboration with Doctor Rui Prediger, in the following institution:

Pharmacology and Experimental Therapeutics, Institute for Biomedical Imaging and Life Sciences, Faculty of Medicine, University of Coimbra, Coimbra

“*Nothing in life is to be feared, it is only to be understood.”*

Marie Curie

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
A	adrenaline
AADC	Aromatic l-amino acid decarboxylase
AC	Adenylyl cyclase
ACh	acetylcholine
ADHD	Attention deficit hyperactive disorder
ATP	adenosine-triphosphate
ATP	Adenosine triphosphate
BBB	Blood brain barrier
CA	Catecholamine
Ca^{2+}	Calcium ion
cAMP	3'-5'-cyclic adenosine monophosphate
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
D1R	D1-like DA receptors
D2R	D2-like DA receptors
DA	Dopamine
DAG	diacylglycerol
DA-Rs	dopamine receptors
DAT	Dopamine transporter
DMV	dorsal motor nucleus of the vagus
DOPAC	3,4-dihydroxyphenylacetic acid
DβH	dopamine-β-hydroxylase
EGF	epidermal growth factor
ENS	enteric nervous system
GI	Gastrointestinal
GPCRs	G protein-coupled receptors
$G\alpha_s$	Adenylyl cyclase stimulatory G protein
HVA	3-methoxy-4-hydroxyphenyl acetic acid; homovanilic acid
i.n.	intranasal

i.p.	intraperitoneal
i.v.	intravenous
ICC	intersticial cells of Cajal
IL4	intracellular loop
IP3	inositol trisphosphate
LBs	Lewy bodies
l-DOPA	l-3,4-dihydroxyphenylalanine
MAO	monoamine oxidase
MAO-B	monoamine oxidase B
MFB	median forebrain bundle
MLC kinase	Myosin light-chain kinase
MLC phosphatase	myosin phosphatase
MMC	migrating motor complex
$MPDP^+$	1-methyl-4-phenyl-2,3-dihydropyridinium
NA	Noradrenaline
NPY	neuropeptide Y
OT	olfactory tubercle
Paraquat	1,1'-dimethyl-4,4'-bipyridinium dichloride
PCD	programmed cell death
PD	Parkinson's Disease
PKA	protein kinase A
PKC	protein kinase C
PNMT	phenylethanolamine-N-methyltransferase
REM	Rapid eye movement
s.c.	subcutaneous
SAM	S-adenosylmethionine
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
TH	tyrosine hydroxylase
VIP	vasoactive intestinal polypeptide
VMAT2	vesicular monoamine transporter 2
VMAT2	vesicular monoamine transporter-2
VTA	ventral tegmental area

α-SYN	α-synuclein
PAELR	parkin-associated endothelin receptor-like receptor
UbCH7	ubiquitin-conjugating enzyme 7
UbCH8	ubiquitin-conjugating enzyme 8
UCHL1	ubiquitin carboxy-terminal hydrolase L1
AC5	Adenylyl cyclase type 5
CREB	cyclic AMP response element binding protein
DARPP-32	dopamine-related phosphoprotein, 32 kDa
MAPK	mitogen-activated protein kinase
NHE	Na^+/H^+ exchanger
PLC	phospholipase C
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
AA	arachidonic acid
AC2	adenylate cyclase type 2
PA	phosphatidic acid
PC	Phosphatidylcholine
PI3K	phosphatidylinositol 3-kinase
PLD	phospholipase D
RTK	receptor tyrosine kinase

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ABSTRACT

It has been thoroughly demonstrated that rodents treated intranasally with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exhibit time-dependent impairments in olfactory, emotional, cognitive and motor functions associated with disruption of dopaminergic neurotransmission across brain structures which possibly recapitulate those observed during different stages of Parkinson's disease (PD). Although autonomic dysfunction, such as constipation, has potential sensitivity as a clinical biomarker of the premotor phase, it has been overlooked.

The aim of this study was to evaluate the intestinal function before motor impairment, using this intranasal MPTP rat PD model. To this end, ileum segments from male Wistar rats (21 weeks old) were collected 7 days following the MPTP administration for functional studies. Isometric contractile concentration-response (CR) curves (cumulative) for dopamine (DA) were performed in both the presence and the absence of sulpiride, which is a selective dopamine D2-like receptors (D2R) antagonist. Ilea samples were also collected for the assessment of the dopaminergic status by measuring total DA content (HPLC) and also tyrosine hydroxylase (TH), which is the rate-limiting enzyme in DA biosynthesis and D2R density (WB). Moreover, immunohistochemical studies were performed on isolated rat ileum not only to analyze the S100β protein (enteroglial intestinal inflammation marker) immunostaining, but also to characterize the cellular location of D2R while assessing possible alterations between treated and untreated animals.

Functional studies showed biphasic DA CR curves for both saline and MPTP-treated rats, evidencing contraction for lower DA concentrations (0.01 to 0.9 µM) and relaxation for higher concentrations (3 to 90 µM). However, MPTP imposed a statistically significant decrease in the maximum contractile effect in the DA CR curves compared to saline. Within saline group, there was a statistically significant difference between DA CR curves performed in the presence and in the absence of the D2R antagonist. On the other hand, no such difference was obtained in the MPTP-treated group. This is highly suggestive that MPTP is perturbing D2R-dependent ileum function. The dopaminergic dysfunction occurs with preserved DA homeostasis as shown by normal DA levels and both TH and D2R ileal expression levels. S100β immunoreactivity was observed within the saline group in smooth muscle cells and ganglion cells of both submucosal and myenteric plexus, while MPTP induced a

tendency to an immunostaining decrease in the submucosal plexus relatively to the myenteric plexus, when comparing to the saline group. In addition, D2R immunoreactivity tends to be higher in the myenteric plexus when compared to submucosal plexus in the saline animals. In turn, within the MPTP-treated group, only representative histological sections of three out of six treated animals showed immunoreactivity in the myenteric plexus.

Overall, the obtained results lead to the existence of an early gut dopaminergic dysfunction that is in place following i.n. MPTP administration. This GI dysfunction may not be directly related to DA total content, since DA homeostasis was preserved in the ileum, but it might be specifically connected to a D2R dysfunction in the myenteric plexus, caused by the neurotoxin.

Keywords: Parkinson's disease, MPTP, GI dysfunction, Dopamine, D2-like Dopamine Receptors.

RESUMO

Tem sido demonstrado que os roedores tratados com 1-metil-4-fenil-1,2,3,6-tetra-hidropiridina (MPTP), por via intranasal, exibem deficiências dependentes do tempo após a exposição à neurotoxina, nas funções olfactiva, emocional, cognitiva e motora associadas a perturbações da neurotransmissão dopaminérgica em estruturas cerebrais, possivelmente mimetizando as alterações observadas durante as diferentes fases da doença de Parkinson (PD). Embora a disfunção autonómica, tal como a obstipação, tenha potencial como biomarcador clínico da fase pré-motora, esta tem sido negligenciada até agora.

O objetivo deste estudo foi avaliar, neste modelo de PD em rato com administração intranasal de MPTP, a função intestinal antes de haver comprometimento motor. Para esse fim, segmentos do íleo de ratos Wistar machos (21 semanas de idade) foram colhidos para estudos funcionais, 7 dias após a administração de MPTP. Foram executadas curvas cumulativas concentração-resposta (CR) de dopamina (DA) (0.01-90 μM) tanto na presença como na ausência de sulpiride, um antagonista selectivo dos recetores dopaminérgicos tipo 2 (D2). Foram também colhidas amostras de íleo para a avaliação da via dopaminérgica, através da medição do conteúdo total de DA (HPLC), da expressão da tirosina-hidroxilase, enzima envolvida no passo limitante da síntese de DA, bem como da expressão do receptor D2 (D2R) da DA (WB). Segmentos de íleo de rato foram ainda utilizados em técnicas de imunohistoquímica quer para analisar a imunomarcação da proteína S100β, um marcador enteroglial de inflamação intestinal, quer para caracterizar a localização celular dos D2R, avaliando simultaneamente possíveis alterações entre os animais tratados e não tratados.

Os estudos funcionais mostraram uma resposta bifásica à DA tanto em ratos salinos como tratados com MPTP, evidenciando-se contração para concentrações mais baixas de DA (0.01 to 0.9 μM) e relaxamento para concentrações mais elevadas (3 to 90 μM). Contudo, o MPTP reduziu significativamente o efeito contráctil máximo nas curvas CR de DA comparativamente ao grupo salino. Houve ainda, no grupo salino, uma diferença estatisticamente significativa entre as curvas CR de DA realizadas na presença e na ausência do antagonista dos D2R. Em contrapartida, nenhuma diferença foi observada no grupo tratado com MPTP, sugerindo que o MPTP perturba a função do íleo dependente dos D2R. A disfunção dopaminérgica ocorre com a homeostase da DA preservada, como o demonstram os níveis de DA, e a expressão de TH e de D2R no

íleo. A imunomarcação para a proteína S100β foi observada no grupo salino em células do músculo liso e células ganglionares de ambos os plexos submucoso e mientérico, enquanto no grupo MPTP se observou tendencialmente uma diminuição no plexo submucoso relativamente ao mientérico, quando comparada com o grupo controlo. É ainda de referir que a imunomarcação dos D2R tende a ser mais elevada no plexo mientérico quando comparado com o plexo submucoso nos animais controlo. Por sua vez, no grupo tratado com MPTP, apenas secções histológicas representativas de três dos seis animais tratados apresentaram imunomarcação no plexo mientérico.

Em suma, os resultados obtidos permitem inferir a existência de uma disfunção dopaminérgica precoce no intestino após a administração de MPTP. Esta disfunção gastrointestinal não estando diretamente relacionada com o conteúdo total de DA, uma vez que a sua homeostase foi preservada no íleo, pode estar especificamente ligada a uma disfunção dos D2R no plexo mientérico, causados pela neurotoxina.

Palavras-chave: Doença de Parkinson, MPTP, disfunção gastrointestinal, dopamina, recetores dopaminérgicos tipo 2

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CHAPTER 1

Introduction

.....

1. Parkinson's Disease

1.1. General considerations of PD

Clinical features of Parkinson's disease (PD) were first described by James Parkinson in 1817 in "An Essay of the Shaking Palsy" (Parkinson, 2002). He reported six patients and described most of the typical clinical features such as bradykinesia, rest tremor, postural instability, stooped posture and micrographia. He did not describe rigidity, which was described by Charcot (1888) and he was Charcot himself who proposed to call this disease as PD (Mizuno et al., 2008).

Clinically, PD is characterized by various motor and non-motor symptoms. Under the motor symptoms, the presence of bradykinesia, resting tremor, rigidity and late postural instability has been established to classically define PD (Lees et al., 2009). On the other hand, many non-motor symptoms are associated to PD, namely cognitive impairment, hallucination, delusion, behavioural abnormalities, depression, disturbances of sleep, loss of smell, pain, and autonomic dysfunctions such as constipation, hypotension, urinary frequency, impotence and sweating (Mizuno et al., 2008). This symptoms are consequence of typical pathological findings which include the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the accumulation of α-synuclein (α-SYN) containing Lewy bodies (LBs) and neurites (Halliday et al., 2011;Beach et al., 2010).

The etiopathogenesis of PD is still not fully understood. Still, it is known that 95% of cases are sporadic, i.e. a multifactorial, idiopathic disorder resulting from contributions of environmental and genetic susceptibility. The remaining 5% are the result of genetic mutations, of which there are several types, many only recently identified (Mounsey and Teismann, 2010).

1.2. Prevalence

PD is among the most frequent neurodegenerative disorders associated with aging (Fahn, 2010), affecting nearly six million people worldwide. Thus, the disorder is particularly prevalent among the elderly population (Litteljohn et al., 2010), affecting 2% of individuals over age 65 and 4–5% over 85 years (Eriksen et al., 2003). Since the

incidence of the disease increases with age (the most important risk factor), it is likely that in the future the number of people suffering from PD steadily increases (Prediger et al., 2011).

Concerning the onset of disease, the mean age is about 60 years of age, however 5-10% of cases, designated as young onset, begin between 20 and 50 years of age (Samii et al., 2004). The incidence of the disease either in men or in women is controversial, as some studies reported that is more common in men, while others failed to found differences between gender (Lau and Breteler, 2006).

1.3. Neuropathology

PD is neuropathologically characterized by a progressive loss of dopaminergic neurons within the substantia nigra (SN), mainly affecting the ventral region of the pars compacta, and by the presence of intracytoplasmic, proteinaceous inclusions termed as Lewy bodies (LBs) (Forno et al., 1996). Analysis of post-mortem brains of individuals displaying PD, showed a loss of 50–70% of nigral neurons in this region when compared to the same region in healthy individuals (Dauer and Przedborski, 2003; Ross et al., 2004).

LBs are intracytoplasmic inclusions, whose principal component is α-synuclein, a small protein (140 amino acids) that is predominantly expressed in the neocortex, hippocampus, substantia nigra, thalamus and cerebellum (Bekris et al., 2010). Moreover, two missense mutations in the gene encoding α-SYN are linked to dominantly inherited PD, thus directly implicating α-SYN in disease pathogenesis (Auluck et al., 2002; Luk et al., 2013).

During the progress of disease, there is a steadily increase in severity of the neuropathological changes, occurring a known distribution pattern through which it is possible to predict alterations (Figure 1). Even in the brains of individuals whose clinical protocols make no mention of motor dysfunctions associated to the disease, this kind of alterations can be developed at some extension. Consequently, it is opportune to subdivide the course of disease into presymptomatic and symptomatic phases (Braak et al., 2003).

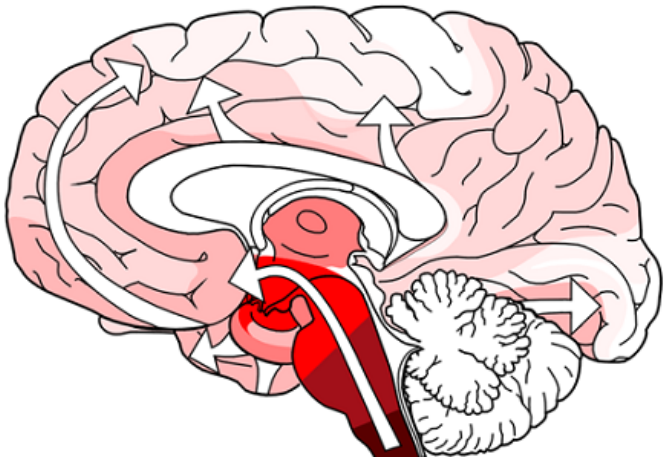

Figure 1 Representation of the ascending pathological process (white arrows). The shading intensity of the colored areas corresponds to the severity of the disease; dark red regions correspond to the initial stages (presymptomatic phase), while light red regions correspond to late stages of the disease. Based on H Braak *et al*., 2003

The neuropathological changes characteristic of PD are thought to evolve sequentially across the brain, hence the recognition of six neuropathological stages (Bratzke, 2004). In the early presymptomatic stages (1-2), pathology is limited to the medulla oblongata and pontine tegmentum (including the dorsal motor nucleus of vagus and the locus coeruleus) and olfactory bulb. As patients become symptomatic (stages 3-4), the substantia nigra and other nuclei within the midbrain and forebrain, such as the nucleus basalis of Meynert, become involved. Finally, in patients who survive into the late stages of the disease (5-6), pathology advances from mesocortex into neocortex.

In addition, the dopaminergic midbrain neurons degeneration in PD leads to loss of striatal DA levels, which can be measured by *in vivo* imaging (Eckert et al., 2007). Since dopamine is implicated in the modulation of movements, mood and motivation, individuals with PD will therefore show characteristic impairments of these functions (Höglinger et al., 2004).

SNpc homeostasis is believed to be vulnerable to different genetic, cellular and environmental factors that independently or concomitantly cause cell death over time (Sulzer, 2007). These factors may lead to mitochondrial dysfunction and oxidative stress, to abnormal protein degradation due to alterations in the ubiquitin system or in chaperone-mediated autophagy, among other forms of subcellular dysfunction (Figure 2). Cell death may be caused by the combination of these alterations, namely α-synuclein aggregation, proteosomal and lysosomal system dysfunction, and reduced

mitochondrial activity. Moreover, gene mutations are associated with impairment of one or several of these mechanisms (Obeso et al., 2010). Which (if any) of these mechanisms is more important to disease pathogenesis is yet to be established.

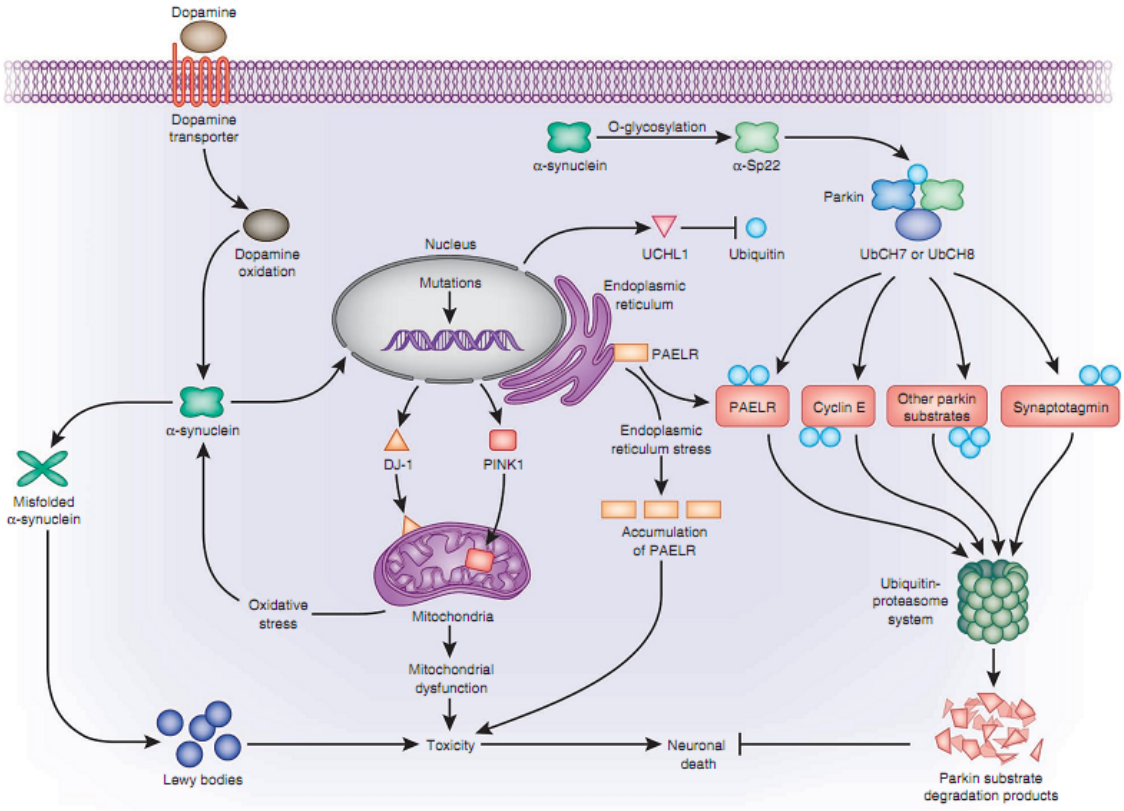

Figure 2: Schematic representation of established etiopathogenic mechanisms and interactions in the dopaminergic cells of the substantia nigra in PD. PAELR, parkin-associated endothelin receptor-like receptor; UbCH7, ubiquitin-conjugating enzyme 7; UbCH8, ubiquitin-conjugating enzyme 8; UCHL1, ubiquitin carboxy-terminal hydrolase L1. Based on Obeso et al. 2010.

1.4. Symptomatology

PD is characterized by four main symptoms, grouped under the acronym TRAP: Tremor at rest, Rigidity, Akinesia (or bradykinesia) and Postural instability (Jankovic 2008). Also included among classic PD features are flexed posture and freezing (motor blocks). Besides the classic motor impairment, the onset and progression of the disease is also associated with nonmotor features (Chaudhuri et al., 2005), including altered behavior, abnormal sleep-waking cycle, and pain, as well as autonomic dysfunctions.

1.4.1. Motor Symptoms

Even though it may also be seen in other disorders, including depression, bradykinesia - slowness of movement - constitutes the most characteristic clinical feature of PD. It is also a hallmark of basal ganglia disorders, and it encompasses difficulties with planning, initiating and executing movement and with performing sequential and simultaneous tasks (Berardelli et al., 2001). The initial manifestation is often slowness of movements while performing daily activities and reaction times (Cooper et al., 1987; Giovannoni et al., 1999).

Rest tremor is also one of the most common and easily recognized of PD motor features (Shahed & Jankovic, 2007). Tremors are unilateral and almost always potentiated in the distal part of an extremity. Although it can also affect the lips, chin, jaw and legs, unlike essential tremor, it rarely involves the neck/head or voice. Typically, rest tremor disappears with action and during sleep (Jankovic, 2002). There's also evidence of essential tremor being a risk factor for PD. Additionally, many PD patients beyond rest tremor also develop postural tremor which is more prominent and disabling than rest tremor and may correspond to the first manifestation of the disease (Shulman et al., 1996; Jankovic et al., 1999).

Usually accompanied by the "cogwheel" phenomenon, rigidity is characterized by increased resistance, specially when associated with an underlying tremor, present throughout the range of passive limb movement (flexion, extension or rotation about a joint). It occurs proximally (e.g., neck, shoulders, hips) and distally (e.g., wrists, ankles) (Jankovic, 2008). Furthermore, neck and trunk rigidity (axial rigidity) may happen resulting in abnormal axial postures. This symptom is often associated with postural deformities which result in flexed neck and trunk posture and flexed elbows and knees (Broussolle et al. 2007; Riley et al. 1989).

As one of the most disabling known symptoms of PD, freezing is a form of akinesia (loss of movement) (Giladi et al., 2001). While this is a characteristic feature of PD, its prevalence is not universal (Bloem et al., 2004). Freezing most commonly affects the legs during walking, but arms and eyelids may also be involved (Boghen, 1997). A sudden and transient inability to move is the typical manifestation, which may include hesitation in the beginning of walk (start hesitation) or a sudden incapacity to move the feet during specific situations (e.g., turning or walking through a narrow passage, crossing busy streets, approaching a destination) (Jankovic, 2008).

Postural instability is generally a manifestation of the late stages of PD, usually occuring after the onset of other clinical features while leading to an increased risk of hip fractures (Williams et al., 2006).

Additionally, there is a number of secondary motor symptoms that may have impact on PD patients while functioning at home, at work or even driving (Singh et al., 2007). In early asymmetric PD, the so-called mirror movements might occur, i.e., voluntary movements may be accompanied by unintended movements in homologous muscles on the opposite side of the body (J. Y. Li et al., 2007). Speech disorders such as the ‘‘tip-of-the-tongue phenomenon’’ are mentioned in PD while referring to monotonous, soft and breathy speech with variable rate and frequent word finding difficulties (Critchley , 1981; Matison et al., 1982).

1.4.2. Non Motor Symptoms

Over the past decade, advances in genetics and neuropathology have provided further insight into the etiopathogenesis of PD (Gasser, 2007; Schapira & Jenner, 2011). At early stages of disease, several non-motor manifestations, such as autonomic, sleep, and olfactory dysfunction, constipation and depression may occur and precede the onset of motor symptoms by more than a decade (Obeso et al. 2010; Chaudhuri et al., 2008; Chaudhuri & Schapira, 2009). Consequently, the concept of premotor PD has gained support (Langston, 2006; Hawkes, 2008).

Early non-motor symptoms of PD include olfactory deficit, rapid eye movement (REM) behavior disorder, excessive daytime somnolence, depression, anxiety, constipation and erectile dysfunction. These early features are consistent with pathological changes using immunohistochemical staining beginning in the dorsal motor nucleus of the glossopharyngeal and vagal nerves and anterior olfactory nucleus and gradually ascend, eventually encompassing the neocortex (Heiko Braak et al., 2003). An early involvement of autonomic neurons in the spinal cord, heart, gastrointestinal and genitourinary tracts has also been hypothesized (Dickson et al., 2008).

The importance of a dopaminergic contribution to non-motor symptoms in PD has recently been reported by a PET study, which showed in vivo evidence of dopamine dysfunction by ^{11}C-raclopride imaging in the hypothalamus of patients with PD (Politis

et al., 2008). Therefore, a dopaminergic contribution to several non-motor symptoms of PD, such as sleep disorders, autonomic dysfunction, and neuro-endocrinal problems, was suggested.

2. Dopaminergic Neurotransmission

2.1. Dopamine (DA)

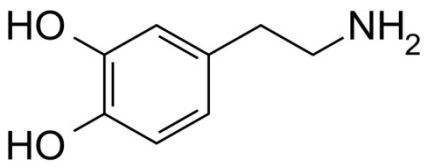

Figure 3: DA chemical structure

DA is the most abundant catecholamine in the brain (about 80% of the catecholamine content) and its involvement and importance as a neurotransmitter in the regulation of different physiological functions in the central nervous system (CNS) is well established. Deregulation of the dopaminergic system has been associated with pathologies such as PD, Tourette's syndrome, schizophrenia, attention deficit hyperactive disorder (ADHD) and generation of pituitary tumours (Vallone et al., 2000).

Their distinctive structural features (Figure 3) are the single amine group, a nucleus of catechol (a benzene ring with two adjacent hydroxyl groups) and a side chain of ethylamine or one of its derivatives (Feldman et al., 1997).

2.2. DA Biosynthesis

Aromatic amino acid tyrosine is the precursor for the synthesis of DA (Vallone et al., 2000).

The transformation of tyrosine into DA includes two reactions (Figure 4):

- The first is catalysed by the enzyme tyrosine hydroxylase (TH) which converts tyrosine into l-3,4-dihydroxyphenylalanine (l-DOPA). TH is

considered the rate-limiting enzyme in this pathway, as inhibition of TH activity induces a reduction of the formation of l-DOPA.

- The second step consists of the cytosolic conversion of l-DOPA, catalysed by the enzyme aromatic l-amino acid decarboxylase (AADC), which produces DA. Therefore, exogenous administration of l-DOPA can increase the synthesis of DA.

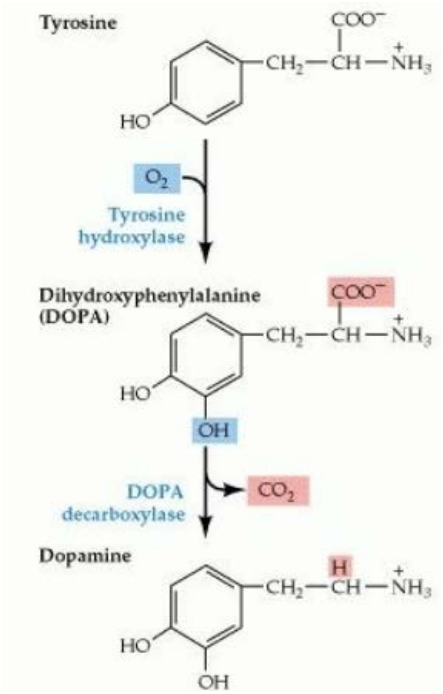

Figure 4: **DA biosynthesis** (adapted from Purves et al., 2001)

In dopaminergic neurons, DA is transported from the cytoplasm to specialized storage vesicles by vesicular monoamine transporter 2 (VMAT2), where the amine concentration is approximately 0.1M (10–1000 times higher than the level in the cytosol). There is also the cytoplasmic free form of DA (although in low abundance), regulated by monoamine oxidase (MAO), an enzyme which catalyzes the oxidation of monoamines (Elsworth & Roth, 1997).

2.3. DA Metabolism

During an action potential, DA's release in the synaptic cleft occurs by exocytosis from the presynaptic terminals, subsequently to the depolarization of the neuron and an increase of cytosolic calcium. The released DA binds to its receptors pre or post-

synaptical. Then, in order not to gradually accumulate in the synaptic cleft and to prevent saturation, DA may follow two pathways: it can be recaptured by the presynaptic neuron through a membrane active mechanism, or suffer enzymatic inactivation. The uptake mechanism may be influenced/blocked by certain drugs, so that the remaining DA remains in the synaptic cleft and is be able to exert its action in the receptors for a longer period of time (Ferraz and Andrade, 1997).

The metabolism of intraneuronal DA occurs by the sequential action of MAO and Catechol-O-methyltransferase (COMT) (Figure 5).

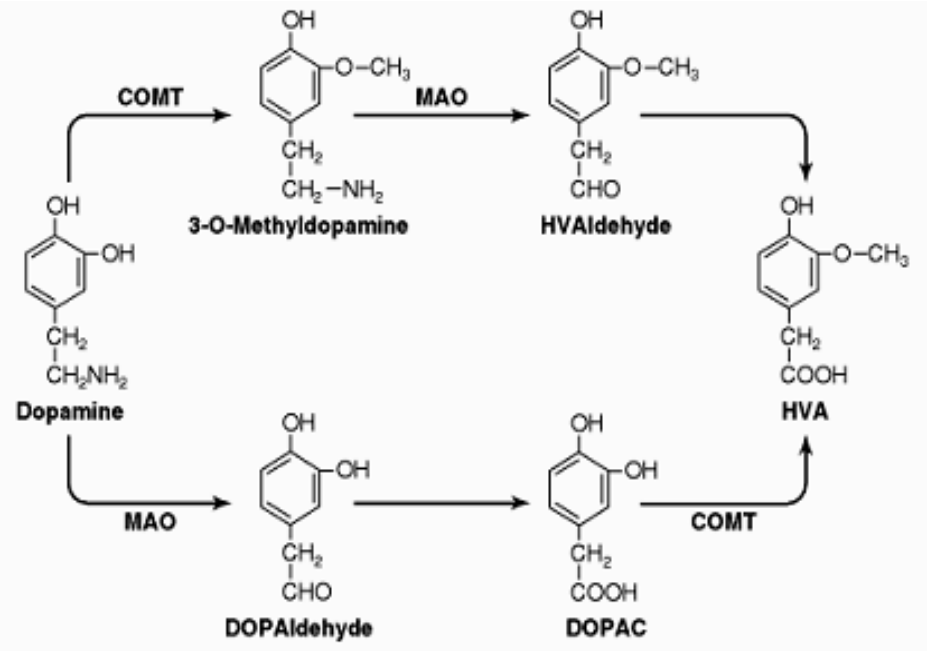

Figure 5: **DA catabolic pathway** (adapted from Siegel et al., 1999).

DA is broken down and originates the two main metabolites of dopamine synthesis in humans, namely, 3,4-dihydroxyphenylacetic acid (DOPAC), by the action of MAO, and 3-methoxy-4-hydroxyphenyl acetic acid, also called homovanilic acid (HVA), by the action of MAO and COMT (Mannisto et al., 1992).

2.4. Dopaminergic Pathways

Projections originating from brain areas that synthesize this neurotransmitter give rise to four axonal pathways: nigrostriatal, mesolimbic, mesocortical and tuberoinfundibular.

The nigrostriatal pathway transmits dopamine from the SNpc to the striatum. This pathway is involved in the control of movement and its degeneration is associated with PD, characterized by tremors, rigidity and akinesia (Gerfen, 1992; Lang and Lozano, 1998).

The mesolimbic pathway arises from the midbrain ventral tegmental area (VTA) and innervates the ventral striatum, the olfactory tubercle (OT) and also parts of the limbic system. It has been implicated in influencing motivated behavior (Koob & Bloom, 1988; Koob, 1992) and also in mediating incentive learning and reinforcement mechanisms associated with positive reward such as palatable food in a hungry animal or addiction (Wang, 2011).

The mesocortical pathway originates from the VTA innervating different regions of the frontal cortex. It appears to be involved in some aspects of learning and memory (Le Moal et al., 1991).

The tuberoinfundibular pathway transmits from cells of the periventricular and arcuate nuclei of the hypothalamus. Projections of this pathway reach the median eminence of the hypothalamus where DA is released into the perivascular spaces of the capillary plexus of the hypothalamic-hypophyseal portal system. Therefore, DA is transported to the anterior pituitary where it acts on the lactotrophs to inhibit the release of prolactin, a hormone which stimulates milk production from mammary glands (Doppler, 1994) and stimulates lactotroph proliferation (Saiardi et al., 1997).

2.5. Dopamine Receptors

Dopamine’s action is due to its binding to specific membrane receptors (Gingrich and Caron, 1993), which belong to the family of seven transmembrane domain G-protein coupled receptors (GPCRs).

The complementary DNAs of five distinct dopamine receptors (DA-Rs) have been isolated and characterized, enabling their subdivision, based on their biochemical and pharmacological properties, into two subfamilies, namely D1- and D2-like. The D1-like subfamily (D1R) includes D1- and D5-R, while the D2-like (D2R) comprises D2-, D3- and D4-R.

Each receptor contains seven stretches of amino acids which are hydrophobic and long enough to span the membrane (Figure 6). Hence, it seems that each of the

dopamine receptors conforms to the general structural model for a GPCR (Kobilka & Schertler, 2008; Palczewski et al., 2000) with an extracellular amino terminus and seven putative membrane spanning α-helices linked by intracellular and extracellular protein loops. On the amino terminus and second extracellular loop, one or more potential sites for glycosylation may be found. In order to form the ligand binding site, the helices are bundled together in the membrane. Also, some information is available on the residues that make contact with ligands. (Chien et al. 2010; Ballesteros et al. 2001). There is an intracellular carboxyl terminus, presumably bearing a palmitoyl group, which may form a further link to the membrane. While the D1R have short third intracellular loops and long carboxyl terminal tails, D2R exhibit long third intracellular loops and short carboxyl terminal tails. Not only does this substantiate a structural basis for the distribution of the receptors into two subfamilies, but it is also expected to have a functional significance, probably related to the specificity of receptor/G protein interaction.

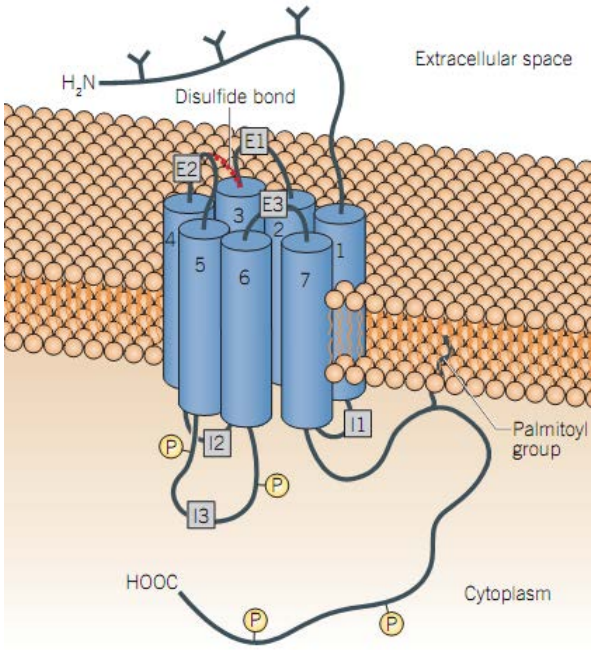

Figure 6: **Schematic representation of a G protein-coupled dopamine receptor** (adapted from Strange and Neve 2013)

Although dopaminergic ligands are able to differentiate between the D1R and D2R receptor subfamilies, most of them do not clearly discriminate between receptors of the same subfamily. For instance, the D1-R antagonist, SCH-23390, or the agonist, SKF-38393, have very similar affinities for both D1- and D5-R (Table I) (Vallone et al. 2000).

Table I: Values for the dissociation constants are given for ligands, determined using ligand binding assays for the five dopamine receptor subtypes. As far as possible, values are given that avoid artefacts present in ligand binding assays with high affinity radioligands. Based on Strange & Neve, 2013.

Drug	**Receptor Affinity, K_i (nM)**				
	D1	**D5**	**D2**	**D3**	**D4**
Chlorpromazine	73	133	0.55	1.2	9.7
Clozapine	141	250	35	83	22
Haloperidol	27	48	0.53	2.7	2.3
Raclopride	>72000	-	1	1.8	240
SCH 23390	0.35	0.3	267	314	3560
SCH 39166	1.2	2	980	-	5520
SKF 83566	0.3	0.4	2000	-	-
(S)-(-)-Sulpiride	36000	77000	2.5	8	1000

2.5.1. D1-like receptors

Both the D1-R and D5-R share pharmacological properties similar to those of the original pharmacologically defined D1-like receptor family, specifically, a high affinity for the benzazepine ligands SCH 23390, SCH 39166, and SKF 83566, which represent selective antagonists for these subtypes. LE 300 is also a potent and useful D1R antagonist due to its distinct structure from the benzazepines, although not as selective for D1R over D2R as the benzazepine antagonists (Zhang, Xiong, Zhen, & Zhang, 2009).

The first D1R agonist developed, SKF 38393, was crucial for discriminating between activation of D1-like and D2-like receptors (Setler, Sarau, Zirkle, & Saunders, 1978). However, it was later realized that the partial agonist nature of SKF 38393 produced an under-appreciation of the contribution of D1-like receptors to behavior. Currently, there are full and/or selective D1-like receptor agonists available such as A 77636, A 68930, SKF 81297, dihydrexidine, and doxanthrine (Strange & Neve, 2013).

While there are higher levels of D1-R in the typical dopamine-rich regions of brain, i.e. in the neostriatum, substantia nigra, nucleus accumbens, and olfactory

tubercle, D5-Rs distribution is much more restricted. This subtype is usually found at much lower levels like hippocampus, thalamus, lateral mamillary nucleus, striatum and cerebral cortex (Missale et al., 1998).

D1-like receptors stimulate adenylyl cyclase (AC) (Figure 7), with the D5-R showing some constitutive activity for this response. As receptors that stimulate AC, the D1R were assumed to couple to the adenylyl cyclase stimulatory G protein ($G\alpha_s$). $G\alpha_s$ bind primarily to the C2 cytosolic domain of AC, bringing the C1 and C2 domains together in a way that enhances the catalytic efficiency of the enzyme. AC catalyzes the conversion of adenosine triphosphate (ATP) to cAMP, which binds to the regulatory subunits of the PKA holoenzyme to disinhibit the catalytic subunits (Neve et al., 2004).

One alternative pathway for D1 signaling is phospholipase C-mediated mobilization of intracellular calcium. Phospholipase C activation and its response has also recently been associated with the D1/D2 receptor heterodimer (Strange and Neve 2013). Moreover, agonists that preferentially stimulate the cAMP response (SKF 83822) or the phospholipase C response (SKF 83959) associated with D1-like receptors have been described (Neve et al., 2004; Strange and Neve, 2013). D1-R plays a crucial role in important actions of DA to control movement, cognitive function as well as cardiovascular function (Zhang et al., 2009).

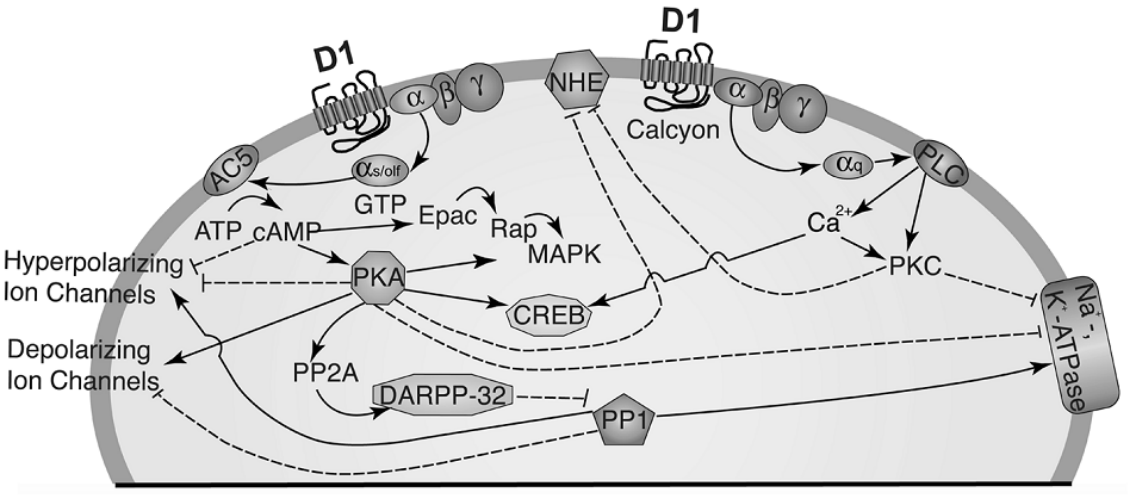

Figure 7: **D1-like receptor signaling pathways.** Stimulatory effects are indicated with a solid line ending in an arrowhead, and inhibitory effects with a dashed line ending in a bar. AC5, adenylate cyclase type 5; CREB, cyclic AMP response element binding protein; DARPP-32, dopamine-related phosphoprotein, 32 kDa; MAPK, mitogen-activated protein kinase; NHE, Na^+/H^+ exchanger; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PP1 or PP2A, protein phosphatase 1 or 2A. Based on Neve et al., 2004.

2.5.2. D2-like receptors

Overall, the D2, D3 and D4 receptors exhibit pharmacological properties similar to those of the originally defined D2 receptor. Thus, their high affinities for drugs such as butyrophenones (haloperidol, spiperone) and substituted benzamides (sulpiride, raclopride), provide selective antagonists for D2-like receptors over D1-like receptors (Kebabian, Tarazi, Kula, & Baldessarini, 1997). A higher affinity for the D2 receptor is shown by the majority of D2 antagonists, when compared with the D3 and D4 receptors (Strange and Neve, 2013). This is explained by D2-R being the predominant D2-like subtype in the brain and corresponding to the pharmacologically defined D2R for which these drugs were developed.

Selective agonists for the D2R relative to the D1R have been developed, specifically N-0437, PHNO and quinpirole. In addition, sumanirole is a full-efficacy agonist and highly selective for the D2 receptor over other DA-Rs (Kebabian et al. 1997; McCall et al., 2005; Strange and Neve, 2013).

High levels of D2-R are found in typical DA rich brain areas. D3 and D4 receptors are less abundant and in a more restricted distribution pattern, located mainly in limbic areas of the brain. Some D3-Rs are also sited in regions associated with motor function like the putamen (Chio et al., 1994). There is also *in vivo* evidence for a gender difference in dopamine D2-like receptor binding potentials in brain regions outside the striatum, particularly in the frontal cortex (Kaasinen et al., 2001; Beaulieu & Gainetdinov, 2011).

The D2-like receptor subtypes have each been shown to inhibit adenylyl cyclase (Figure 8) when expressed in recombinant cells. However, the signal via the D3-R has proven more difficult to demonstrate and is generally lower than for the other two subtypes, which may be associated with preferential coupling of the D3-R to specific adenylyl cyclase isoforms. Therefore, D2-like receptors will, when activated, stimulate a series of processes including acute signaling events (inhibition of adenylyl cyclase, stimulation of K^+ channels, inhibition of Ca^{2+} channels and stimulation of arachidonic acid release) and longer term events (MAP kinase and β-arrestin-2/Akt/GSK-3 signaling, and mitogenesis) (Beaulieu and Gainetdinov, 2011).

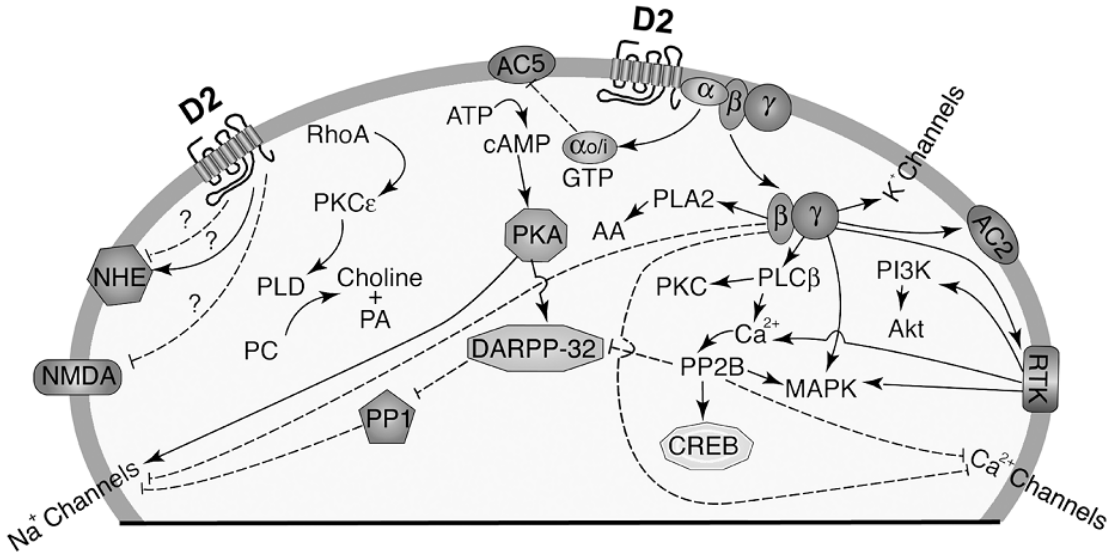

Figure 8: **D2-like receptor signaling pathways.** Stimulatory effects are indicated with a solid line ending in an arrowhead, and inhibitory effects with a dashed line ending with a bar. AA, arachidonic acid; AC2 or AC5, adenylate cyclase type 2 or 5; CREB, cyclic AMP response element-binding protein; DARPP-32, dopamine- and cyclic AMP-regulated phosphoprotein, 32 kDa; MAPK, mitogen-activated protein kinase; NHE, Na+/H+ exchanger; PA, phosphatidic acid; PC, phosphatidylcholine; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; PP1 or PP2A, protein phosphatase 1 or 2A; RTK, receptor tyrosine kinase. Based on Neve et al., 2004.

The D2-R has an important role mediating the effects of dopamine to control movement, certain aspects of behavior in the brain and prolactin secretion from the anterior pituitary gland. Even though D3 and D4 receptors functions are currently unknown, their localizations in limbic areas of brain suggest an involvement in cognitive, emotional, and behavioral function (Missale et al., 1998). Hence, D2R represent attractive potencial targets for selective antipsychotic drugs design. Most drugs used to treat schizophrenia (antipsychotics) and PD (Beaulieu and Gainetdinov, 2011) are reported to have high affinities for D2-like receptors, being selective D2R antagonists and agonists, respectively.

3. Noradrenergic neurotransmission

3.1. Noradrenaline (NA)

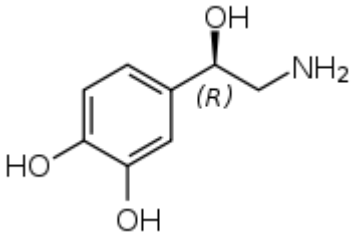

Figure 9: NA chemical structure

NA, adrenaline (AD) and DA belong to the catecholamine (CA) class of monoamines with NA and DA representing the two primary CAs in the mammalian brain (Bloom, 2010) . NA (Figure 9) as well as adrenaline have been established to be released by various neurons serving as a classical synaptic neurotransmitter in the CNS (Fuxe, 1965). A range of studies states that projections of NA neurons can also be non-synaptic, releasing it nonspecifically within areas of brain-tissues, where it exhibits a hormone-like behavior (Smeets & González, 2000). Overall, NA release is acknowledged to contribute to a variety of functions such as long-term synaptic plasticity, pain modulation, motor control, local blood flow, sleep wake cycles, arousal, task performance optimization and energy homeostasis (Aston-Jones and Cohen, 2005; Samuels and Szabadi, 2008a;b).

3.2. NA Biosynthesis

The neurotransmitter NA synthesis (Figure 10) includes three steps starting with the amino acid tyrosine, which in a first step is converted to l-DOPA by the TH, as aforesaid when DA synthesis was described. L-DOPA represents the direct precursor for DA, and DA is then converted into NA by the enzyme dopamine-β-hydroxylase (DβH). This is a Cu^{2+}-containing glycoprotein which needs O_2 and ascorbic acid for its catalytic activity. DβH exist in vesicles within chromaffin cells of the adrenal medulla as well as in transmitter storage vesicles in the terminals of neurons that release NA or adrenaline. There is no DβH in the cytoplasm of neurons or chromaffin cells, which

leads to the knowledge that NA synthesis follows DA uptake from the cytoplasm into the vesicles. Methylation by phenylethanolamine-N-methyltransferase (PNMT) finally converts NA to adrenaline, a process which predominantly takes place in the medullae of the adrenal glands. (Smeets and González, 2000). PNMT constitutes a cytoplasmic enzyme that requires the methyl donor, S-adenosylmethionine (SAM), as a cofactor.

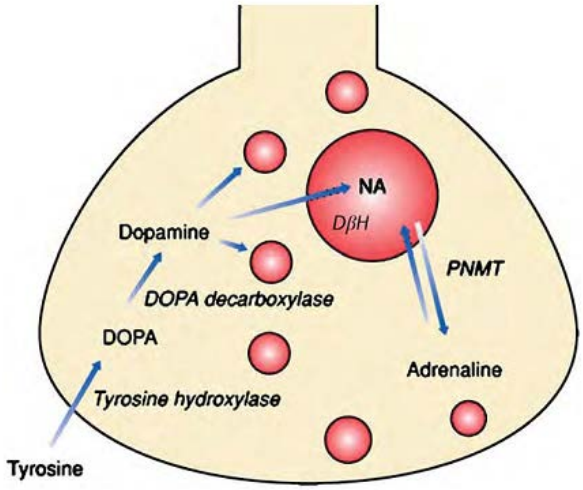

Figure 10: The synthetic pathway for NA and Adrenaline in neuron terminals and chromaffin cells. (adapted from Stanford, 2001)

Adrenaline can also be formed from NA by a less specific enzyme, N-methyl-transferase, which is prevalent in the bronchi, liver and kidney. However, whether neurons in these tissues actually synthesize and release adrenaline is still unclear (Stanford, 2001).

NA is found in the majority, but not all, postganglionic sympathetic neurons. Its concentration is the highest in these neurons terminals, where it is stored in membrane-bound vesicles of about 50–90 nm diameter. These vesicles constitute the location of the final step in NA synthesis, as they are assembled in the neuronal cell body and transported to the terminals, via the axons. NA release from the vesicles into the synapse involves a cascade of enzymatic reactions which leads to the docking and fusion of the vesicles with the neuronal membrane. Then, the formation of a pore and extrusion of neurotransmitter into the synapse occurs (Stanford, 2001).

Noradrenergic neurons cell bodies in the brain are found within seven distinct, bilateral clusters in the pons/medulla region of the brainstem. These groups of cell

bodies (known as nuclei A1–A7) are subdivided into the *locus coeruleus* system (A4 and A6) and the lateral tegmental nuclei (A1, A3, A5 and A7). Of all these nuclei, it was the A6 nucleus, in the pons, that has been more carefully studied because it is the source of over 40% of all the NA-releasing neurons in the brain. As a matter of fact, neurons from this nucleus innervate almost every region of the CNS. From the A5 nucleus are known to derive most of the NA-releasing neurons in the spinal cord. Even though the dorsomedial medullary A2 NA-containing neurons are sometimes included in the lateral tegmental group, it is also often regarded as a separate system within the *nucleus tractus solitarius* (Stanford, 2001).

Therefore, the distribution of NA and adrenaline in the brain and periphery suggests the fundamental role of these catecholamines in the regulation of the autonomic nervous system.

4. PD Animal Models

Since PD is exclusively human, it does not develop spontaneously in animals. Therefore, in order to observe and study this pathology in animals, there is the need to artificially induce the disease by administration of neurotoxic agents (Dauer and Przedborski, 2003). For that reason, various animal models have been developed which enable the study of the mechanism and the therapeutic intervention in PD, for instance MPTP, 6-hydroxydopamine, rotenone and paraquat administration (Dauer & Przedborski, 2003; Meredith et al., 2008; Newhouse et al., 2004; Przedborski et al., 2001).

Experimental models of PD attempt to artificially replicate in the animal, the nigrostriatal dopaminergic degeneration observed in PD, simulating the pathological, biochemical and histological changes of the disease as well as functional disturbances. The aims of these models are not only to assist in understanding the mechanisms that favor the onset of the degenerative process, but also to develop therapeutic strategies. They also serve as support for a better understanding of the anatomy and the functionality of the brain structures (Dauer & Przedborski, 2003).

4.1. MPTP Model

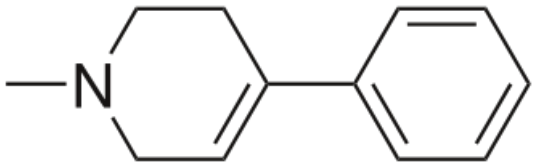

Figure 11: Chemical Structure of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine or MPTP

MPTP, the abbreviation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Figure 11) constitutes a by-product of the chemical synthesis of a meperidine analog with potent heroin-like effects. MPTP is also known for its capacity of inducing a parkinsonian syndrome in humans almost indistinguishable from PD (Langston & Irwin, 1986). Early in 1982 MPTP was recognized as a neurotoxin, when several young drug addicts mysteriously developed a profound parkinsonian syndrome after the intravenous use of street preparations of meperidine analogs which, unknown to anyone, were contaminated with MPTP (Langston and Ballard, 1983).

This neurotoxin is able to produce an irreversible and severe parkinsonian syndrome in humans and non-human primates, which replicates almost all of the symptoms of PD, including tremor, rigidity, slowness of movement, postural instability and even freezing. It is believed that while in PD the neurodegenerative process occurs over several years, after MPTP administration the most active phase of neurodegeneration is completed within a few days (Jackson-Lewis et al., 1995; Langston, 1987)

Neuropathological data shows that MPTP administration causes damage to the nigrostriatal dopaminergic pathway matching that one seen in PD (Agid and Ruberg 1987). Nevertheless, the MPTP model has, until now, been lacking two typical neuropathologic features of PD: firstly, according to most published reports, except for SNpc, pigmented nuclei such as *locus coeruleus* have been spared; secondly, Lewy bodies, so characteristic of PD, have not been compellingly observed in MPTP-induced parkinsonism so far (Forno et al., 1992). In MPTP-injected monkeys, intraneuronal inclusions reminiscent of LBs have been described though (Forno et al., 1986).

4.1.1. MPTP Metabolism

MPTP has a complex multistep metabolism (Figure 12) (Tipton & Singer, 1993). Due to being highly lipophilic, it freely and rapidly crosses the Blood brain barrier (BBB) and within a minute after MPTP injection, the toxin levels are detectable in the brain (Markey et al., 1984). Once in the brain, MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) inside non-dopaminergic cells. Then, $MPDP^+$ is oxidized to the active MPTP metabolite, MPP^+, which is subsequently released into the extracellular space, taken up by the dopamine transporter (DAT) and concentrated within dopaminergic neurons, where it exerts its toxic effects (Reith, 2001).

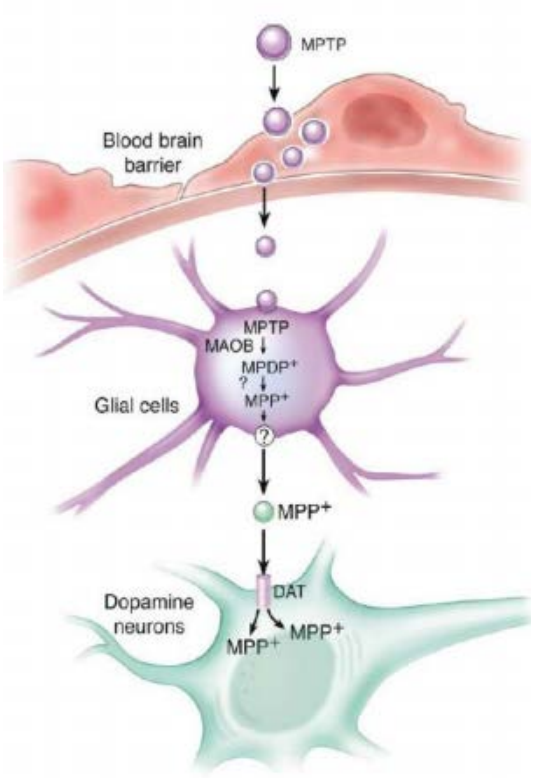

Figure 12: Schematic Representation of MPTP metabolism (adapted from Dauer and Przedborski, 2003)

The importance of these metabolic steps in MPTP-induced neurotoxicity and the fact that MPP^+ is the actual perpetrator are corroborated by the following remarks:

1. pretreatment with MAO-B inhibitors such as deprenyl prevents MPTP biotransformation to MPP^+, blocking dopaminergic toxicity (Heikkila et al., 1984; Markey et al., 1984);

2. pretreatment with DA uptake inhibitors (e.g. mazindol) prevents MPP^+ entry into dopaminergic neurons also blocking dopaminergic toxicity (Javitch et al., 1985), at least in mice;

3. striatal MPP^+ content is linearly correlated with dopaminergic toxicity in mice (Giovanni et al., 1991).

Inside dopaminergic neurons (Figure 13), MPP^+ can follow one of at least three routes:

1. it can bind to the vesicular monoamine transporter-2 (VMAT2), which translocates MPP^+ into synaptosomal vesicles (Liu et al, 1992);

2. it can be concentrated within the mitochondria by a mechanism that depends on the mitochondrial transmembrane potential (Ramsay & Singer, 1986);

3. it can remain in the cytosol and interact with cytosolic enzymes, especially those carrying negative charges (Klaidman et al., 1993).

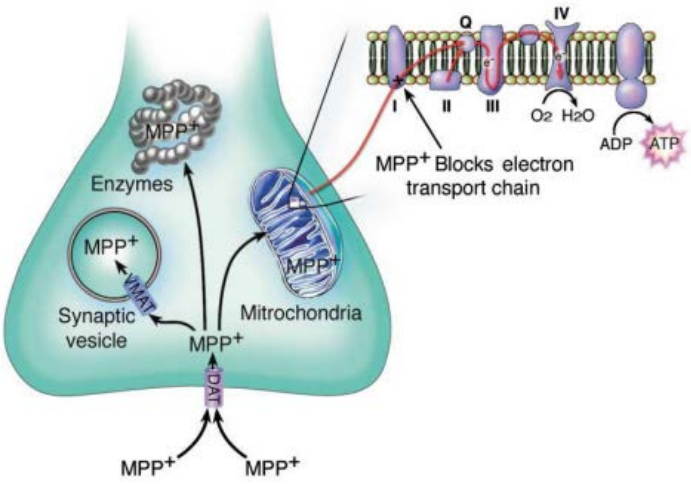

Figure 13: Schematic Representation of MPP^+ intracellular pathways (adapted from Dauer e Przedborski, 2003)

The sequestration of MPP^+ into synaptosomal vesicules seems to have a protective effect on cells from MPTP-induced neurodegeneration as it prevents the toxin from accessing mitochondria, its likely site of action.

Once inside the mitochondria, MPP^+ inhibits the multienzyme complex I of the mitochondrial electron transport chain and, consequently, impairs oxidative phosphorylation (Nicklas et al., 1985). This blockade rapidly leads to decreases in tissue

ATP content, particularly in the striatum and ventral midbrain (Chan et al., 1991; Fabre et al., 1999), the most sensitive brain regions to MPTP.

Oxidative stress may be another early effector of the complex I inhibition due to MPP^+. In fact, MPP^+ can stimulate the production of ROS, particularly superoxide, by hampering the flow of electrons through complex I (Hasegawa et al., 1997).

MPTP administration also induces accumulation and nitration of α-synuclein in the cytosol of SNpc dopaminergic neurons (Przedborski et al. 2000; Przedborski et al. 2001), and ablation of α-synuclein in mutant mice is established to prevent MPTP-induced dopaminergic neurodegeneration (Dauer et al., 2002). While whether α-synuclein plays any direct role in regulating programmed cell death (PCD) is unclear, the expression of mutant α-synuclein in cell cultures might promote apoptosis (Xu et al., 2002), and cytochrome c has been reported to stimulate *in vitro* aggregation of α-synuclein (Hashimoto et al., 1999). Together, these aspects lead to the knowledge that the activation of PCD is fundamental in MPTP toxicity. Furthermore, PCD alterations have been reported in PD postmortem samples that are of pathological significance and targeting specific PCD molecules are suggested to be a valuable neuroprotective strategy for the treatment of PD (Vila & Przedborski, 2003).

4.1.2. MPTP Administration

Currently, the most frequently used animals for MPTP studies are monkeys, mice and rats (Petzinger and Langston, 1998; Kopin and Markey, 1988; Giovanni et al., 1994 a; b). The administration of MPTP through numerous different routes, which use varied dosing regimens, has led to the development of several distinct models, each characterized by some unique behavioral and/ or biochemical features. These models were developed based on the concept of delivering MPTP in a way that creates both the most severe and stable form of SNpc damage, while avoiding undesirable consequences such as acute death, dehydration and malnutrition. Even though MPTP can be administrated through many different routes, including gavage and stereotaxic injection into the brain, the most frequent, reliable, and reproducible lesion is provided by its systemic administration, i.e. intravenous (i.v.), subcutaneous (s.c.), and intraperitoneal (i.p.) or intramuscular.

4.1.2.1 Intranasal MPTP Administration

The nasal cavity represents a relatively unexplored route of penetration of neurotoxins. However, several factors make this a promising alternative to MPTP administration: the nasal mucosa exhibits a large surface area; it has a porous endothelial membrane; olfactory receptors are directly exposed to environment chemicals; the distribution of olfactory receptors is contiguous with the CNS; high total blood flow; avoidance of first-pass metabolism; and a weak BBB (Dluzen & Kefalas, 1996; Prediger et al., 2006; Rojo et al., 2006).

Since the i.v., s.c. and i.p. injections of MPTP lead to degeneration of mesencephalic dopaminergic neurons (Ballard et al., 1985; Kim et al., 2005; Smeyne & Jackson-Lewis, 2005; Takahashi et al., 1997), the necessity for a credible alternative arises. Recently, intranasal (i.n.) administration of MPTP has been demonstrated to also trigger neurodegeneration in the SN, supporting the utilization of the nasal route by environmental neurotoxins to reach the basal ganglia and develop parkinsonism (Aguiar Jr et al., 2013; Prediger et al., 2006; Rojo et al., 2006).

In this context, a new experimental model of PD has been recently proposed consisting of a single i.n. administration of MPTP in rodents (Figure 14) (Prediger et al., 2011). Previous findings demonstrate that rats (Castro et al., 2012; Moreira et al., 2010; Prediger et al., 2006) and mice (Aguiar Jr et al., 2013; Prediger et al., 2010) infused with i.n. MPTP manifest impairments in olfactory, cognitive, emotional and motor functions comparable to those associated with the different stages of PD. Furthermore, such infusion causes time-dependent loss of TH in the olfactory bulb and SNpc of rats, leading to a significant dopamine depletion in different brain areas (Castro et al., 2012; Moreira et al., 2010; Prediger et al., 2006). Consequently, the i.n. MPTP administration has been recognized as an ecologically valid mean for assessing novel neuroprotective and palliative treatments for both the motor and non-motor symptoms of PD.

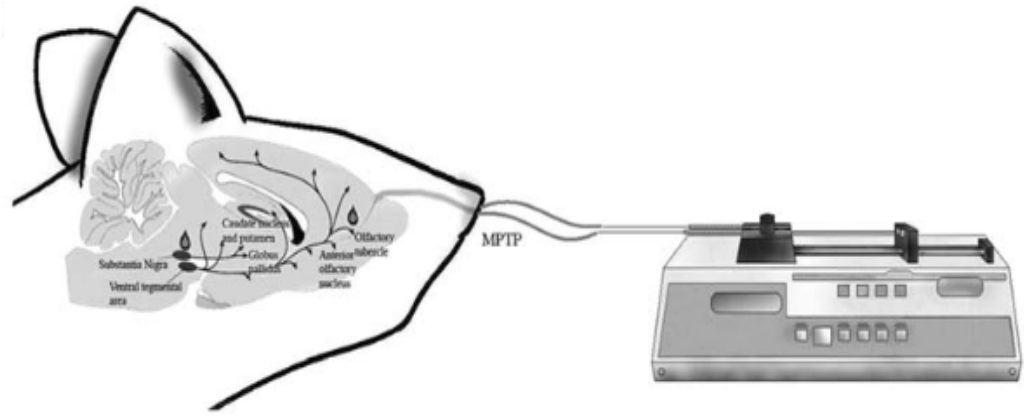

Figure 14: Schematic procedures of the intranasal administration of MPTP (adapted from Prediger et al.2010)

4.2. 6-OHDA Model

6-hydroxydopamine (6-OHDA) (Figure 15) corresponds to the first animal model of PD associated with SNpc dopaminergic neuronal death and was introduced more than 30 years ago (Ungerstedt and Arbuthnott, 1970).

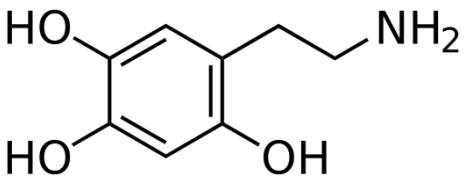

Figure 15: Chemical Structure of 6-OHDA

The 6-OHDA neurotoxin, is structurally similar to DA and NA, having a high affinity for the plasma membrane transporters of these catecholamines (Breese & Traylor, 1971). Once inside the neurons, it is rapidly oxidized, producing hydrogen peroxide and paraquinone, both highly toxic (Kelemen, 1978). Inside neurons, 6-OHDA accumulates in the cytosol, generating ROS and inactivating biological macromolecules by generating quinones that attack nucleophilic groups.

Given that 6-OHDA cannot cross the BBB, it must be administered by local stereotaxic injection into the SN, median forebrain bundle (MFB) or striatum in order to target the nigrostriatal dopaminergic pathway (Javoy et al., 1976; Jonsson, 1983). When administrated into SN or the MFB, dopaminergic neurons start degenerating within 24

hr dying without apoptotic morphology (Jeon et al., 1995). After injection into the striatum, though, 6-OHDA produces a more protracted retrograde degeneration of nigrostriatal neurons, which lasts between 1 and 3 weeks (Jackson-Lewis et al., 1995; Sauer & Oertel, 1994). However, none of the models of 6-OHDA intoxication have led to the formation of LB-like inclusions so far.

This neurotoxin may be created within the brain by a nonenzymatic reaction of DA, hydrogen peroxide, and free iron (Jellinger et al., 1994; Linert et al., 1996; Slivka & Cohen, 1985). Auto-oxidation of DA by nitrite ions or manganese can also generate 6-OHDA (Garner & Nachtman, 1989; Palumbo et al., 1999). Moreover, oxidative damage via hydrogen peroxide and derived $^{\bullet}OH$ are associated with the neurotoxic mechanism by 6-OHDA.

4.3. Paraquat Model

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) (Figure 16) is a potent, widely used herbicide and also an analogue of MPP^{+}, which is the active metabolite of MPTP, as described above. Since an epidemiological association has been found between its use in agriculture and incidence of PD, this compound may be involved in PD pathogenesis (Di Monte et al., 2002).

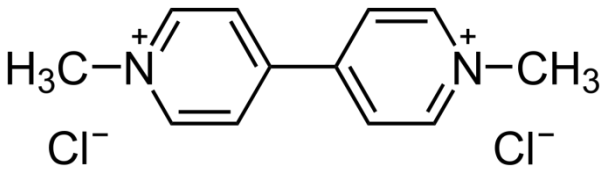

Figure 16: Chemical Structure of Paraquat

This compound is transported to the brain by a specific neutral amino acid transporter (K. Shimizu et al., 2001), where it reaches levels 50–200 times lower than in the kidney (Barlow et al., 2003). This leads to the suggestion that paraquat may not only produce toxicity in peripheral tissues but also in the CNS, which has been supported by in vitro studies that show that paraquat kills dopaminergic neurons in the rat organotypic midbrain culture (Shimizu et al,, 2003). In addition, *in vivo* studies have shown its moderate deteriorative influence on dopaminergic neurons and transmission in rodents (McCormack et al., 2002; K Shimizu et al., 2003; Thiruchelvam et al., 2003)

Paraquat does not easily penetrate the BBB (Shimizu et al., 2001), and its CNS distribution does not match any known enzymatic or neuroanatomic distribution (Widdowson et al. , 1996). Its toxicity seems to be mediated by the formation of superoxide radicals (Day et al., 1999). Systemic administration of paraquat to mice is known to cause SNpc dopaminergic neuron degeneration, which is accompanied by α-synuclein containing inclusions, as well as increases in α-synuclein immunostaining in frontal cortex (Manning-Bog et al., 2002).

Regardless the outcome of those researchers, the ability to induce dopaminergic neuronal loss and α-synuclein-positive inclusions in a reliable approach may demonstrate to be valuable for studies of the role of α-synuclein in neurodegeneration.

4.4. Rotenone Model

Rotenone (Figure 17) is the most potent member of the rotenoids, a family of natural cytotoxic compounds extracted from tropical plants. It is extensively used as an insecticide and fish poison. By being highly lipophilic, rotenone readily gains access to all organs (Talpade et al., 2000). Rotenone binds at the same site as MPP^+ and inhibits mitochondrial complex I.

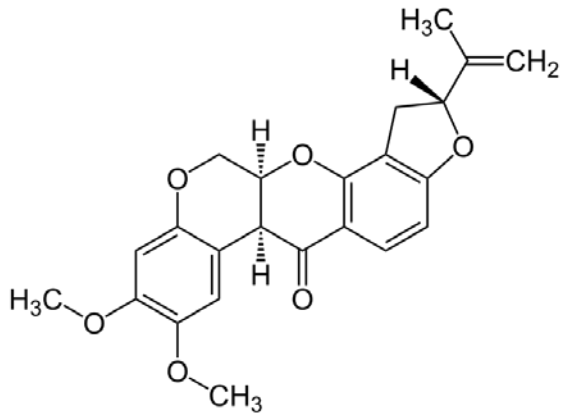

Figure 17: Chemical Structure of Rotenone

It is important to remark that dying DA neurons contain cytoplasmic inclusions, which like Lewy bodies, are immunopositive for α-synuclein and ubiquitin. There are also other pathological features present, including elevations in oxidative damage,

microgliosis and increased iron deposits. Behaviorally, the rats display prominent motor deficits (Fleming et al., 2004).

The rotenone model has clear advantages over more acute administration of other toxins, namely the progressive nature of degeneration and the presence of neuronal inclusions. Nevertheless, even with identical experimental conditions, rotenone causes either selective damage to DA neurons or more widespread cell loss (Betarbet et al., 2000; Sherer et al., 2003). Thus, while DA neurons may be most vulnerable to rotenone exposure, other unrelated populations can be also damaged.

Rats treated daily with a i.p. 2.75–3.0 mg/kg dose, display high variability of other symptoms of PD, including SN accumulation and aggregation of α-synuclein, microgliosis, iron accumulation, loss of enteric neurons and cardiac sympathetic denervation (Greenamyre et al, 2001).

Nevertheless, the characteristic of LB-associated dopaminergic neurodegeneration present in this model should enable performing a novel series of experiments in order to explore the correlation between aggregate formation and neuronal death.

5. The Gastrointestinal System

The Gastrointestinal (GI) System includes not only the GI tract, comprising mouth, pharynx, esophagus, stomach, small intestine and large intestine, but also the accessory organs such as salivary glands, liver, gallbladder and pancreas, that while not part of the tract, secrete substances into it via connecting ducts (Vander et al., 2003).

5.1. Structure of the GI Tract Wall

The GI tract wall includes specialized structures, which aim to increase the surface area available for absorption, namely villi and microvilli. The first are fingerlike projections extending from the luminal surface of the small intestine. The surface of each villus is covered with a layer of epithelial cells whose surface membranes form small projections called microvili. There are also four layers which compose the GI tract wall, namely mucosa, submucosa, muscularis externa and serosa (Figure 18). The combination of the epithelium, lamina propria, and muscularis mucosa forms a secretory and absorptive layer called mucosa. The epithelium consists of a simple layer

of exocrine cells that secrete mucus into the lumen of the tract and endocrine cells that release hormones into the blood. Just below the epithelium there is a layer of connective tissue, the lamina propria, through which pass small blood vessels, nerve fibers, and lymphatic ducts. The lamina propria is separated from underlying tissues by a thin layer of smooth muscle, the muscularis mucosa. Moreover, the submucosa, located beneath the mucosa, is a connective tissue layer containing a network of nerve cells, such as the submucous plexus, and blood and lymphatic vessels whose branches penetrate into both the overlying mucosa and the underlying layers of smooth muscle called the muscularis externa. The muscularis externa is responsible for the contractile activity of the GI tract wall and has two distinct layers: a relatively thick inner layer of circular muscle, whose fibers are oriented in a circular pattern around the tube such that contraction produces a narrowing of the lumen, and a thinner outer layer of longitudinal muscle, whose contraction shortens the tube. The myenteric plexus, located between these two muscle layers, is a second network of nerve cells, including neurons and ganglia to both sympathetic and parasympathetic nervous systems of the autonomic nervous system. Finally, surrounding the outer surface of the tube is a thin layer of connective tissue called serosa, supporting the GI tract in the abdominal cavity (Vander et al., 2001; Sherwood, 2010).

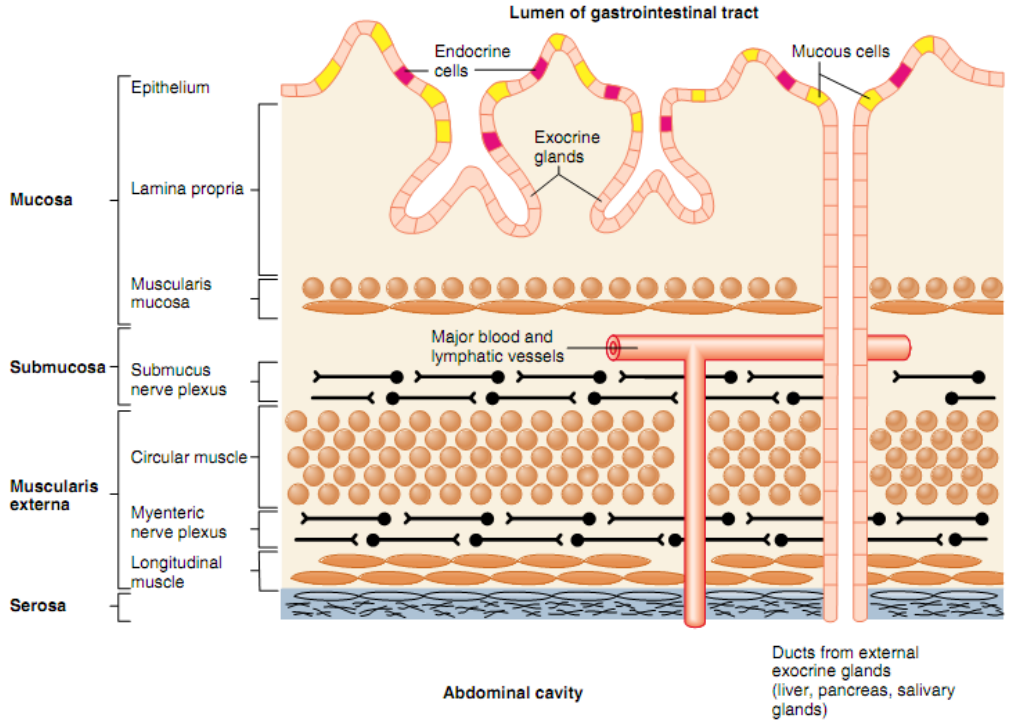

Figure 18: Structure of the gastrointestinal wall in longitudinal section. (withdrawn from Vander et al., 2003)

5.2. Intestinal smooth muscle contraction

Smooth muscle cells of the GI Tract Wall exhibit an elongated fusiform morphology, having only one nucleous and behaving as unitary types. In contrast to skeletal muscle cells, a single smooth muscle cell does not extend around the muscle length, typically being arranged in sheets. Each cell contains three types of filaments: the thick myosin filaments which are thicker in the skeletal muscle; fine filaments of actin, which contain tropomyosin, but not the regulatory protein troponin; and intermediate desmin filaments, which do not participate directly in the contraction, but are part of the cytoskeleton, allowing transmission of contractile force throughout the cell. These filaments intertwine with each other. (Olsson and Holmgren, 2001; Hansen, 2003). Smooth muscle does not have a striated appearance due to the fact that the filaments are not grouped in sarcomeres or organized into myofibrillas, contrary to skeletal muscle. Due to the lack of sarcomeres, smooth muscle has no Z-disks, but has dense bodies which contain the same protein found in Z-discs. These dense bodies are positioned along the smooth muscle cell and attached to the inner surface of the plasma membrane (sarcolemma). Thus, the actin filaments are anchored to the dense bodies which are distributed in the sarcoplasm and the sarcolemma and whose cohesion is maintained by the intermediate filament desmin (Figure 19). Besides the dense bodies, the sarcolemma includes caveolae, which are membrane invaginations that contain calcium deposits. Smooth muscle cells are attached through gap junctions and desmosomes. Gap junctions are especially present in the inner muscle layer and allow the flow of intracellular regulatory molecules and ionic currents involved in the generation of action potentials, thereby facilitating direct electric conduction between the various cells. On the other hand, desmosomes not only contribute to intercellular adhesion, but also allow the spread of the contractile force to the adjacent cells. Thus, the smooth muscle cells form an electrical syncytium that is innervated by hundreds of excitatory and inhibitory neurons, i.e. a connected group, mechanically and electrically functioning as a unit (Sherwood, 2010).

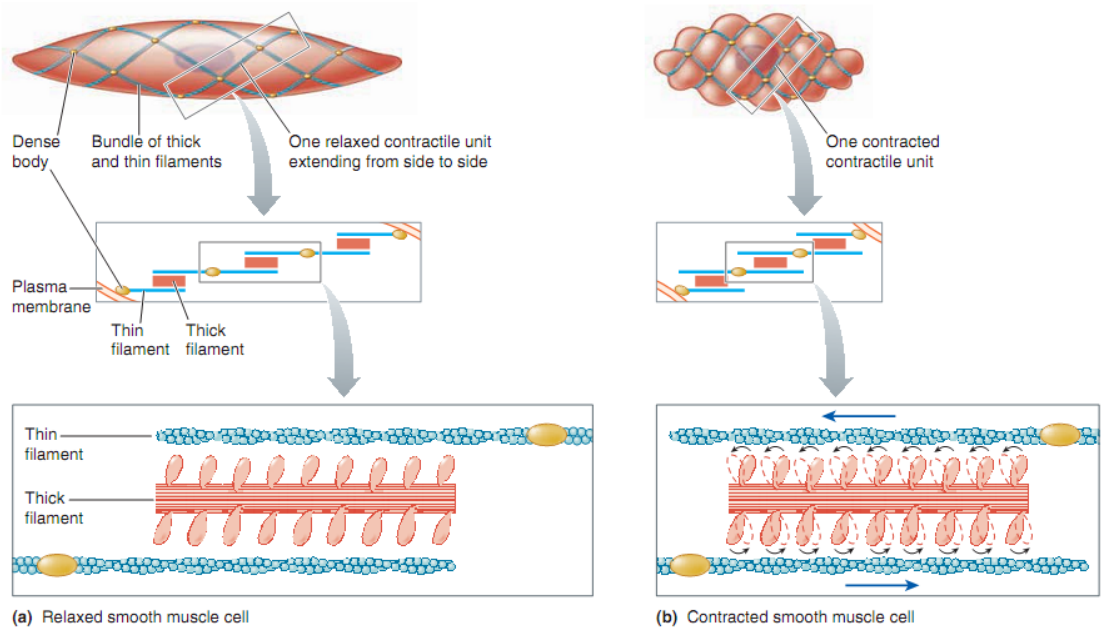

Figure 19: Arrangement of thick and thin filaments in a smooth muscle cell in relaxed (a) and contracted (b) states (Sherwood, 2008).

5.2.1. Smooth muscle contraction mechanism

At the origin of smooth muscle contraction process may be one of the following mechanisms: mechanical activation of myosin and actin; receptor activation through the action of hormones or neurotransmitters; change in membrane potential by the activation of ion channels or spreading action potentials; action of interstitial cells of Cajal (ICC). Contraction of smooth muscle is initiated by a Ca^{2+}-mediated change in the thick filaments. In response to specific stimuli in smooth muscle, the intracellular concentration of Ca^{2+} increases and this activator Ca^{2+} combines with the acidic protein calmodulin. This complex activates myosin light-chain kinase (MLC kinase) to phosphorylate the light chain of myosin. Cytosolic Ca^{2+} is increased due to the Ca^{2+} release from intracellular stores (sarcoplasmic reticulum) as well as entry from the extracellular space through Ca^{2+} channels (Sherwood, 2010; Webb et al., 2003). Agonists binding to serpentine receptors, coupled to a heterotrimeric G protein, stimulate phospholipase C activity. This enzyme is specific for the membrane lipid phosphatidylinositol 4,5-bisphosphate to catalyze the formation of two potent second messengers: inositol trisphosphate (IP3) and diacylglycerol (DAG). The binding of IP3 to receptors on the sarcoplasmic reticulum results in the release of Ca^{2+} into the cytosol. DAG, along with Ca^{2+}, activates protein kinase C (PKC), which phosphorylates specific

target proteins. There are several isozymes of PKC in smooth muscle, and each has a tissue-specific role (e.g., vascular, uterine, intestinal, etc.) (Figure 20).

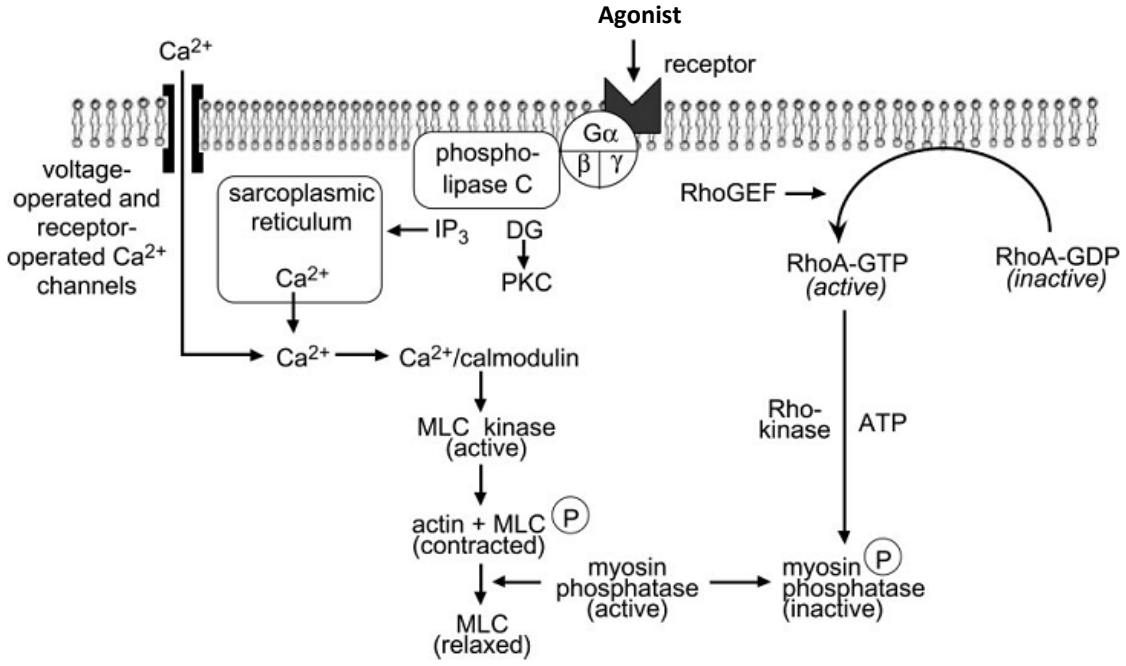

Figure 20: Regulation of smooth muscle contraction. (adapted from Webb, 2003)

In addition to the Ca^{2+}-dependent activation of MLC kinase, the state of myosin light chain phosphorylation is further regulated by myosin phosphatase (MLC phosphatase), which removes the high-energy phosphate from the light chain of myosin to promote smooth muscle relaxation. The myosin-binding subunit, when phosphorylated, inhibits the enzymatic activity of MLC phosphatase, allowing the light chain of myosin to remain phosphorylated, thereby promoting contraction.

5.2.2. Smooth muscle relaxation mechanism

Smooth muscle relaxation occurs either through the removal of the contractile stimulus or by the direct action of a substance which stimulates inhibition of the contractile mechanism. In any case, this process requires a decreased in intracellular Ca^{2+} concentration and increased MLC phosphatase activity (Figure 21).

Several mechanisms are implicated in the removal of cytosolic Ca^{2+} and involve both the sarcoplasmic reticulum and the plasma membrane. Ca^{2+} uptake into the sarcoplasmic reticulum is dependent on ATP hydrolysis. When phosphorylated, this sarcoplasmic reticular Ca,Mg-ATPase, binds two Ca^{2+} ions, which are subsequently

translocated to the luminal side of the sarcoplasmic reticulum and released. Mg^{2+} is required for the activity of the enzyme as it binds to the catalytic site of the ATPase to mediate the reaction. The plasma membrane also contains Ca,Mg-ATPases, which provides an additional mechanism for reducing the concentration of activator Ca^{2+} in the cell. This enzyme differs from the sarcoplasmic reticular protein as, by having an autoinhibitory domain that can be bound by calmodulin, it causes stimulation of the plasma membrane Ca^{2+} pump. Na^{+}/ Ca^{2+} exchangers are also located on the plasma membrane and aid in decreasing intracellular Ca^{2+}. Consequently, Ca^{2+} unbinds from calmodulin and MLC kinases remains inactive. In turn, the MLC phosphatase is activated, catalyzing the MLC dephosphorylation and promoting the smooth muscle relaxation(Webb et al., 2003; Sherwood, 2010).

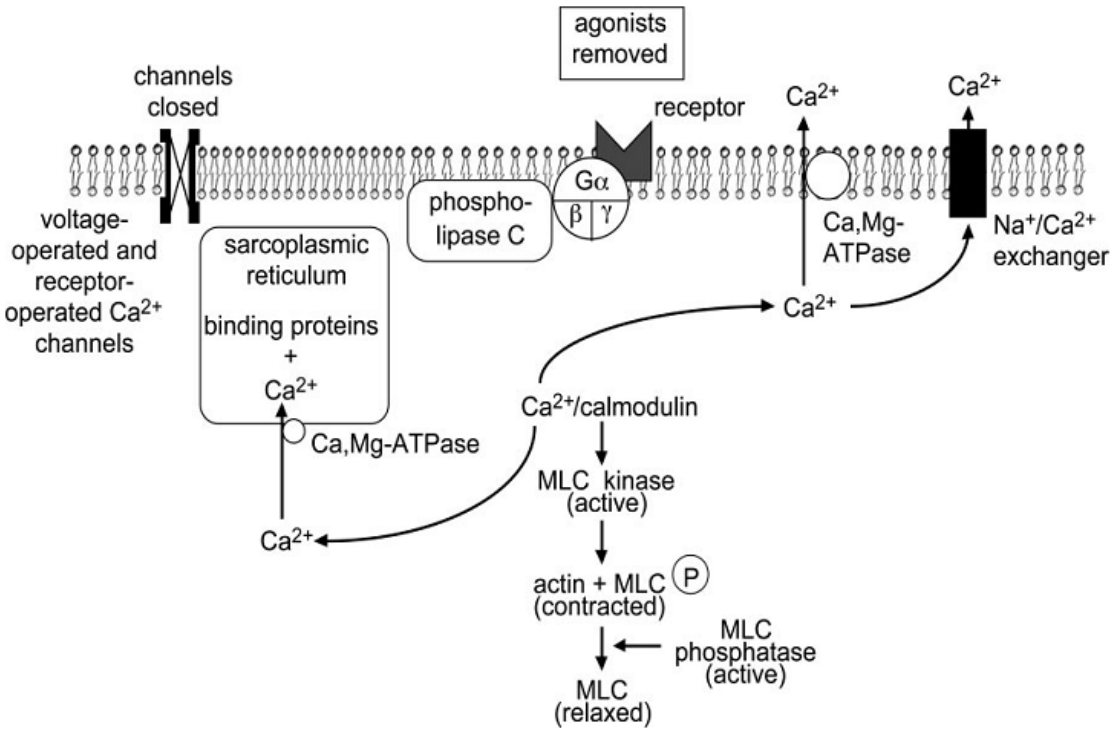

Figure 21: Regulation of smooth muscle relaxation. (Webb, 2003)

5.2.3. Phasic and Tonic contraction processes

Smooth muscle contraction can be classified in various ways, depending on the timing and means of increasing cytosolic Ca^{2+}: phasic or tonic (Hansen, 2003; Sherwood, 2010). Tone exists because this type of smooth muscle has a relatively low resting potential of -55 mV to -40 mV. Some surface-membrane voltage-gated

Ca^{2+}channels are open at these potentials. The resultant Ca^{2+} entry maintains a state of partial contraction. The rhythmic phasic contractions produce mixing and slow distal propulsion of luminal content (peristalsis). The maximal frequency and direction of propagation of these contractions are regulated by slow waves originated by the pacemaker activity of the ICC in the myenteric plexus and the inner layer of circular muscle. (Camborová, Hubka, Sulková, & Hulín, 2003; Hansen, 2003; Hirst & Edwards, 2004). These cells contact with each other and also with muscle cells and the nerve terminals, initiating rhythmic electrical activity, thereby facilitating the enhancement of current, i.e the slow wave (Hansen, 2003). Moreover, these pacemaker cells are spontaneously active at resting potential values, while the smooth muscle cells continue with the same potential values. Under experimental conditions, the influx currents of voltage-sensitive calcium channel in the ICC are activated at lower potentials (more negative) than the muscle cells channels. However, depolarization of the ICC has to occur, in order for an activation of pacemaker current, (Čamborová et al., 2003).

The basic electrical rhythms in the gut are fairly constant and characterized by above mentioned slow waves. A typical slow wave consists of the following sequence: rapid depolarization (upstroke), partial depolarization, a sustained plateau, and complete repolarization to the resting membrane potential. In most cases when threshold is reached, spike potentials occur and are superimposed on the slow waves plateau phase, underlying rhythmic phasic contractions (Camborová et al., 2003; Hansen, 2003; Huizinga & Lammers, 2009; Sherwood, 2010).

5.3. Regulation of gut motility

The control of motility and secretion in the gastrointestinal tract depends on extrinsic parasympathetic and sympathetic innervations and intrinsic innervation, provided by the enteric nervous system (ENS). Hormones are also involved, including secretin, insulin, epidermal growth factor (EGF), gastrin, tachykinin and somatostatin. Some of these peptides are released from the endocrine cells of the GI mucosa after a meal or in response to a mechanical stimulus/chemical content of GI.

The linking between CNS and GI tract is established by extrinsic autonomic innervation: the parasympathetic pathways favor the organ activity through the acetylcholine (ACh) action, while sympathetic pathways contribute to its reduction due

to the action of NA. Even though the ENS controls the motility and intestinal secretion in an independent way of extrinsic innervation (feature which earned it the epithet of mini-brain (Benarroch, 2007)), it has a modulatory role in ENS activity. The ENS controls motility and secretion via local reflexes which are triggered by diverse stimuli, including local distension of the intestinal wall, mucosal distortion and chemical content of the lumen. The circular and longitudinal muscle layer, secretory glands and the vasculature are considered effector systems in the ENS, reflecting the integrated neuronal activity of these systems. Enteric neurons are classified into different categories based on their functional, electrophysiological and histochemical properties. They include intrinsic primary afferent neurons (IPANs), interneurons, motor neurons, secretomotor neurons, and vasomotor neurons. The neurochemical signaling within the ENS is extremely complex. Although most ENS neurons use ACh, many also use nitric oxide (NO), vasoactive intestinal polypeptide (VIP), substance P, neuropeptide Y (NPY), in various combinations. There is also a small proportion of neurons that synthesize dopamine, serotonin or γ-aminobutyric acid (GABA). ATP can have both inhibitory and excitatory actions, depending on the location and type of receptors on which it operates. The IPANs, present in both the myenteric and submucosal plexuses, have long dendritic processes that ramify extensively and project both in oral and anal directions to make synapses with interneurons, motor neurons, and other sensory neurons within the ENS. Intestinofugal afferent neurones (IFANs), a subset of myenteric ganglion neurons, relay mechanosensory information to the sympathetic prevertebral and pelvic ganglia and mediate a peripheral reflex that inhibits gut activity.

The peristaltic reflex allows the normal propulsion of the contents of the gut and involves an ascending excitatory and a descending inhibitory component. The ascending excitatory reflex involves myenteric motor neurons that use ACh and substance P and induce contraction of the smooth muscle located orally to the site of stimulation. On the other hand, the descending inhibitory reflex involves inhibitory motor neurons that utilize NO, VIP, ATP, and NPY, in a variety of combinations, and elicit relaxation of the smooth muscle located anally to the site of stimulation. Thus, the peristaltic reflex is coordinated by the action of cholinergic interneurons that receive inputs from IPANs and project to either the excitatory or the inhibitory motoneurons. The excitatory and inhibitory influences of these motor neurons are relayed to the intestinal smooth muscle cells via the CIC. The ENS has neural mechanisms capable of controlling the activity of

the CIC and secretory cells that are located in the intestinal mucosa regulating secretion of fluid, electrolytes and rhythmicity. Although dopaminergic neurons constitute only a small proportion of ENS neurons, they may also be important in the modulation of gastrointestinal motility. Studies in knock-out mice reveal that these neurons inhibit intestinal motility, probably via presynaptic D2 receptors in cholinergic myenteric neurons, as D2 antagonists, such as metoclopramide or domperidone, facilitate gastrointestinal motility by enhancing ACh release. (Benarroch, 2007; Costa et al., 2000; Hirst & Edwards, 2004; Olsson & Holmgren, 2001).

5.4 Gastrointestinal Dysfunction in PD

There is an increasing recognition that gastrointestinal (GI) dysfunction occurs in PD (Cersosimo and Benarroch, 2012; Jost, 2010; Pfeiffer, 2011). Taking into account that all parts of the gastrointestinal tract can be affected and in some cases this might occur early in the disease course, the acknowledgement of these gastrointestinal complications may lead to earlier and potentially more effective diagnostic and therapeutic intervention.

In 1817, in the former description by James Parkinson it was already stated that GI dysfunction represented a common syndrome, which often preceded the onset of motor disabilities (Natale et al., 2010).

Besides the classic motor symptoms, the onset and progression of the disease is also linked to non-motor features, for instance, altered behavior, abnormal sleep-waking cycle, and pain as well as autonomic dysfunctions (Jost, 2010).

Whereas experimental modeling of PD pathology in the CNS has been extensively carried out, peripheral autonomic dysfunction has been neglected (Natale et al., 2010). However, the pathophysiology of GI dysfunction still remains unclear and even experimental models are still under development while an effective cure for PD is lacking.

Strong evidence indicates that autonomic involvement in PD actually starts in the periphery (Braak et al., 2003). Hence, both its early involvement and the accessibility of peripheral tissues make the autonomic nervous system an attractive target for detection of early biomarkers of the disease.

While the entire GI tract may be affected in PD, the most common symptom consists of constipation. GI dysfunction was hypothesized to depend on a degenerative

process involving the enteric nervous system (ENS), equivalently to what occurs in the CNS. Hence, the lack of knowledge on the physiological role of NA/DA in the gut makes it opportune to approach the GI dysfunctions in PD (Natale et al., 2010).

5.4.1. Pathophysiology of GI dysfunction in PD

While dopaminergic deficiency and nigrostriatal damage may be accountable for some aspects of PD, it is also quite evident that additional sites of involvement, both within the CNS and beyond it, also play significant roles. Additionally, the dorsal motor nucleus of the vagus (DMV), which provides parasympathetic innervation to much of the GI tract, is strongly involved in PD (Jellinger, 1987; Halhday et al., 1990). Actually, Braak and colleagues have recently verified that the DMV is one of the two earliest sites of pathology within the CNS in PD (Tredici et al., 2002) which can translate into GI dysfunction.

The knowledge that the pathology of PD is not exclusive to the CNS has also become very clear and the extensive involvement of the ENS is now assumed. For instance, LBs were first identified within the ENS in 1984 (Qualman et al., 1984) and afterward their presence within the ENS were confirmed by several researchers (Kupsky et al., 1987; Wakabayashi et al., 1988). Not a long ago, α-synuclein deposition within the ENS in the stomach has been suggested to be the earliest site of pathology in PD (Braak et al., 2006). Even if ENS involvement in PD indeed includes dopaminergic neurons (Singaram et al., 1995), other neuronal systems within the gut are also affected (Wakabayashi et al., 1990).

5.4.2. ENS and MPTP

In analogy to MPTP use in the nigrostriatal system, consequent GI malfunction can be systematically studied via this PD animal model. In this context, Anderson et al. have shown, by an *in vivo* evaluation of the effects of MPTP in the mouse colon, that 10 days after i.p. MPTP administration, there is a 40% reduction of TH-positive neurons (Anderson et al., 2007). Indirectly other authors using MPTP and alternative toxic agents (e.g. rotenone) in comparable animal models have suggested the same. For example, the loss of myenteric neurons was replicated in the rat following rotenone (Drolet et al., 2009).

MPTP decreases myenteric plexus DA levels and has distinct effects on GI motility such as an acute reversible inhibition of migrating motor complex (MMC) activity and slow wave frequency in the duodenum and jejunum (accompanied by intestinal hypothermia) and also a chronic disruption of the MMC in the proximal jejunum of rats (Eaker et al., 1987; Mangla et al.,1989).

Acute and chronic upper GI hypomotility observed in the rat model were thought to induce a delay in intestinal transit and believed to induce upper GI ulcerations, even in the presence of gastric hyposecretion (Szabo, 1990). However, Anderson et al. found similar gastric emptying patterns for both solids and liquids, before and after MPTP administration, and the small intestinal transit times were also identical.

These conflicting results confirm the necessity to clarify (Schemann and Neunlist, 2004):

- which characteristics of the ENS are conserved throughout different species;
- which are exclusive to humans ;
- which of these discrepancies result from the PD model used.

In addition, MPTP only induces alterations in dopaminergic transmission and associated GI function, thereby generating a model restricted to the isolated deficiency of DA in intestinal motility.

5.4.3. DA role in modulating GI function

It is well acknowledged that catecholamines modulate GI motility. Sympathetic nerves through NA inhibit ACh release from motor neurons (via α2-adrenoceptors) (Scheibner, Trendelenburg, Hein, Starke, & Blandizzi, 2002) and relax smooth muscle (Gershon, 1967). Nonetheless, the gut also contains DA, which has only recently been confirmed as an intrinsic neurotransmitter of the ENS (Li et al. 2004; Anlauf et al. 2003). While enteric dopaminergic neurons express TH and the DAT, they lack dopamine β-hydroxylase, the enzyme that converts DA to NA as mentioned above. Also, enteric dopaminergic neuros have been identified in mouse, guinea pig (Li et al., 2004) and human (Anlauf et al., 2003).

The role of DA in modulating bowel motility remains controversial and, thus, far from being clear. However, the morphology shows that MPTP administration leads to

neurodegeneration in the ENS, in both myenteric and submucosal plexus. This was evidenced by haematoxylin histochemistry of enteric neurons joined with TH immunostaining and the alterations were described as being more severe in the small intestine (Natale et al., 2010).

A reduction in the DAT immunoreactivity from MPTP-treated animals was also observed in mice, which suggests a specific involvement of dopaminergic neurons. Also, DA neurons are described as abundant in control mice, when compared to MPTP-treated mice, leading to the role of DA in GI function modulation. Analogously to the CNS, the physiology of DA-R stimulation in the gut is completely altered due to the loss of enteric DA neurons (Natale et al., 2010).

Furthermore, the effects of DA on mouse ileum contractility were pharmacologically analyzed in an attempt to characterize the receptor subtypes involved as well as the related transduction mechanism. This analysis indicated that DA endogenously released modulates small intestine contractility in mouse through interaction on its own receptors, in which D1-Rs play a crucial role as mediators of DA effects in this species ileum (Zizzo et al., 2010).

However, another study was carried out, in which the expression of all DA-R subtypes in adult and fetal mouse gut was analyzed. Its aim was to identify DA receptors, determine their location and oral–anal distribution and, also, to determine which receptors mediate actions of dopaminergic neurons. Since the D2 and D3 receptor subtypes were found in the ENS, the relevance of D2- and D3-mediated dopaminergic responses in enteric physiology was investigated by measuring GI motility in transgenic mice lacking one or both of these receptors. Hence, D2 receptors were suggested to inhibit intestinal motility and do so physiologically. The phenotype of mice that lack D2R implies that D2R constitute a key mediator of the effects of endogenous DA in the ENS. Therefore, it is proposed that endogenous DA exerts a net inhibitory effect on intestinal motility and does so primarily via enteric neuronal D2R (Zhi Shan Li et al., 2006).

Recently, after systematically studying the DA effects on the longitudinal smooth muscle of the rat small intestine, a striking regional dependence of DA leading to a contraction in the early phase of DA receptor-dependent response was described, which was largest in the duodenum, but smaller in the jejunum and ileum (Kirschstein et al., 2009).This not only confirmed previous results in the rat and guinea-pig jejunum and

ileum showing a DA-induced relaxation (Boselli et al. 1987; Lucchelli et al. 1986), but also led to a specific DA receptor-dependent contraction in the duodenum. After a pharmacological characterization of D1R and D2R with selective antagonists, both contraction and relaxation were evidenced, suggesting the involvement of both DA receptors (Kirschstein et al., 2009).

Consequently, the logic question would be: how can both dopamine D1-like and D2-like receptors activation induce smooth muscle contraction if they have opposite intracellular pathways? One hypothesis is that they act at different sites. Therefore, D1-like receptors are suggested to be active presynaptically, while D2-like receptors act postsynaptically. D1-like receptors (D1-R and D5-R) couple to G-proteins activating adenylyl cyclase and increasing intracellular 3'-5'-cyclic adenosine monophosphate (cAMP), followed by protein kinase A (PKA) activation. This would relax smooth muscle cells by phosphorylation of phospholamban which disinhibits the sarco(endo)plasmic reticulum Ca^{2+} ATPase. Instead of a relaxation, however, a contraction was observed. This could be explained by a presynaptic action of D1-like receptors, where cAMP-dependent pathways would stimulate transmitter release. In turn, D2-like receptors (D2, D3 and D4), via direct postsynaptic mechanisms, couple to the G-protein inhibiting adenylyl cyclase, exhibiting the opposite effects, which results in a contraction. (Kirschstein et al., 2009).

CHAPTER 2

Aims of this thesis

Aims of this Thesis

The general goal of this study is to evaluate the intestinal function and biochemical alterations before motor impairment occurs by using an intranasal MPTP rat PD model.

To accomplish the main purpose, specific approaches have been devised as follows:

- Evaluate possible DA's modifications on the isolated rat ileum function after a single i.n. MPTP administration by comparing to untreated control animals;
- To better understand and pharmacologically characterize the effect of MPTP in the mechanism of action of DA by studying the contractile response of isolated rat ileum to DA in the presence of a D2R antagonist;
- Evaluate the involvement of cholinergic mechanisms in the contractile response of isolated rat ileum to DA by using a non-selective muscarinic receptors antagonist;
- Assess the dopaminergic status in rat ileum, 7 days after i.n. MPTP or saline administration as a measure of evaluating neurotoxicity: measurement of total DA content (HPLC) and TH and D2R protein expression (WB);
- Perform immunohistochemical studies on isolated rat ileum in order to analyze the immunostaining of protein S100β (enteroglial intestinal inflammation marker) of both saline and MPTP-treated animals;
- Characterize the cellular location of D2R in the isolated rat ileum and assess possible alterations in its immunostaining 7 days after i.n. MPTP administration compared to untreared animals.

CHAPTER 3

Material and Methods

1. Animal model and groups

In this study, male Wistar rats with 11 weeks age, obtained from Charles River Laboratories (Barcelona, Spain), were used. Rats were housed under controlled temperature (22±1ºC) and relative humidity (50-60%) and a 12-h light-12-h dark cycle. The animals were housed in pairs and fed *ad libitum* with distilled water and rodent maintenance chow (A-04 Panlab, Barcelona, Spain) containing 15.4% of protein and 2.9% of lipids. All procedures involving animals were performed according to the National and European Communities Council Directives on Animal Care.

At 21 weeks of age, rats were divided into two groups (n=6 rats per group) and were submitted to the i.n. MPTP protocol as described by Prediger (Prediger et al., 2009) or administered with i.n 0.9% NaCl (saline) (Table II). Thus, rats from the MPTP group were lightly anaesthetized and a 10-mm piece of PE-50 tubing was inserted through the nostrils. The tubing was connected to a peristaltic pump set at a flow rate of 12.5 µl/min. The MPTP HCl (2 mg/ml)) was infused for 4 min (0.1 mg/nostril). To regain normal respiratory function animals were given a 1-min interval and then the procedure was repeated with infusions administered through the contralateral nostrils. Animals remained in the procedure room for 24 h under surveillance before being replaced in the animal room. Rats were kept under controlled environmental conditions with no food or water restrictions after that. Technical MPTP safety guidelines were implemented.

Table II. Groups of animals used in the experimental work.

Group	**Age**
Saline: 0.9% NaCl	21 Weeks
MPTP: i.n. administration	21 Weeks

1.1. Sample collection

Animals were previously anaesthetized and then sacrificed by decapitation 7 days following the MPTP administration. The intestinal portions which correspond to the ileum were isolated and immediately immersed in a cold, carbogen aerated (95% O2/5% CO2) Krebs–Henseleit solution of the following composition (mmol/L): NaCl

118.67; KCl 5.36; MgSO4.7H2O 0.57; CaCl2 1.90; KH2PO4.2H2O 0.90; NaHCO3 25.0; glucose 11.1; pH 7.4.

The samples were washed and the intestinal contents as well as the portions of the adjacent mesentery were removed. The ileum was then divided into several segments of approximately 15 mm to be immediately used in functional studies; others were fixed in formaldehyde, buffered to pH 6.9, and conventionally processed to the techniques of histology and immunohistochemistry; others were frozen at -80°C for subsequent lysis and homogenization.

2. Functional Studies

2.1. Organ isolation and assembly

Ileum segments were suspended on stainless steel hooks under a basal tension of 29.6 mN (Santos, 2005) in 15 mL organ baths filled with Krebs–Henseleit solution aerated with 5% CO2–95% O2 and maintained at 37 °C (Figure 22). The optimal basal tension was determined in previously performed experiments in which the contractile response of the organ to 100 μM ACh was studied under different tensions. The basal tension in each experiment was that which occurred spontaneously in tissue submitted to the optimal tension, after the equilibrium state had been reached, following the organ assembly. Quiescent tissues with little or no spontaneous activity were used.

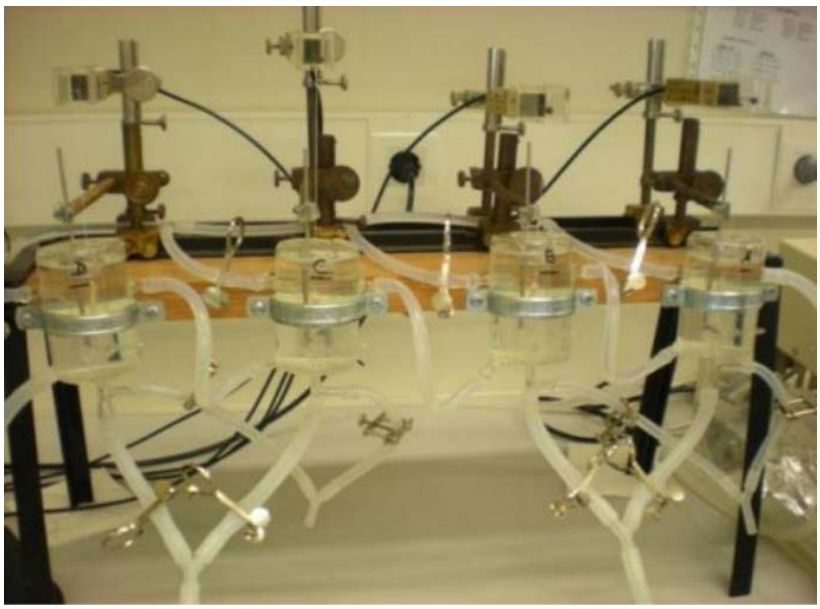

Figure 22: **Ileum segments assembled in 15 ml organ baths filled with Krebs–Henseleit**.

2.2. Experimental protocol

Following a 2-hour equilibration period with periodic washings, tonic isometric contractions were recorded with a Letica Scientific Instruments isometric transducer connected to a four channel polygraph (Polygraph 4006; Panlab, Letica Scientific Instruments, Barcelona, Spain). Firstly, all intestinal segments were submitted to 100 μM of exogenous ACh in order to directly induce the maximum contraction of ileum smooth muscle, which enables the comparison of the different experiments results. Cumulative concentration–response (CR) curves for DA (0.01-90μM) were then performed (Figure 23) in the absence and the presence of 10 μM sulpiride, a selective antagonist at dopamine D2 and D3 receptors, added to the organ bath 30 minutes before the DA CR curve. Also, cumulative CR curves for DA in the absence and in the presence of 1 μM atropine, a competitive non selective antagonist at the muscarinic acetylcholine receptors, were performed in a saline group of animals.

In all experiments control segments were used to which only the appropriate solvent to the antagonist was added.

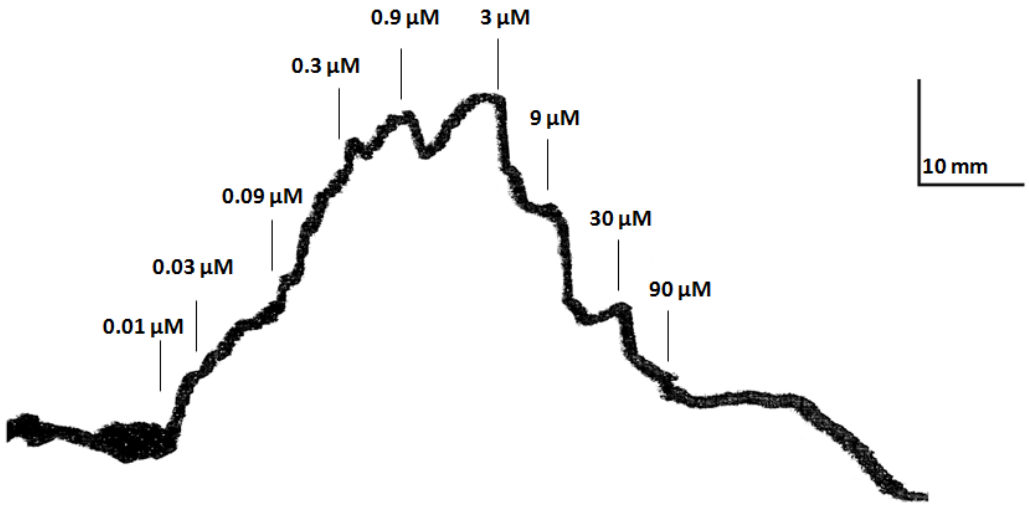

Figure 23: **Example of a cumulative concentration–response (CR) curve for DA**. 10 mm = 3,9 mN, 40 s.

2.3. Data processing and statistical analysis

The final results of each experiment were expressed as a percentage of the maximum response obtained for Ach. Differences between the contractile responses induced by each concentration of the CR curves for the agonist, performed in the absence (control) or in the presence of the antagonist were evaluated by *t*-test for unpaired data. Values of $P < 0.05$ were considered to indicate significant differences.

All results are presented as mean ± standard error of mean (SEM) of the number of experiments (n) indicated. All statistical analyzes were performed using IBM SPSS Statistics software.

3. HPLC analysis of dopamine content

The dopaminergic status was accessed by measuring total DA content of ileum segments of both animal groups. For that purpose, we resorted to a high pressure liquid chromatography method (HPLC) with reverse phase and electrochemical detection (amperometric) (Morgadinho et al, 2004). The equipment needed included a Gilson pump (model 307) a Gilson auto-injector (Model 234; 50µl loop), a Gilson electrochemical detector (model 142) and UniPoint software v5.11 (Figure 24).

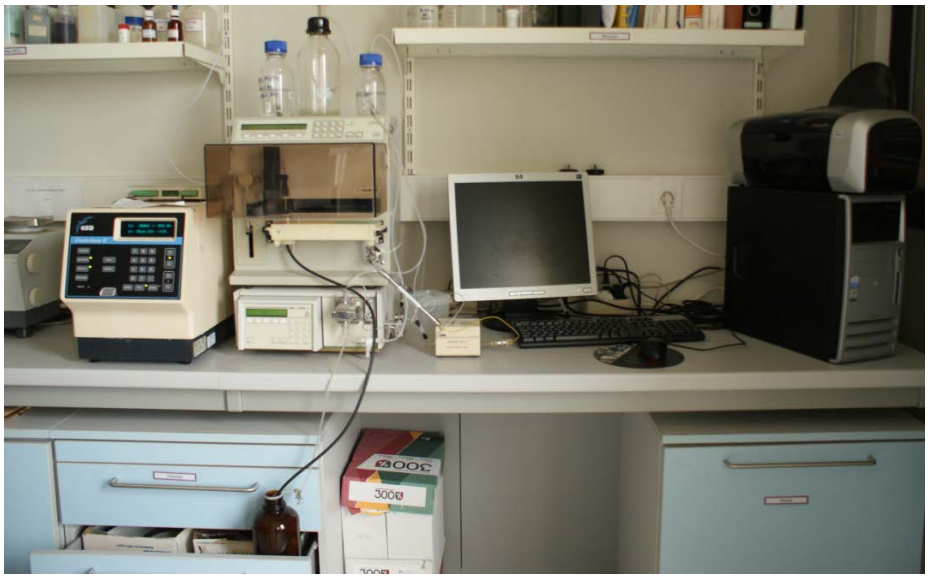

Figure 24: HPLC system used in this study for the quantification of monoamines.

3.1. Sample preparation for the DA levels determination by HPLC

Ileum sections were cut into small pieces and homogenized by mechanical dissociation using a Potter-Elvehjem, at 4°C, in 500 µL of perchloric acid ($HClO_4$) 0.2 M, followed by homogenization with the aid of a sonicator, wherewith 3 pulses of 10 seconds for each sample were carried out. Subsequently, the samples were centrifuged at 13,000 rpm (15,493xg), for 7 min, at 4°C. The supernatant was removed to tubes containing a microfilter 0.22 µM Nylon (SPIN-X® Centrifuge Tube Filter, Costar) and the samples centrifuged again at 13,000 rpm for 4 min, at 4°C. The samples were frozen at -80°C for later analysis by HPLC.

To the obtained pellet 500 µL of 1 M sodium hydroxide (NaOH) were added followed by the homogenization of the samples with a sonicator as previously described. Samples were centrifuged at 13,000 rpm for 15 min and at 4°C. The supernatant was removed from each sample into new tubes and frozen at -80 ° C for later protein quantification.

3.2. Chromatographic Conditions

For the separation and quantification of DA levels it was used a Waters 2 Spherisorb® ODS column (4.6 x 250 mm; particle size: 5 µm). The mobile phase, filtered and degassed, consisted of sodium acetate trihydrate (0.1 M), citric acid monohydrate (0.1M), sodium octyl sulfate (0.5 mM), EDTA (0.15 mM) triethylamine (1 mM) and methanol (10%) (pH 3.8). The flow rate was 0.3 ml/minute and the sensitivity was maintained at 100 nA/V. The retention time obtained to DA was 16.20 ± 0.05 minutes. The concentration of DA in each sample was calculated having the standard curves of DA as reference (Figure 25). The results were expressed in ng/mg protein. Differences between saline and MPTP rats ileum DA levels were evaluated by *t*-test for unpaired data. Results were statistically analyzed using IBM SPSS Statistics software and expressed as mean ± standard errors of the mean (SEM) of the number of experiments (n) indicated.

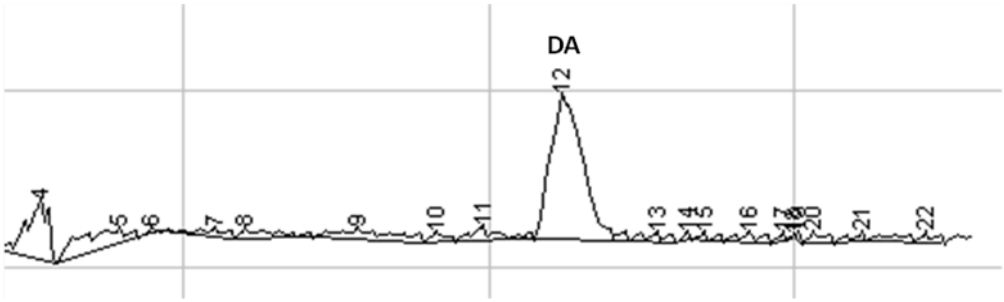

Figure 25: Representative chromatogram of DA 25 ng/ml obtained at a flow rate of 0.3 ml/minute and sensitivity at 100 nA/V.

3.3. Determination of total concentration of protein using BCA Assay

The quantification of total protein was obtained by using the bicinchoninic acid Assay (bicinchoninic acid - BCA) (Pierce™ BCA Protein Assay Kit, Pierce Biotechnology, Rockfor, IL, USA), a simple, fast and accurate assay for colorimetric quantification of total proteins. This method is based on the ability of proteins to reduce the Cu^{2+} to Cu^{+} under alkaline conditions. The resulting purple color of the chemical reaction is the consequence of interaction between the BCA and Cu^{+}. This water-soluble complex strongly absorbs light at a wavelength of 562 nm and the absorbance measurements may occur between 540 and 590nm. One of the fundamental characteristics of this assay is the linearity of the results, which enables the determination of total concentration of proteins for an enlarged scale (20-2000 µg/ml). The absorbance was measured at 570 nm using the Gen5 Data Analysis software.

For this quantitative analysis, 1:32 diluted samples were used. For the standard curve, 6 dilutions of bovine serum albumin (BSA) were prepared and also a standard blank (no protein). The two reagents A and B used were mixed in a 50 A:1 B ratio and applied to each sample (including standards). Subsequently, all samples were incubated at 37°C for 30 minutes. The absorbance was measured at 570 nm using the Gen5 Data Analysis software. Duplicates of all samples and standards were prepared.

4. Protein detection by Western blotting

4.1. Protein extraction from rat ileum segments

In order to evaluate the presence of TH (60 kDa) and D2R (48/51 kDa) in the ileum of saline and MPTP-treated animals, proteins from lysates of ileum segments were analyzed by Western blotting.

Ileum sections were cut into small pieces and homogenized by mechanical dissociation using a Potter-Elvehjem, at 4°C, in 300µl of RIPA lysis buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 5 mM ethylene glycol tetraacetic acid (EGTA), 1% (v/v) Triton X-100 (Tx-100), 0.5% (m/v) sodium deoxycholate (DOC) and 0.1% (m/v) sodium dodecyl sulfate (SDS), supplemented with 1x protease inhibitor cocktail (Roche, Indianapolis, IN, USA)). After incubation on ice for 1h, the lysates were sonicated and then centrifuged at 13000 rpm, for 15 min, at 4°C.

After centrifugation, the resulting supernatant fraction (corresponding to total extract) was collected and protein concentration was determined using the BCA assay (Pierce™ BCA Protein Assay Kit, Pierce Biotechnology, Rockfor, IL, USA). Samples were then denatured with 2x Laemmli buffer (0.25 M Tris-HCl (pH 6.8), 20% (m/v) glycerol, 4% (m/v) SDS, 200 mM DL-dithiothreitol (DTT) and bromophenol blue) for 30 minutes at 70°C.

4.2. Polyacrylamide gel electrophoresis and immunodetection

For the western blot analysis, 80 µg of protein were loaded per lane and separated by electrophoresis on SDS-10% polyacrylamide gel in buffer 100 mM Tris-HCl (pH 8.0-8.5) containing 100 mM bicine and 0.1% (m/v) SDS, at 130 V, for 70 minutes. After electrophoresis, proteins were electro-transferred to polyvinylidene difluoride PVDF (Immobilon®-P PVDF transfer membranes 0,2 µm, Millipore) membranes. Western blot transfer was performed using 12,5 mM Tris-HCl (pH 8.0-8.5) containing 96 mM glycine and 20% (v/v) methanol, for 210 minutes at 350 A, at 4°C. After transfer, membranes were blocked with 5% (m/v) non-fat milk in Tris-buffered saline (TBS: 20 mM Tris (pH 7.6) and 150 mM NaCl, containing 0.1% (v/v) Tween-20 (TBS-T)), for 1h with agitation, at room temperature. Membranes were incubated with

primary antibodies (Table II), diluted in TBS-T supplemented with 5% non-fat milk, overnight at 4°C.

Membranes were washed six times with TBS-T for 30 min and then incubated with adequate secondary antibodies (Table III) with agitation for 1h, at room temperature. After secondary antibody incubation, membranes were washed again for 30 min with TBS-T and the intensity of the bands was detected by a chemifluorescence enhancer substrate (ECF) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in the Fluorescent Image Analyzer FLA 900 Typhoon detector (GE Healthcare Bio-Sciences).

To confirm equal protein loading and sample transfer, membranes were reprobed with mouse anti-β-actin or mouse anti-GAPDH antibodies. The optical density of the bands was quantified by densitometry, using the Image Quant 5.0 software, and results were normalized on the basis of the results of the corresponding loading protein, actin or GAPDH.

Once again differences between saline and MPTP rats ileum TH or D2R levels were evaluated by *t*-test for unpaired data. Results were statistically analyzed using IBM SPSS Statistics software and expressed as mean ± standard errors of the mean (SEM) of the number of experiments (n) indicated.

Table III: Primary and secondary antibodies used for Western Blotting analysis.

Antibody	**Dilution**	**Company**
Mouse anti-TH	1:1000	Millipore (MAB318)
Mouse anti-D2R	1:200	Santa Cruz Biotechnology, Inc. (sc-5303)
Mouse anti-GAPDH	1:1000	Millipore (MAB374)
Mouse anti-β-Actin	1:1000	Sigma-Aldrich (A2228)
HRP secondary anti-mouse	1:5000	Sigma-Aldrich (B3582)

5. Immunohistochemistry (IHC)

In order to evaluate possible signs of inflammation in saline or MPTP rats ileum segments histochemical (hematoxylin/eosin - HE) and immunohistochemical (IHC) studies were performed, using a marker of enteroglial-sustained intestinal inflammation - the S100β protein. The S100β protein, belonging to the S100 superfamily of proteins

that bind Ca^{2+}-Zn^{2+}, is a homodimer of β subunit, which in the brain, at nanomolar concentrations, promotes neuronal survival and proliferation of astrocytes. There is also increasing evidence that S100β acts as a cytokine or damage-associated molecular pattern protein not only in inflammatory but also in neurodegenerative diseases. In the human intestine this protein is expressed by enteric glial cells. Recent data show the correlation between overexpression and release of S100β with intestinal inflammatory conditions. This protein is considered an easily diffusible pro-inflammatory cytokine which gains access to the extracellular space especially at immune-inflammatory reaction sites in the gut (Cirillo et al., 2011).

The ileum segments were isolated and fixed in formalin buffered at pH 6.9 and conventionally processed, with subsequent paraffin inclusion. Histological sections with 3 μm thickness were cut and initially subjected to HE staining.

Concerning the immunohistochemical studies, sections were, firstly, dewaxed, rehydrated (xylene, 100% ethanol and 95% ethanol) and blued in running water. Subsequently, epitope retrieval was performed via microwaving the slides in sodium citrate buffer 10% (v/v) (Leica Microsystems), pH 6, for 20 minutes at 480 Watts. As soon as the buffer cooled, and after washing with phosphate buffered saline (PBS, LabVision), endogenous peroxidase was inhibited by incubation of the sections with 3% H_2O_2 for 5 minutes at room temperature. The sections were then incubated with the primary mouse monoclonal antibody anti-S100β (Millipore Corporation, Billerica, MA, USA) at a 1:250 dilution for 30 minutes, followed by the post-primary, which activates the polymer penetration and contains animal serum 10% (v/v) in buffered serum 0.09%TBS/ProClin ™ 950, for 8 minutes. Incubation with the polymer linked to the peroxidase (Novolink ™ Polymer, HRP Poli-IgG of anti-mouse/rabbit at 8μ/ml, containing animal serum 10% (v/v) in TBS/ProClin ™ 950 -0,09%) was held at room temperature in a humid chamber and lasted 8 minutes, according to the manufacturer's recommendations. After washing with PBS the sections were revealed by using a 1.74% diaminobenzidine (DAB) solution, in a stabilizing solution for 10 minutes. Finally, the slides were contrasted with 0.02% hematoxylin for 5 minutes, followed by diaphanization, dehydration and mounting in a suitable synthetic medium.

Each structure in each ileal section representative of the two animal groups was A relatively compared according to the intensity of immunostaining.

Additionally, a characterization of D2R cellular localization was considered a useful complement to the other techniques used in this research. In order to achieve this, fully automated IHCs were performed, using Leica BOND-MAX (Leica Biosystems Nussloch, Germany). Briefly, there was an antigenic retrieval in sodium citrate buffer for 20 minutes, followed by a peroxide block for 5 minutes. Then the sample were incubated with the primary mouse monoclonal antibody anti-D2R (Santa Cruz Biotechnology, Inc., CA, USA) at a 1:50 dilution for 30 minutes, followed by the post-primary for 8 minutes, polymer also for 8 minutes, a mix DAB refine for 10 minutes and hematoxylin for 5 minutes. Finally, diaphanization, dehydration and mounting in a suitable synthetic medium were performed.

6. Reagents and drugs used

All reagents used in the preparation of physiological Krebs-Henseleit solution and buffer solutions for protein extraction and western blot analysis were purchased from Panreac (Barcelona, Spain) and were pro analysi products. The following drugs were used for functional studies: dopamine hydrochloride, acetylcholine chloride, sulpiride and atropine (Sigma-Aldrich, St. Louis, USA). The solutions were prepared with the solvents listed by the manufacturers, and in particular dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA).

CHAPTER 4

Results

1. Contractile response to Dopamine in the Wistar rat ileum

1.1. Influence of MPTP treatment on the concentration–response curve for Dopamine

In the gut, DA is released by enteric neurons and modulates motility of small intestine smooth muscle cells. Thus, a systematically analysis of the dopamine-induced effects on the isolated rat ileum was carried out.

In order to pharmacologically characterize the influence of MPTP treatment on the contractile response to DA in the Wistar rat ileum, cumulative concentration-response curves for DA (0.01-90μM) in both the presence and the absence of the referred treatment were performed.

Firstly, all intestinal segments were submitted to 100 μM of exogenous ACh in order to directly induce the maximum contraction of ileum smooth muscle. The analysis of Figure 26 shows that even if there is a slightly tendency to an increased contractile response to ACh after MPTP treatment, no statistically significant changes were observed between both groups, therefore enabling the comparison of the different experiments results, that were traduced in percentage of 100 μM Ach induced maximum contraction.

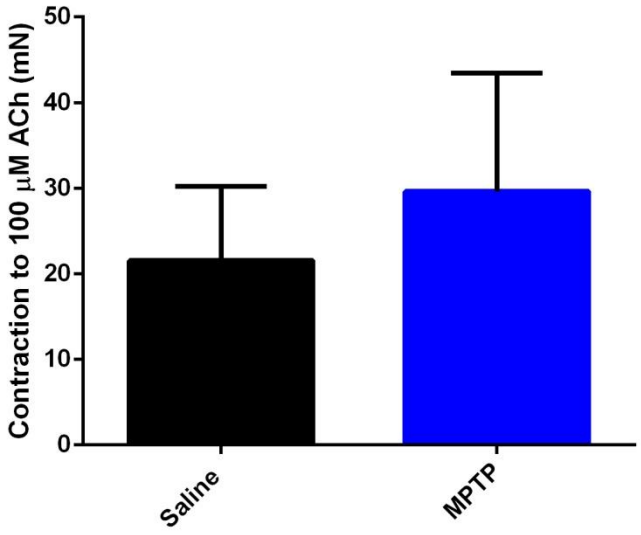

Figure 26: Contractile response to ACh of both Saline (●) and MPTP-treated (●) Wistar rats (n=16 per group); the results are expressed as average values ± S.E.M; vertical bars represent S.E.M. The significance of the statistical differences was analyzed by Student's *t* test.

Functional studies showed biphasic CR curves for DA, tested in concentrations of 0.01 to 90 µM, on both saline and MPTP-treated rats. Consequently, there was a clear evidence of contraction for lower DA concentrations (0.01 to 0.9 µM) and relaxation for higher concentrations (3 to 90 µM), demonstrating a contractile concentration-dependent effect on the isolated rat ileum (Figure 27).

In addition, MPTP imposed a statistically significant decrease on the maximum contractile effect of the DA CR curves, as well as a significant reduction on the maximum relaxation, compared to saline, confirming DA's modifications on the rat ileum function, seven days after a single intranasal MPTP administration.

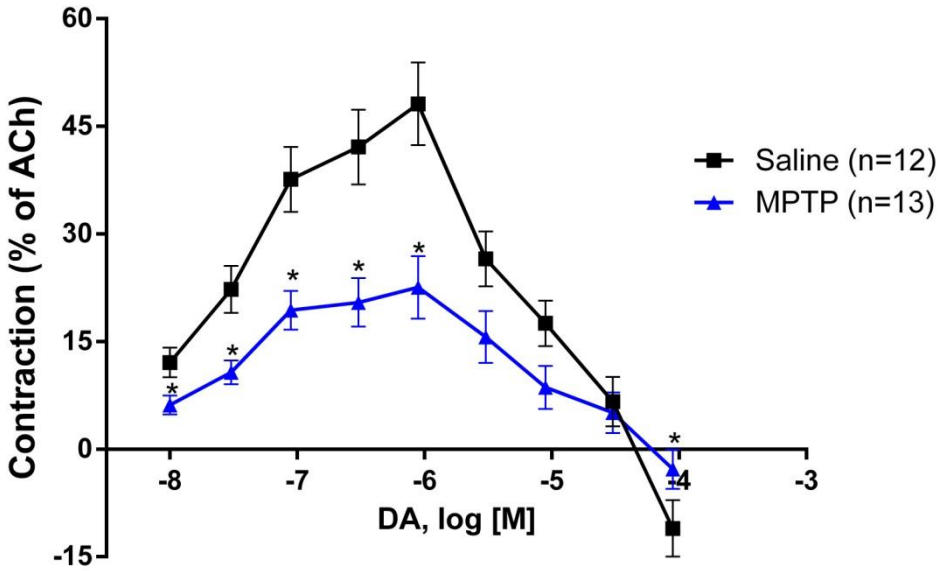

Figure 27: Contractile response of the Wistar rat ileum at 21 weeks of age to DA, in the absence (■ - Saline) and the presence of MPTP treatment (▲); the results are expressed as average values ± S.E.M; vertical bars represent S.E.M. The significance of the statistical differences was analyzed by Student's *t* test. * *P* <0.05 vs. Saline.

1.2. Effect of D2R antagonist on the concentration–response curve for Dopamine in both the presence and the absence of the MPTP treatment

Endogenous dopamine is established to play a physiological role in the GI tract, but whether dopamine receptors are involved remains controversial (Walker et al.

2000). Therefore, further studies on the involvement of dopamine receptors in the intestinal motility are warranted. Here, we focused on the D2-like receptors and its blockade implications on cumulative DA CR curves. Sulpiride (10µM) was the selective antagonist of dopamine D2-like receptors used as it exhibits minimal nonspecific effect on other receptor systems.

Within saline group (Figure 28), there was a statistically significant difference between DA CR curves performed both in the absence and in the presence of the sulpiride, showing a decrease in the DA-induced maximum contraction with D2R blockade. Nonetheless, no such difference was obtained in the MPTP-treated group, where no statistically significant alterations were observed on contraction phase of the biphasic curve in the presence of the D2R antagonist (Figure 29). However there was a reduction in the DA-induced maximum relaxation. While comparing both saline and MPTP-treated groups in the presence of sulpiride (Figure 30), it was established that even if no differences were found for lower exogenous DA concentrations, i.e. when the ileum contracts, there was a decrease in relaxation in the MPTP group for higher DA concentrations. This is highly suggestive that MPTP is perturbing D2R-dependent ileum function.

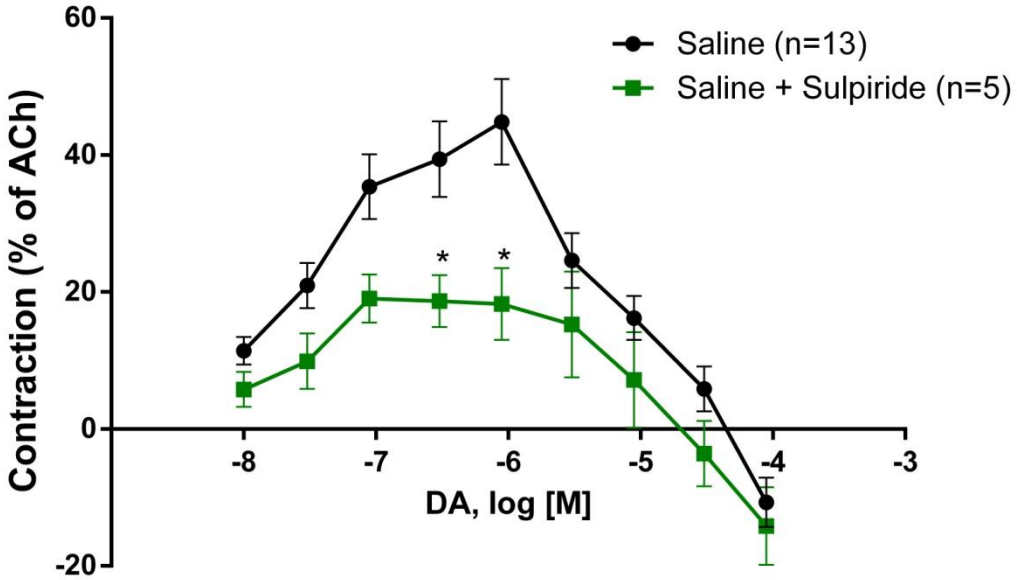

Figure 28: Contractile response of the Wistar rat ileum at 21 weeks of age to DA, in the absence (● - Saline) and in the presence of 10µM Sulpiride (■); the results are expressed as average values ± S.E.M; vertical bars represent S.E.M. The significance of the statistical differences was analyzed by Student's *t* test. * *P* <0.05 vs. Saline.

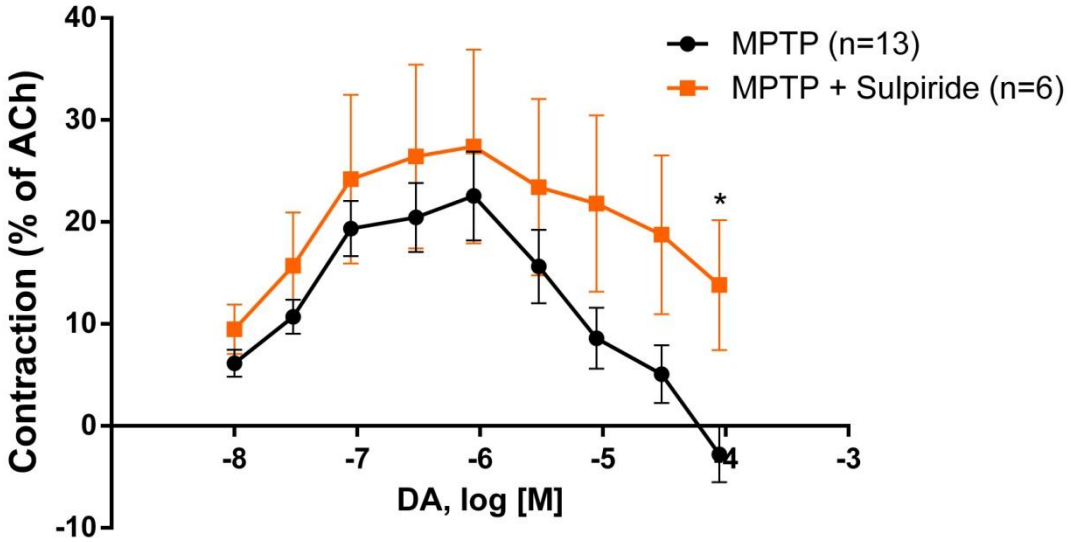

Figure 29: Contractile response of the Wistar rat ileum at 21 weeks of age to DA, in MPTP-treated rats in both the absence (● - MPTP) and in the presence of 10µM Sulpiride(■); the results are expressed as average values ± S.E.M; vertical bars represent S.E.M. The significance of the statistical differences was analyzed by Student's *t* test. * *P* <0.05 vs. Saline

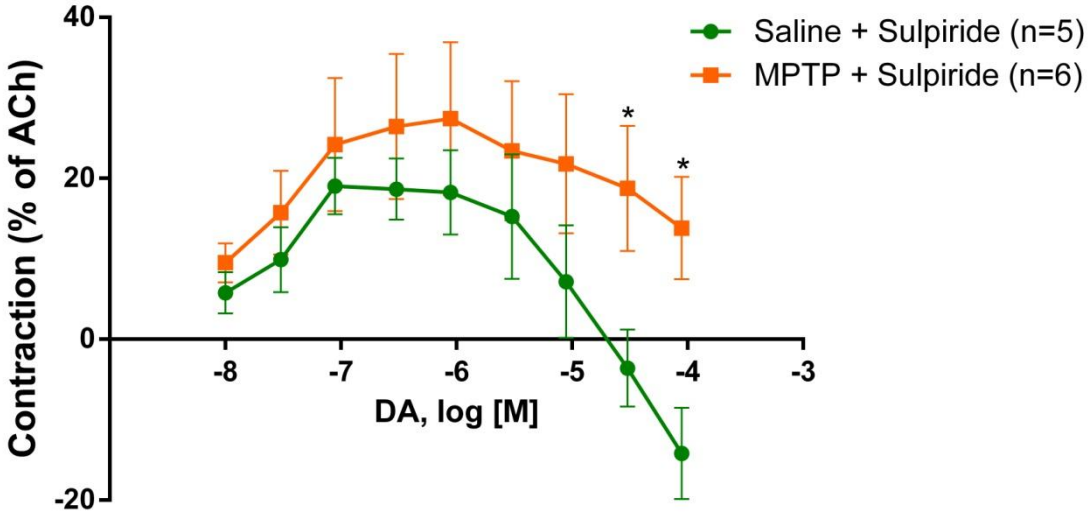

Figure 30: Contractile response of the Wistar rat ileum at 21 weeks of age to DA, in Saline(●) and MPTP-treated rats (■), both in the presence of Sulpiride (10µM); the results are expressed as average values ± S.E.M; vertical bars represent S.E.M. The significance of the statistical differences was analyzed by Student's *t* test. * *P* <0.05 vs. Saline**.**

1.3. Atropine effect on the concentration–response curve for Dopamine

Taking into account that ACh is a major excitatory neurotransmitter in the ENS, cholinergic mechanisms play an important role in the regulation of intestinal motility (Furness, 2000). Thus, DA CR curves were performed within the saline group in the presence of 1 µM atropine, a non-selective muscarinic receptors antagonist (Figure 31). No statistically significant changes were observed between the control CR curves and the ones performed in the presence of the antagonist, leading to the conclusion that the contractile response to DA is not mediated by muscarinic receptors.

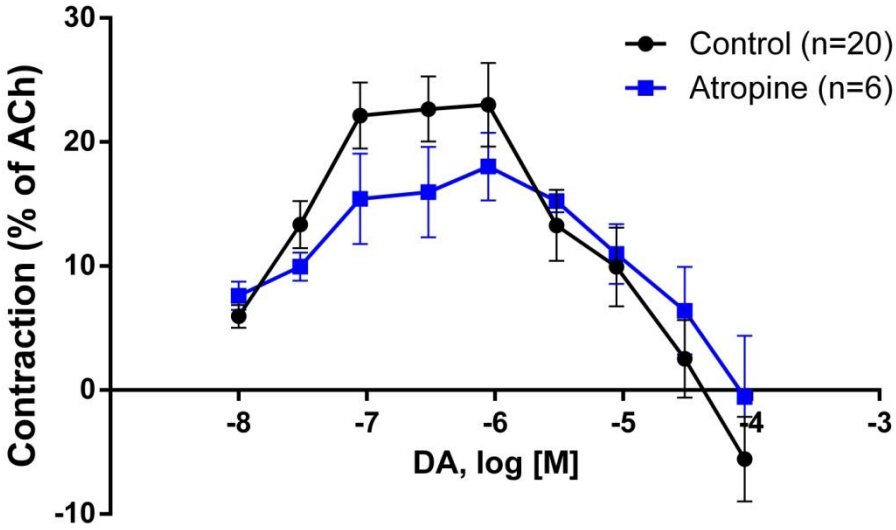

Figure 31: Contractile response of the Wistar rat ileum to DA, in both the absence (● - Control) and in the presence of 1µM Atropine (■); the results are expressed as average values ± S.E.M; vertical bars represent S.E.M. The significance of the statistical differences was analyzed by Student's *t* test.

2. Effect of MPTP treatment in the total content of DA in the Wistar rat ileum

GI dysfunction in PD was hypothesized to depend on a degenerative process involving the ENS, similarly to what occurs in the CNS, and consequently anticipating a decrease of DA levels in the small intestine (Natale et al., 2010).

To quantitatively assess the dopaminergic status, the total DA content from ileum tissue of saline and MPTP-treated rats was measured (Figure 32). MPTP treatment did not induce a significant change in the tissue levels of DA (1.21±0.20 ng/mg of protein) in comparison with controls (1.03±0.22 ng/mg of protein), implying that the dopaminergic dysfunction occurs with preserved DA homeostasis.

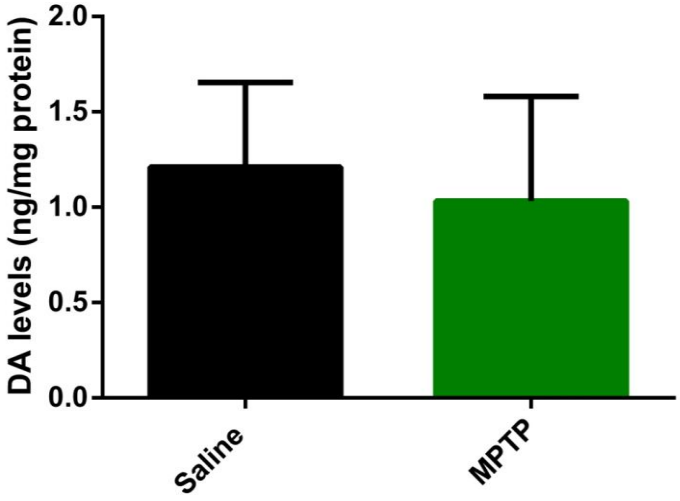

Figure 32: Effect of i.n. administration MPTP on DA content in the rat ileum; the results are expressed as average values ± S.E.M; vertical bars represent S.E.M. (Saline: n= 5; MPTP: n=6). The significance of the statistical differences was analyzed by Student's *t* test.

3. Effect of MPTP treatment in TH and D2R ileal levels

3.1. TH density analysis

TH catalyzes the rate-limiting step in catecholamine biosynthesis, implying that changes in its activity are primarily responsible for changes in the rate of catecholamine synthesis. Therefore, TH levels were assessed by Western Blotting analysis (Figure 33) in an attempt to further confirm the results obtained for DA total content by HPLC. In MPTP animals, TH levels show a slightly tendency to decrease (84.90±17.38%) compared to the saline group (100.00±28.05%), of about 15.10%. However, MPTP did not induce statistically significant differences in the protein levels, as expected.

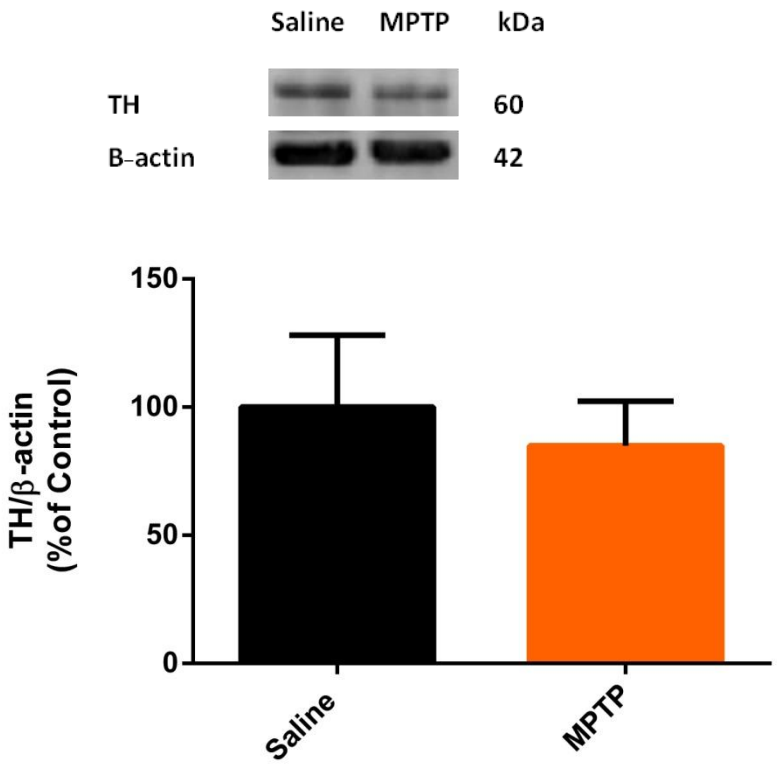

Figure 33: Evaluation of protein levels of TH in total ileum cell lysates by Western Blotting. Data are expressed as percentage of control and represent the mean ± SEM (Saline: n= 5; MPTP: n=6).The significance of the statistical differences was analyzed by Student's *t* test.

3.2. D2R density analysis

DA-induced contraction of the small intestine is believed to be largely, albeit not entirely, mediated by D1-like and D2-like dopamine receptors (Kirschstein et al., 2009). Having in consideration the results obtained from the functional studies, which suggested that MPTP is perturbing D2R-dependent ileum motility, D2R levels were also analyzed by Western Blotting (Figure 34). In MPTP animals, DA levels show a tendency to decrease (77.45±7.81%) compared to the saline group (100.00±18.37%), of about 22.55%, but fail to induce statistically significant differences.

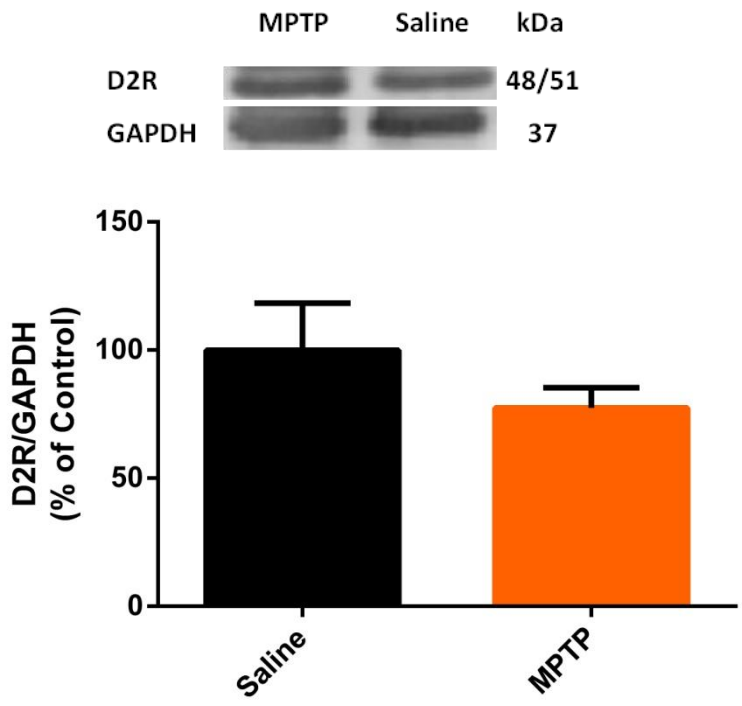

Figure 34: Evaluation of D2R levels in total ileum cell lysates by Western Blotting. Data are expressed as percentage of control and represent the mean ± SEM (n= 5 per group).The significance of the statistical differences was analyzed by Student's *t* test.

4. Histological and immunohistochemical studies of Wistar rat ileum segments

4.1. Immunoreactivity for S100β

In figure 35 it is possible to analyse the ileum sections of Wistar rats belonging to the two groups – Saline and MPTP-treated rats. Six ileum samples of each group were submitted to immunohistochemical techniques in order to evaluate possible alterations of S100β protein expression. A human colon sample was used as a positive control for the S100β protein.

A relative comparison between MPTP-treated and saline rats was achieved. Thus, the saline group showed immunostaining for smooth muscle cells and ganglion cells of both submucosal and myenteric plexus. In adition, S100β immunoreactivity was observed in dendritic cells of a Peyer's patch. In contrast, the MPTP-treated group evidenced a decrease in the S100β immunoreactivity in the submucosal plexus relatively to the myenteric plexus, when compared to the saline group.

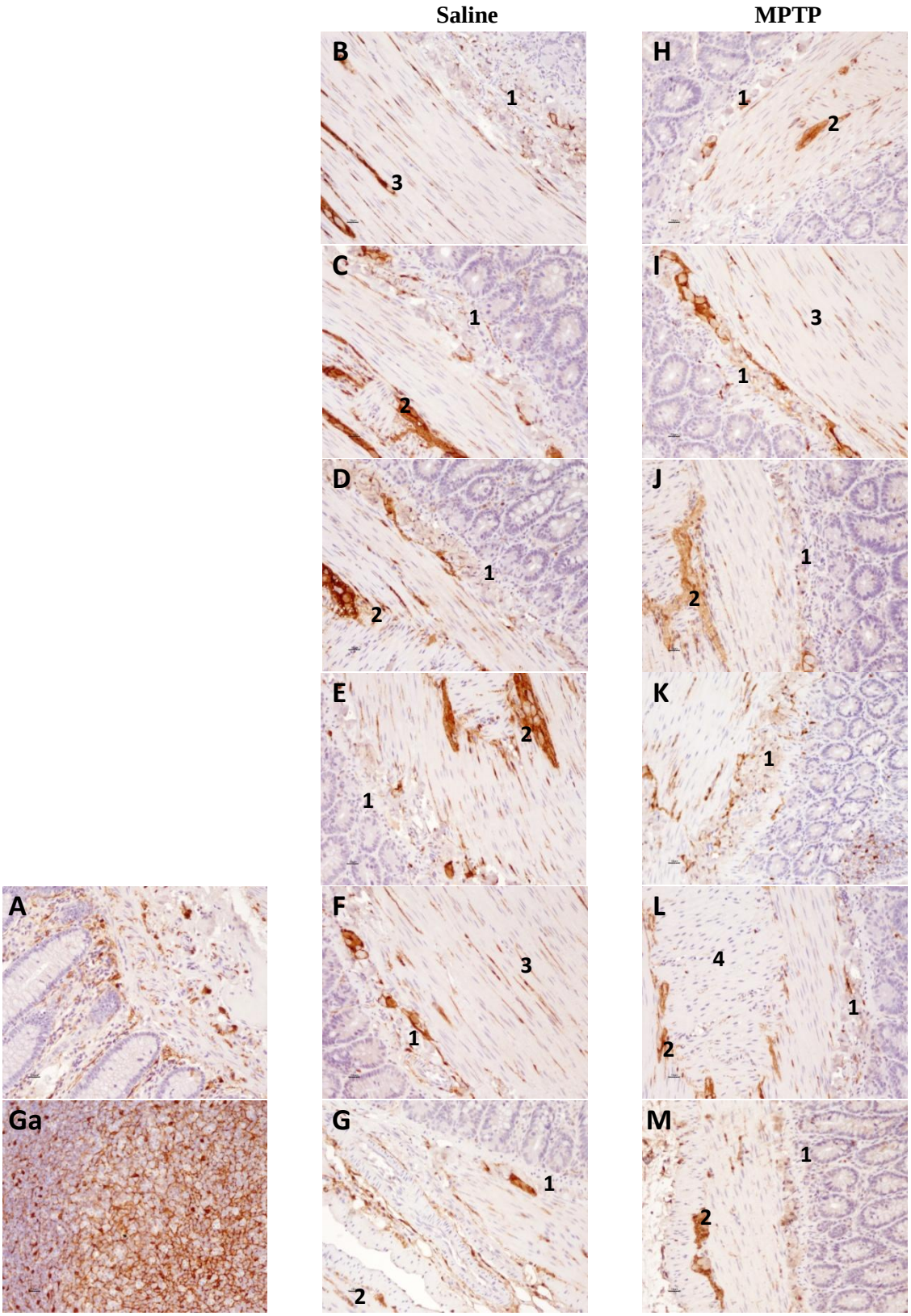

Figure 35: Representative S100β immunohistochemical findings on isolated ileum from saline and MPTP treated rats. S100β imunoreactivity appears in brown. **Panel A** X200 refers to a section of human colon (positive control); **Panels B-G**, X200 refer to saline group ileum sections. **Panels H-M**, X200 refer to MPTP-treated group ileum sections. **Panel Ga**, X200 refers to a Peyer's patch. S100β positive cells in ganglion cells of both the submucous (1) and myenteric (2) plexuses and smooth muscle cells of circular (3) and longitudinal layers (4).

4.2. Immunoreactivity for D2R

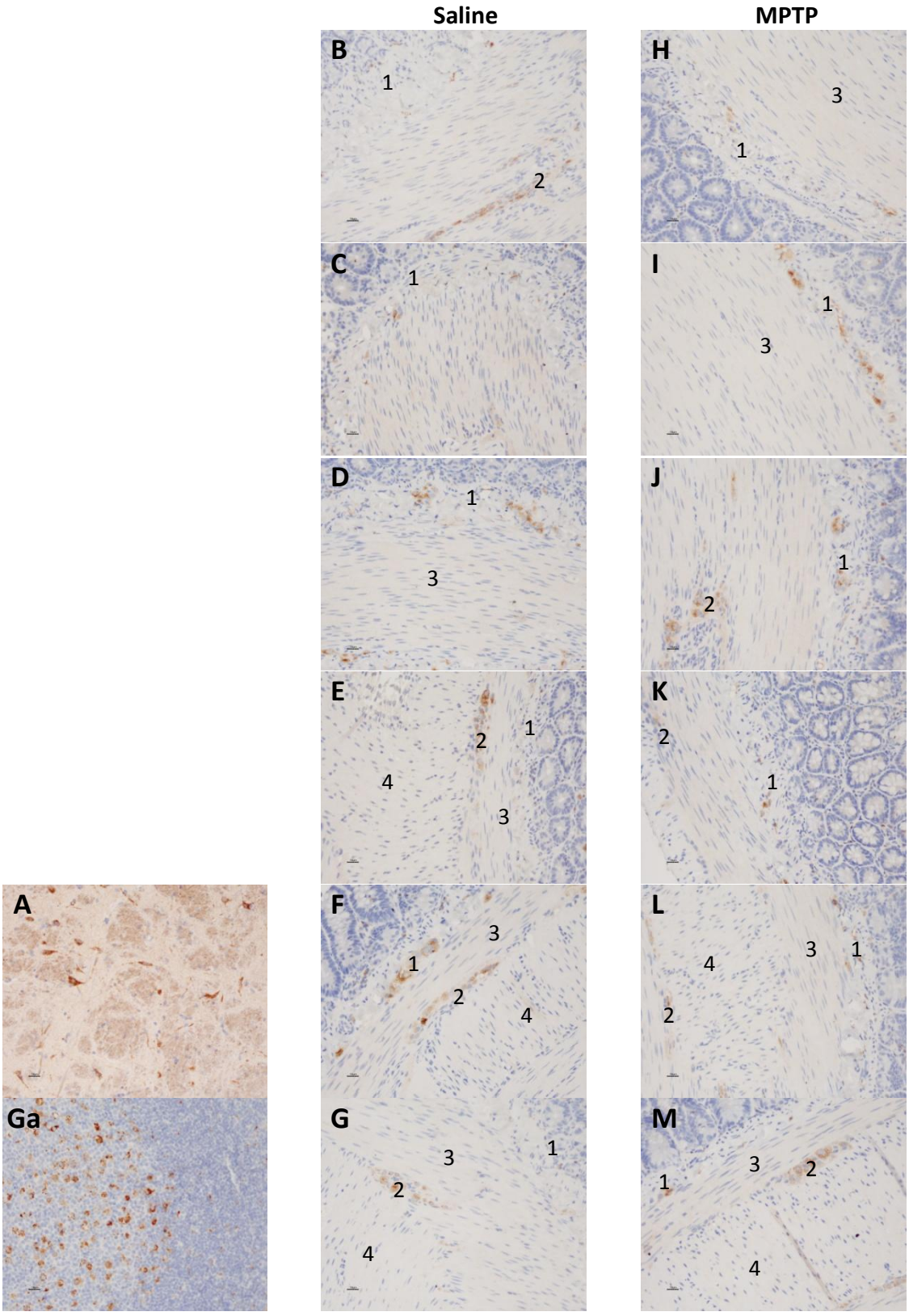

Figure 36: Representative D2R immunohistochemical findings on isolated ileum from saline and MPTP treated rats. D2R imunoreactivity appears in brown. **Panel A** X200 refers to a section of rat striatum (positive control); **Panels B-G**, X200 refer to saline group ileum sections. **Panels H-M**, X200 refer to MPTP-treated group ileum sections. **Panel Ga**, X200 refers to a Peyer's patch. D2R positive cells in ganglion cells of both the submucous (1) and myenteric (2) plexuses but not on smooth muscle cells of circular (3) and longitudinal layers (4).

As shown in figure 36, a characterization of the cellular location of D2R in the isolated rat ileum was carried out, while assessing possible alterations in its immunostaining for this PD model. Yet again, six ileum samples of rats of each group were submitted to immunohistochemical techniques. A sample of rat striatum was used as a positive control for the D2R.

IHC studies showed some variability in the D2R distribution within the ileum. Smooth muscle cells and epithelial cells showed no immunoreactivity. Dendritic cells, which have the same embryonic origin (ectoderm) as neuronal cells, were also immunopositive for D2R on a Peyer's patch. Immunoreactivity tends to be higher in the myenteric plexus when compared to submucosal plexus in the saline animals. On the other hand, within the MPTP-treated group only three out of six samples showed immunoreactivity in the myenteric plexus.

CHAPTER 5

Discussion

PD is among the most frequent neurodegenerative disorders associated with aging and its clinical diagnosis rests on the identification of the characteristics related to DA deficiency that are a consequence of degeneration of the SNpc. It is reasonably well established that prior to the motor phase of classical PD, which includes motor symptoms as bradykinesia, resting tremor, rigidity and late postural instability, there is a prodromal period of several years duration (Hawkes et al., 2010). Non-motor symptoms, which involve derangement of behaviour, sleep, sensation, and autonomic function, are surprisingly common and may represent initial clinical features of PD (Chaudhuri et al., 2006). Although autonomic dysfunction, such as constipation, has potential sensitivity as a clinical biomarker of the premotor phase, it has been overlooked. In addition, rodents treated intranasally with MPTP have been thoroughly demonstrated to exhibit time-dependent impairments in olfactory, emotional, cognitive and motor functions associated with disruption of dopaminergic neurotransmission across brain structures which possibly recapitulate those observed during different stages of PD.

The aim of this study was to evaluate the intestinal function before motor impairment, seven days after a single i.n. MPTP administration. Firstly, in order to observe the influence of MPTP treatment on the contractile response of the Wistar rat isolated ileum to exogenous DA, cumulative concentration-response curves for DA in both the presence and the absence of the referred treatment were performed. During a second phase, in an attempt to explain the pharmacological basis underlying the impairment of function previously established between both groups of animals, we focused on the D2-like receptors and their blockade implications on the cumulative DA CR curves. The utility of this type of *in vitro* pharmacological studies, when compared with *in vivo* studies, is that not only does it allow the manipulation of the effective concentration of the drug in the organ, but it also facilitates the quantification the organ's response. The preference for male animals in these functional studies was due to the fact that females' cycles are difficult to determine/forecast and the underlying high levels of estrogen could alter the GI response indirectly. The ileum was used for biological preparation as an experimental *in vitro* model on the grounds that it represents the intestinal portion with greater pharmacological reactivity, in conjunction with the fact that it is considered a standard model for studying the pharmacological action of compounds within the intestine (Liu & Coupar, 1997). Moreover, cumulative

CR curves for exogenous DA were performed due to the fact that the tonic isometric contractions recorded displayed a stable plateau which enabled the successive addition of doses.

Following a 2-hour equilibration period with periodic washings, all intestinal segments were submitted to 100 μM of exogenous ACh in order to directly induce the maximum contraction of ileum smooth muscle. This, combined with the repetition of this step after concluding the CR curves, allowed us to assess whether the maximum contraction of the organ remained unaffected or if there was any deterioration over time. Organ contraction induced by ACh (100 mM) presented similar amplitudes before and after the functional study, confirming the obtained results.

Functional studies showed biphasic CR curves for DA (0.01 to 90 μM), on both saline and MPTP-treated rats, demonstrating a contractile concentration-dependent effect on the isolated rat ileum. Consequently, there was a clear evidence of contraction for lower DA concentrations (0.01 to 0.9 μM) and relaxation for higher concentrations (3 to 90 μM). Also, MPTP imposed a statistically significant decrease on the contraction phase of the DA CR curves, as well as a significant reduction on the maximum relaxation, compared to saline treated group, confirming DA's modifications on the rat ileum function, seven days after a single intranasal MPTP administration.

Afterward, the D2-like receptors blockade implications on the cumulative DA CR curves became our main focus and for that we have used a D2R blocker, sulpiride (D2R blocker) in a concentration in which it remains selectivity to D2 receptors over D1 receptors. There was a statistically significant difference between DA CR curves performed both in the absence and in the presence of the sulpiride, showing a decrease in the DA-induced maximum contraction with D2R blockade, within saline group, without affecting the relaxation phase. These results *per si* reveal that D2R are mediating DA-induced ileal contraction. In the MPTP-treated group, sulpiride did not significantly change the contractile profile of DA CR curves, but there was a statistically reduction in the DA-induced maximum relaxation. Moreover, both saline and MPTP-treated groups in the presence of sulpiride showed no differences for lower exogenous DA concentrations, suggesting that the DA-induced contraction is mediated by D2R and that these are not functional after MPTP treatment. Once again, in the presence of D2R blockade, there was a decrease in relaxation in the MPTP group for

higher DA concentrations, indicating that MPTP appear to affect pathways that are involved in relaxation not mediated by D2R.

Endogenous dopamine plays a physiological role in the GI tract, but whether dopamine receptors are involved remains debatable (Walker et al., 2000). Here we further demonstrate their importance, since dopamine-induced contraction is mediated by D2-like dopamine receptors. Our results suggest MPTP affects what is mediated by sulpiride while showing a greater involvement of D2R during the contraction phase of the CR curve, as opposed to a smaller influence during the phase of the CR curve where relaxation occurs. D2R activation appears to lead to smooth muscle contraction, which is explained by D2R being active postsynaptically. Thus D2R, couple to Gi-protein, inhibits adenylyl cyclase, decreases intracellular cAMP, leading to smooth muscle cells contraction. On the other hand, if active presynaptically, it can induce neurotransmitter release through phospholipase C activation, also a signaling pathway associated to this type of receptor (Neve et al., 2004; Kirschstein et al., 2009).

As mentioned before, ACh is a major excitatory neurotransmitter in the ENS, which explains the important role that cholinergic mechanisms play in the regulation of intestinal motility. As a result, a strengthening of ACh action at postsynaptic level induced by DA was also hypothesized. In order to discard that possibility, atropine was used as a non-selective muscarinic receptors antagonist, allowing the blockade of all those receptors in the rat ileum preparations. Atropine did not produce significant differences between the cumulative curves CR for DA within the saline group, hence excluding the involvement of muscarinic receptors in the DA contractile mechanism.

Briefly, dopamine-induced contraction is mediated by D2-like receptors and our results are highly suggestive that MPTP is perturbing that D2R-dependent ileum function. The relaxation part of the CR curves to DA still warrants further investigation. The role of D1-like dopamine receptors should not be overlooked either. As dopamine can also directly activate α and β adrenoceptors, their possible influence in cumulative CR curves for DA should be accessed (Kirschstein et al., 2009).

PD patients have been reported to present a lower level of DA in the muscularis externa of the colon (Singaram et al., 1995), suggesting that the damage of the enteric dopaminergic system might be one of the unrecognized factors causing GI dysfunction in the course of PD. However, it is still unclear whether the GI dysfunction is predominantly associated with central or peripheral dopaminergic deficiency, or

whether it is mainly caused by the decrease (or increase) of enteric DA content. In rodents, it has been previously described that 10 days after three i.p. MPTP injections, mice have shown persistently delays in colonic motility, constipation and a reduction of TH-positive neurons, as a result of the loss of DA neurons within both myenteric and submucosal plexus in the intestine (Natale et al., 2010). In another study, 10 days after four i.p. injections MPTP administration, there was a reduction of DA neurons in the mouse ENS (Anderson et al., 2007). Accordingly, evaluating the isolated rat ileum neurotoxicity in our PD model, 7 days after a single i.n. MPTP administration, became imperative. For that, DA total content (HPLC) and TH and D2R protein expression (WB) were measured. MPTP treatment did not induce a significant change in the tissue levels of DA (1.21±0.20 ng/mg of protein) in comparison with controls (1.03±0.22 ng/mg of protein). TH levels show a slightly tendency to decrease in MPTP-treated animals (84.90±17.38%) when compared to the saline group (100.00±28.05%), of about 15.10%. However, no statistically significant differences in the protein levels were observed. These results imply that, in this premotor rat PD model, the dopaminergic dysfunction occurs with preserved intestinal DA homeostasis. It is also worth mentioning, that not only there are marked species differences in the susceptibility to the neurotoxic effects of MPTP, but there are also variations depending on whether is it a single administration or chronic neurotoxin (Giovanni et al., 1994).

Enteric glia participates on the homeostasis of the GI tract. In the CNS, increased expression of astroglial-derived S100β protein has been associated with the onset and maintaining of inflammation. However, little is known about the role of this enteroglial intestinal inflammation protein marker during neuronal damage caused by MPTP, especially in the ENS. In order to comparatively evaluate possible alterations of S100β protein expression, immunohistochemical studies on isolated rat ileum on both Saline and MPTP-treated samples were performed. The expression and release of S100β protein have been correlated with inflammation in the intestine (celiac disease and ulcerative colitis). In the human intestine, only S100β protein is indeed physiologically and specifically expressed by enteric glial cells (Esposito et al., 2007, Cirillo et al., 2009, Cirillo et al., 2011). In general, we were able to establish a relative comparison between MPTP-treated and saline rats. The saline group showed immunostaining for smooth muscle cells and ganglion cells of both submucosal and myenteric plexus. S100β immunoreactivity was also observed in dendritic cells of a Peyer's patch. On the

other hand, in the MPTP-treated group, the results provided evidence that S100β immunoreactivity was decreased in the submucosal plexus relatively to the myenteric plexus, when comparing to the saline group. Consequently, this decrease shows a disparity between the CNS and the ENS, since S100β protein levels have been described, in humans, to be higher in post-mortem substantia nigra of patients with PD compared with control tissue (Sathe et al., 2012). However, an increase of immunopositive cells was found in the striatum and substantia nigra of control mice when compared to mice one and three days after MPTP treatment, but not to animals seven days after the i.p. MPTP administration (Muramatsu et al., 2003), suggesting that the activation of S100β immunopositive cells may reflect reactive astrocytes after MPTP treatment.

Very little is known about the characterization of D2 receptors in the rat GI tract and its role in intestinal motility. Transcripts encoding D1R, D2R, D3R, and D5R were detected in RNA extracted from the longitudinal muscle with adherent myenteric plexus, whereas those encoding D1R, D3R, D4R, and D5R were identified in the mucosa, which is consistent with the possibility that the enteric D2R is neuronal. Transcripts encoding D2 were also detected in myenteric neurons of mouse ileum by *in situ* hybridization (Li et al., 2006). Therefore, we attempted to characterize the cellular location of D2R in the isolated rat ileum while assessing possible alterations in its immunostaining for this PD model. The immunohistochemical analysis showed some variability in the distribution of D2R within the ileum. Firstly, smooth muscle cells and epithelial cells showed no immunoreactivity. Immunoreactivity tends to be higher in the myenteric plexus when compared to submucosal plexus in the saline animals. In turn, within the MPTP-treated group, only representative histological sections of three out of six treated animals showed immunoreactivity in the myenteric plexus. This heterogeneous distribution of the D2R immunoreactivity between both saline and MPTP-treated animals might be underlying the different DA CR curves profiles in the presence of D2R blockade, namely the decrease in relaxation in the MPTP group for higher DA concentrations.

CHAPTER 6

Conclusion and Future Studies

In the present study, we confirm the deleterious effects of a premotor PD model in the ileum function, specifically 7 days after an i.n. MPTP administration. In addition to characterizing the biphasic DA CR curves for both saline and MPTP-treated rats, i.e., contraction for lower DA concentrations and relaxation for higher concentrations, the functional studies evidenced GI dysfunction for MPTP-treated Wistar rats, as there was a statistically significant decrease in the DA maximum contractile effect, when compared to saline.

Moreover, an impairment of the DA effect similar to the one found for the MPTP-treated group was imposed in the presence of the D2R antagonist, sulpiride. While, within the saline group, the presence of sulpiride induced significant differences in comparison to its absence, the MPTP-treated rats showed no such disparity in the same conditions. Not only does this sustain the importance of D2R in the GI function, but it also leads to the conclusion that MPTP has a harmful effect in D2R-dependent ileum function.

The small intestine dopaminergic dysfunction was accompanied by preserved DA homeostasis, since DA and TH ileal levels were identical for both groups. Also, D2R density, measured by WB, showed no differences. However, IHC allowed us to establish a tendency to a decrease in D2R immunoreactivity in the myenteric plexus within the MPTP group, when compared to the saline group. Since generically myenteric plexus is associated to contractility while submucosal is linked to secretion, these results summed to the functional study observations in the presence of D2R blockade, on the MPTP-treated group, suggest that this dopaminergic neutotoxin can affect both the contraction and relaxation phases of DA CR curves.

Finally, further investigation is needed to understand this distance between functional studies results and the actual D2R expression, mainly in what concerns the overlooked D1R and the possible MPTP-induced alterations in the transduction pathways coupled to D2R and D1R receptors.

CHAPTER 7

References

Agid, Y., Javoy-Agid, F., & Ruberg, M. (1987). Biochemistry of neurotransmitters in Parkinson's disease. *Movement Disorders*, 2(7), 166–230.

Aguiar Jr, A. S., Tristão, F. S. M., Amar, M., Chevarin, C., Lanfumey, L., Mongeau, R., Raisman-Vozari, R. (2013). Parkin-knockout mice did not display increased vulnerability to intranasal administration of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP). *Neurotoxicity Research*, 24(2), 280–287.

Anderson, G., Noorian, A. R., Taylor, G., Anitha, M., Bernhard, D., Srinivasan, S., & Greene, J. G. (2007). Loss of enteric dopaminergic neurons and associated changes in colon motility in an MPTP mouse model of Parkinson's disease. *Experimental Neurology*, 207(1), 4–12. doi:10.1016/j.expneurol.2007.05.010

Andrade L. A. F., Ferraz H. B., (1997), Inibição Enzimática, Neuroprotecção e Tratamento da Doença de Parkinson. *Rev. Neurociências*. Vol.5, 27-33;

Anlauf, M., Schäfer, M. K., Eiden, L., & Weihe, E. (2003). Chemical coding of the human gastrointestinal nervous system: cholinergic, VIPergic, and catecholaminergic phenotypes. *Journal of Comparative Neurology*, 459(1), 90–111.

Aston-Jones, G., & Cohen, J. D. (2005). An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. *Annual Review of Neuroscience*, 28, 403–450. doi:10.1146/annurev.neuro.28.061604.135709

Auluck, P. K., Chan, H. Y. E., Trojanowski, J. Q., Lee, V. M. Y., & Bonini, N. M. (2002). Chaperone suppression of alpha-synuclein toxicity in a Drosophila model for Parkinson's disease. *Science* (New York, N.Y.), 295(MARCH), 865–868. doi:10.1126/science.1067389

Ballard, P. A., Tetrud, J. W., & Langston, J. W. (1985). Permanent human parkinsonism due to 1-methy 1–4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) Seven cases. *Neurology*, 35(7), 949.

Ballesteros, J. a, Shi, L., & Javitch, J. a. (2001). Structural mimicry in G protein-coupled receptors: implications of the high-resolution structure of rhodopsin for structure-function analysis of rhodopsin-like receptors. *Molecular Pharmacology*, 60(1), 1–19. doi:10.1124/mol.60.1.1

Barlow, B. K., Thiruchelvam, M. J., Bennice, L., Cory-Slechta, D. a, Ballatori, N., & Richfield, E. K. (2003). Increased synaptosomal dopamine content and brain concentration of paraquat produced by selective dithiocarbamates. *Journal of Neurochemistry*, 85, 1075–1086. doi:10.1046/j.1471-4159.2003.01773.x

Beach, T. G., Adler, C. H., Sue, L. I., Vedders, L., Lue, L., White Iii, C. L., … Walker, D. G. (2010). Multi-organ distribution of phosphorylated alpha-synuclein histopathology in subjects with Lewy body disorders. *Acta Neuropathologica*, 119(6), 689–702. doi:10.1007/s00401-010-0664-3

Beaulieu, J.-M., & Gainetdinov, R. R. (2011). The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacological Reviews*, 63(1), 182–217. doi:10.1124/pr.110.002642.182

Bekris, L. M., Mata, I. F., & Zabetian, C. P. (2010). The genetics of Parkinson disease. *Journal of Geriatric Psychiatry and Neurology*, 23(4), 228–42. doi:10.1177/0891988710383572

Benarroch, E. E. (2007). Enteric nervous system: Functional organization and neurologic implications. *Neurology*, 69, 1953–1957. doi:10.1212/01.wnl.0000281999.56102.b5

Berardelli, A., Rothwell, J. C., Thompson, P. D., & Hallett, M. (2001). *Pathophysiology of bradykinesia in Parkinson ' s disease*, 2131–2146.

Bertrand PP, Kunze WA, Furness JB, Bornstein JC. The terminals of myenteric intrinsic primary afferent neurons of the guinea-pig ileum are excited by 5-hydroxytryptamine acting at 5-hydroxytryptamine-3 receptors. *Neuroscience*. 2000;101(2):459-69.

Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, a V, & Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nature Neuroscience*, 3, 1301–1306. doi:10.1038/81834

Bloem BR, Hausdorff JM, Visser JE, et al. (2004). Falls and freezing of gait in Parkinson's disease: a review of two interconnected, episodic phenomena. *Mov Disord*.; 19: 871-884.

Bloom, F. E. (2010). The catecholamine neuron: Historical and future perspectives. *Progress in Neurobiology*, 90, 75–81. doi:10.1016/j.pneurobio.2009.10.014

Boghen D. (1997). Apraxia of lid opening: a review. *Neurology*; 48: 1491-1494.

Boselli, C., Lucchelli, A., & Grana, E. (1987). Receptors involved in the dopamine-induced relaxation of the isolated rat ileum. *Pharmacological Research Communications*, 19(12), 953–956.

Braak, H., de Vos, R. A. I., Bohl, J., & Del Tredici, K. (2006). Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged

for Parkinson's disease-related brain pathology. *Neuroscience Letters*, 396(1), 67–72. doi:10.1016/j.neulet.2005.11.012

Braak, H., Del Tredici, K., Rüb, U., De Vos, R. a I., Jansen Steur, E. N. H., & Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of Aging*, 24, 197–211. doi:10.1016/S0197-4580(02)00065-9

Braak, H., Ghebremedhin, E., Rüb, U., Bratzke, H., & Del Tredici, K. (2004). Stages in the development of Parkinson's disease-related pathology. *Cell and Tissue Research*, 318(1), 121–34. doi:10.1007/s00441-004-0956-9

Braak, H., Rüb, U., Gai, W. P., & Del Tredici, K. (2003). Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *Journal of Neural Transmission* (Vienna, Austria : 1996), 110(5), 517–36. doi:10.1007/s00702-002-0808-2

Breese, G. R., & Traylor, T. D. (1971). Depletion of brain noradrenaline and dopamine by 6-hydroxydopamine. *British Journal of Pharmacology*, 42, 88–99. doi:10.1111/j.1476-5381.1971.tb07089.x

Broussolle, E., Krack, P., Thobois, S., Xie-Brustolin, J., Pollak, P., & Goetz, C. G. (2007). Contribution of Jules Froment to the study of parkinsonian rigidity. *Movement Disorders : Official Journal of the Movement Disorder Society*, 22(7), 909–14. doi:10.1002/mds.21484

Camborová, P., Hubka, P., Sulková, I., & Hulín, I. (2003). The pacemaker activity of interstitial cells of Cajal and gastric electrical activity. *Physiological Research / Academia Scientiarum Bohemoslovaca*, 52, 275–284.

Castro, A. a., Ghisoni, K., Latini, A., Quevedo, J., Tasca, C. I., & Prediger, R. D. S. (2012). Lithium and valproate prevent olfactory discrimination and short-term memory impairments in the intranasal 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) rat model of Parkinson's disease. *Behavioural Brain Research*, 229(1), 208–215. doi:10.1016/j.bbr.2012.01.016

Cersosimo, M. G., & Benarroch, E. E. (2012). Pathological correlates of gastrointestinal dysfunction in Parkinson's disease. *Neurobiology of Disease*, 46(3), 559–64. doi:10.1016/j.nbd.2011.10.014

Chan, P., Delanney, L. E., Irwin, I., Langston, J. W., & Monte, D. Di. (1991). Tetrahydropyridine in Mouse Brain. *Journal of Neurochemistry*, 348–351.

Chaudhuri, K. R., & Martinez-Martin, P. (2008). Quantitation of non-motor symptoms in Parkinson's disease, 15, 2–8.

Chaudhuri, K. R., & Schapira, A. H. (2009). Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. *The Lancet Neurology*, 8(5), 464–474. doi:10.1016/S1474-4422(09)70068-7

Chaudhuri, K. R., Healy, D. G., & Schapira, a H. (2006). Non-motor symptoms of Parkinson's disease: diagnosis and management. *Lancet Neurol*, 5(March), 235–245. doi:10.1016/s1474-4422(06)70373-8

Chaudhuri, K. R., Yates, L., & Martinez-Martin, P. (2005). The non-motor symptom complex of Parkinson's disease: A comprehensive assessment is essential. *Current Neurology and Neuroscience Reports*, 5(4), 275–283. doi:10.1007/s11910-005-0072-6

Chien, E. Y. T., Liu, W., Zhao, Q., Katritch, V., Won Han, G., Hanson, M. A., Stevens, R. C. (2010). Structure of the Human Dopamine D3 Receptor in Complex with a D2/D3 Selective Antagonist. *Science*, 330 (6007), 1091–1095. doi:10.1126/science.1197410

Chio, C. L., Lajiness, M. E., & Huff, R. M. (1994). Activation of heterologously expressed D3 dopamine receptors: comparison with D2 dopamine receptors. *Molecular Pharmacology*, 45, 51–60.

Cirillo C, Sarnelli G, Esposito G, Grosso M, Petruzzelli R, Izzo P, Cali G, D'Armiento FP, Rocco A, Nardone G, Iuvone T, Steardo L, Cuomo R (2009) Increased mucosal nitric oxide production in ulcerative colitis is mediated in part by the enteroglial-derived S100B protein. *Neurogastroenterology and motility: the official journal of the European Gastrointestinal Motility Society* 21:1209-e1112.

Cirillo C, Sarnelli G, Esposito G, Turco F, Steardo L, Cuomo R (2011) S100B protein in the gut: the evidence for enteroglial-sustained intestinal inflammation. *World journal of gastroenterology:* WJG 17:1261-1266.

Cooper, J. A., Sagar, H. J., Tidswell, P., & Jordan, N. (1987). Slowed central processing in simple and go/no-go reaction time tasks in Parkinson's disease, 517–529.

Costa, M., Brookes, S. J. H., & Hennig, G. W. (2000). Anatomy and physiology of the enteric nervous system. *Gut*, 47(suppl 4), iv15–iv19.

Critchley, E. M. (1981). Speech disorders of Parkinsonism: a review. *Journal of Neurology, Neurosurgery, and Psychiatry*, 44, 751–758. doi:10.1136/jnnp.44.9.751

Dauer, W., & Przedborski, S. (2003). Parkinson's Disease : Mechanisms and Models, 39, 889–909.

Dauer, W., Kholodilov, N., Vila, M., Trillat, A.-C., Goodchild, R., Larsen, K. E., Hen, R. (2002). Resistance of alpha -synuclein null mice to the parkinsonian neurotoxin MPTP. *Proceedings of the National Academy of Sciences of the United States of America*, 99(22), 14524–14529. doi:10.1073/pnas.172514599

Day, B. J., Patel, M., Calavetta, L., Chang, L.-Y., & Stamler, J. S. (1999). A mechanism of paraquat toxicity involving nitric oxide synthase. *Proceedings of the National Academy of Sciences*, 96(22), 12760–12765.

Del Tredici, K., Rüb, U., de Vos, R. A. I., Bohl, J. R. E., & Braak, H. (2002). Where does parkinson disease pathology begin in the brain? *Journal of Neuropathology & Experimental Neurology*, 61(5), 413–426.

Di Monte, D. A., Lavasani, M., & Manning-Bog, A. B. (2002). Environmental factors in Parkinson's disease. *Neurotoxicology*, 23(4), 487–502.

Dickson, D. W., Fujishiro, H., DelleDonne, A., Menke, J., Ahmed, Z., Klos, K. J., … Ahlskog, J. E. (2008). Evidence that incidental Lewy body disease is pre-symptomatic Parkinson's disease. *Acta Neuropathologica*, 115, 437–444. doi:10.1007/s00401-008-0345-7

Dluzen, D. E., & Kefalas, G. (1996). The effects of intranasal infusion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) upon catecholamine concentrations within olfactory bulbs and corpus striatum of male mice. *Brain Research*, 741, 215–219. doi:10.1016/S0006-8993(96)00934-1

Doppler, W. (1994). Regulation of gene expression by prolactin. *Reviews of Physiology, Biochemistry and Pharmacology*, 124, 93–130.

Drolet, R. E., Cannon, J. R., Montero, L., & Greenamyre, J. T. (2009). Chronic rotenone exposure reproduces Parkinson's disease gastrointestinal neuropathology. *Neurobiology of Disease*, 36(1), 96–102. doi:10.1016/j.nbd.2009.06.017

Eckert, T., Feigin, A., Lewis, D. E., Dhawan, V., Frucht, S., & Eidelberg, D. (2007). Regional metabolic changes in parkinsonian patients with normal dopaminergic imaging. *Movement Disorders : Official Journal of the Movement Disorder Society*, 22(2), 167–73. doi:10.1002/mds.21185

Elsworth, J. D., & Roth, R. H. (1997). Dopamine synthesis, uptake, metabolism, and receptors: relevance to gene therapy of Parkinson's disease. *Experimental Neurology*, 144(144), 4–9. doi:10.1006/exnr.1996.6379

Eriksen, J. L., Dawson, T. M., Dickson, D. W., & Petrucelli, L. (2003). Caught in the Act : α -Synuclein Is the Culprit in Parkinson ' s Disease, 453–456.

Esposito G, Cirillo C, Sarnelli G, De Filippis D, D'Armiento FP, Rocco A, Nardone G, Petruzzelli R, Grosso M, Izzo P, Iuvone T, Cuomo R (2007) Enteric glial-derived S100B protein stimulates nitric oxide production in celiac disease. *Gastroenterology* 133:918-925.

Fabre, E., Monserrat, J., Herrero, A., Barja, G., & Leret, M. L. (1999). Effect of MPTP on brain mitochondrial H2O2 and ATP production and on dopamine and DOPAC in the striatum. *Journal of Physiology and Biochemistry*, 55(4), 325–331.

Fahn, S. (2010). Parkinson's disease: 10 years of progress, 1997-2007. *Movement Disorders : Official Journal of the Movement Disorder Society*, 25 Suppl 1, S2–14. doi:10.1002/mds.22796

Feldman RS, Meyer JS, Quenzer LF. (1997) Catecholamines, Principles of neuropsychopharmacology. *Sunderland, MA: Sinauer Associates,* Inc, 277–344.

Fleming, S. M., Zhu, C., Fernagut, P. O., Mehta, A., DiCarlo, C. D., Seaman, R. L., & Chesselet, M. F. (2004). Behavioral and immunohistochemical effects of chronic intravenous and subcutaneous infusions of varying doses of rotenone. *Experimental Neurology*, 187, 418–429. doi:10.1016/j.expneurol.2004.01.023

Forno LS. (1996). Neuropathology of Parkinson's disease. *J. Neuropathol. Exp Neurol.*; 55: 259-272.

Forno, L. S., DeLanney, L. E., Irwin, I., & Langston, J. W. (1992). Similarities and differences between MPTP-induced parkinsonsim and Parkinson's disease. Neuropathologic considerations. *Advances in Neurology*, 60, 600–608.

Forno, L. S., Langston, J. W., DeLanney, L. E., Irwin, I., & Ricaurte, G. a. (1986). Locus ceruleus lesions and eosinophilic inclusions in MPTP-treated monkeys. *Annals of Neurology*, 20, 449–455. doi:10.1002/ana.410200403

Fuxe, K. (1965). Evidence for the existence of monoamine neurons in the central nervous system. *Zeitschrift Für Zellforschung Und Mikroskopisch*, 596, 573–596. Retrieved from http://link.springer.com/article/10.1007/BF00337069

Garner, C. D., & Nachtman, J. P. (1989). Manganese catalyzed auto-oxidation of dopamine to 6-hydroxydopamine in vitro. *Chemico-Biological Interactions*, 69(4), 345–351.

Gasser, T. (2007). Update on the genetics of Parkinson's disease. *Movement Disorders : Official Journal of the Movement Disorder Society*, 22 Suppl 1(May), S343–50. doi:10.1002/mds.21676

Gerfen, C. R. (1992). The neostriatal mosaic: multiple levels of compartmental organization. *Journal of Neural Transmission.* Supplementum, 36, 43–59. doi:10.1146/annurev.neuro.15.1.285

Gershon, M. D. (1967). Inhibition of gastrointestinal movement by sympathetic nerve stimulation: the site of action. *The Journal of Physiology*, 189(2), 317–327.

Giladi N, McDermott MP, Fahn S, et al. (2001). Freezing of gait in PD: prospective assessment in the DATATOP cohort. *Neurology*; 56: 1712-1721.

Gingrich, J. a, & Caron, M. G. (1993). Recent advances in the molecular biology of dopamine receptors. *Annual Review of Neurosciences*, 16, 299–321.

Giovanni, a, Sonsalla, P. K., & Heikkila, R. E. (1994). Studies on species sensitivity to the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Part 2: Central administration of 1-methyl-4-phenylpyridinium. *The Journal of Pharmacology and Experimental Therapeutics*, 270, 1008–1014.

Giovanni, A., Sieber, B.-A., Heikkila, R. E., & Sonsalla, P. K. (1991). Correlation between the neostriatal content of the 1-methyl-4-phenylpyridinium species and dopaminergic neurotoxicity following 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine administration to several strains of mice. *Journal of Pharmacology and Experimental Therapeutics*, 257(2), 691–697.

Giovanni, A., Sieber, B.-A., Heikkila, R. E., & Sonsalla, P. K. (1994). Studies on species sensitivity to the dopaminergic neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine. Part 1: Systemic administration. *Journal of Pharmacology and Experimental Therapeutics*, 270(3), 1000–1007.

Giovannoni, G., van Schalkwyk, J., Fritz, V. U., & Lees, a J. (1999). Bradykinesia akinesia inco-ordination test (BRAIN TEST): an objective computerised assessment of upper limb motor function. *Journal of Neurology, Neurosurgery & Psychiatry*, 67(5), 624–629. doi:10.1136/jnnp.67.5.624

Greenamyre, J. T., Sherer, T. B., Betarbet, R., & Panov, a V. (2001). Complex I and Parkinson's disease. *IUBMB Life*, 52, 135–141. doi:10.1080/15216540152845939

Halhday, G. M., Li, Y. W., Blumbergs, P. C., Joh, T. H., Cotton, P. R. G. H., Howe, P. R. C., Geffen, L. B. (1990). Neuropathology of Immunohstochemically Idendied Brainstem Neurons in Parkinson ' s Disease, 373–385.

Halliday, G., Lees, A., & Stern, M. (2011). Milestones in Parkinson's disease--clinical and pathologic features. *Movement Disorders : Official Journal of the Movement Disorder Society*, 26(6), 1015–21. doi:10.1002/mds.23669

Hansen, M. B. (2003). Neurohumoral control of gastrointestinal motility. *Physiological Research*, 52, 1–30. doi:10.1051/rnd:19800701

Hasegawa, E., Kang, D., Sakamoto, K., Mitsumoto, a, Nagano, T., Minakami, S., & Takeshige, K. (1997). A dual effect of 1-methyl-4-phenylpyridinium (MPP+)-analogs on the respiratory chain of bovine heart mitochondria. *Archives of Biochemistry and Biophysics*, 337(1), 69–74. doi:S0003-9861(96)99726-X [pii]\n10.1006/abbi.1996.9726

Hashimoto, M., Takeda, a, Hsu, L. J., Takenouchi, T., & Masliah, E. (1999). Role of cytochrome c as a stimulator of alpha-synuclein aggregation in Lewy body disease. *The Journal of Biological Chemistry*, 274, 28849–28852.

Hawkes, C. H. (2008). The prodromal phase of sporadic Parkinson's disease: does it exist and if so how long is it? *Movement Disorders : Official Journal of the Movement Disorder Society*, 23(13), 1799–807. doi:10.1002/mds.22242

Hawkes, C. H., Del Tredici, K., & Braak, H. (2010). A timeline for Parkinson's disease. *Parkinsonism and Related Disorders*, 16(2), 79–84. doi:10.1016/j.parkreldis.2009.08.007

Heikkila, R., Manzino, L., Cabbat, F., & Duvoisin, R. (1984). Protection agains the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine by monoamine oxidase inhibitors. *Nature*, 311, 467–469.

Hirst, G. D. S., & Edwards, F. R. (2004). Role of interstitial cells of Cajal in the control of gastric motility. *Journal of Pharmacological Sciences*, 96, 1–10. doi:10.1254/jphs.CRJ04002X

Höglinger, G. U., Rizk, P., Muriel, M. P., Duyckaerts, C., Oertel, W. H., Caille, I., & Hirsch, E. C. (2004). Dopamine depletion impairs precursor cell proliferation in Parkinson disease. *Nature Neuroscience*, 7(7), 726–35. doi:10.1038/nn1265

Huizinga, J. D., & Lammers, W. J. E. P. (2009). Gut peristalsis is governed by a multitude of cooperating mechanisms. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 296(1), G1–G8.

Jackson-Lewis, V., Jakowec, M., Burke, R. E., & Przedborski, S. (1995). Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Neurodegeneration : A Journal for Neurodegenerative Disorders, Neuroprotection, and Neuroregeneration*, 4, 257–269.

Jankovic, J. (2002). Essential tremor: a heterogenous disorder. *Movement Disorders : Official Journal of the Movement Disorder Society*, 17(4), 638–44. doi:10.1002/mds.10221

Jankovic, J. (2008). Parkinson's disease: clinical features and diagnosis. *Journal of Neurology, Neurosurgery, and Psychiatry*, 79(4), 368–76. doi:10.1136/jnnp.2007.131045

Jankovic, J., Schwartz, K. S., & Ondo, W. (1999). Re-emergent tremor of Parkinson's disease. *Journal of Neurology, Neurosurgery & Psychiatry*, 67(5), 646–650. doi:10.1136/jnnp.67.5.646

Javitch, J. A., D'Amato, R. J., Strittmatter, S. M., & Snyder, S. H. (1985). Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proceedings of the National Academy of Sciences*, 82(7), 2173–2177.

Javoy, F., Sotelo, C., Herbet, A., & Agid, Y. (1976). Specificity of dopaminergic neuronal degeneration induced by intracerebral injection of 6-hydroxydopamine in the nigrostriatal dopamine system. *Brain Research*, 102(2), 201–215.

Jellinger, K. (1987). Overview of morphological changes in Parkinson's disease. *Advances in Neurology*, 45, 1.

Jellinger, K., Linert, L., Kienzl, E., Herlinger, E., & Youdim, M. B. (1994). Chemical evidence for 6-hydroxydopamine to be an endogenous toxic factor in the pathogenesis of Parkinson's disease. *Journal of Neural Transmission. Supplementum*, 46, 297–314.

Jeon, B. S., Jackson-Lewis, V., & Burke, R. E. (1995). 6-Hydroxydopamine lesion of the rat substantia nigra: time course and morphology of cell death. *Neurodegeneration : A Journal for Neurodegenerative Disorders, Neuroprotection, and Neuroregeneration*, 4, 131–137. doi:10.1006/neur.1995.0016

Jonsson, G. (1983). Chemical lesioning techniques: monoamine neurotoxins. *Handbook of Chemical Neuroanatomy*, 1, 463–507.

Jost, W. H. (2010). Gastrointestinal dysfunction in Parkinson's Disease. *Journal of the Neurological Sciences*, 289(1-2), 69–73. doi:10.1016/j.jns.2009.08.020

Kaasinen, V., Någren, K., Hietala, J., Farde, L., & Rinne, J. O. (2001). Sex differences extrastriatal dopamine D2-like receptors in the human brain. *American Journal of Psychiatry*, 158(February), 308–311. doi:10.1176/appi.ajp.158.2.308

Kebabian, J. W., Tarazi, F. I., Kula, N. S., & Baldessarini, R. J. (1997). Compounds selective for dopamine receptor subtypes. *Drug Discovery Today*, 2(8), 333–340. doi:10.1016/S1359-6446(97)01075-1

Kelemen, S. R., & Kelemen, S. R. (1978). Model Experiments on the Molecular Mechanism of Action of 6-Hydroxydopamine. *Journal of Catalysis*, 34, 24–34.

Kim, R. H., Smith, P. D., Aleyasin, H., Hayley, S., Mount, M. P., Pownall, S., … Horne, P. (2005). Hypersensitivity of DJ-1-deficient mice to 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyrindine (MPTP) and oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America*, 102(14), 5215–5220.

Kirschstein, T., Dammann, F., Klostermann, J., Rehberg, M., Tokay, T., Schubert, R., & Köhling, R. (2009). Dopamine induces contraction in the proximal, but relaxation in the distal rat isolated small intestine. *Neuroscience Letters*, 465(1), 21–6. doi:10.1016/j.neulet.2009.08.080

Klaidman, L. K., Adams, J. D., Leung, a C., Kim, S. S., & Cadenas, E. (1993). Redox cycling of MPP+: evidence for a new mechanism involving hydride transfer with xanthine oxidase, aldehyde dehydrogenase, and lipoamide dehydrogenase. *Free Radical Biology & Medicine*, 15, 169–179. doi:10.1016/0891-5849(93)90056-Z

Kobilka, B., & Schertler, G. F. X. (2008). New G-protein-coupled receptor crystal structures: insights and limitations. *Trends in Pharmacological Sciences*, 29(January), 79–83. doi:10.1016/j.tips.2007.11.009

Koob, G. F. (1992). Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends in Pharmacological Sciences*, 13, 177–184. doi:10.1016/0165-6147(92)90060-J

Koob, G. F., & Bloom, F. E. (1988). Cellular and molecular mechanisms of drug dependence. *Science* (New York, N.Y.), 242, 715–723.

Kopin, I. J., & Markey, S. P. (1988). MPTP toxicity: implications for research in Parkinson's disease. *Annual Review of Neuroscience*, 11, 81–96. doi:10.1146/annurev.neuro.11.1.81

Kupsky, W. J., Grimes, M. M., Sweeting, J., Bertsch, R., & Cote, L. J. (1987). Parkinson's disease and megacolon Concentric hyaline inclusions (Lewy bodies) in enteric ganglion cells. *Neurology*, 37(7), 1253.

Lang, A., & Lozano, A. (1998). Parkinson's Disease: First of Two Parts. *New England Journal of Medicine*, 339(15), 1044–1053.

Langston, J. W. (1987). MPTP: the promise of a new neurotoxin. *Movement Disorders*, 2, 73–90.

Langston, J. W. (2006). The Parkinson's complex: parkinsonism is just the tip of the iceberg. *Annals of Neurology*, 59(4), 591–6. doi:10.1002/ana.20834

Langston, J. W., & Ballard, P. A. J. (1983, August). Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *The New England Journal of Medicine.* UNITED STATES.

Langston, J. W., & Irwin, I. (1986). MPTP: Current Concepts and Controversies. Clinical Neuropharmacology, 9(6). Retrieved from http://journals.lww.com/clinicalneuropharm/Fulltext/1986/12000/MPTP__Current_Concepts_and_Controversies_.1.aspx

Lau, L. M. L. De, & Breteler, M. M. B. (2006). Epidemiology of Parkinson ' s disease, 525–535.

Le Moal M, Simon H. Mesocorticolimbic dopaminergic network:functional and regulatory roles. *Physiol Rev*, 71:155–234.

Lees, A. J., Hardy, J., & Revesz, T. (2009). Parkinson's disease. *Lancet*, 373(9680), 2055–66. doi:10.1016/S0140-6736(09)60492-X

Li, J. Y., Espay, A. J., Gunraj, C. a., Pal, P. K., Cunic, D. I., Lang, A. E., & Chen, R. (2007). Interhemispheric and ipsilateral connections in Parkinson's disease: Relation to mirror movements. *Movement Disorders*, 22(6), 813–821. doi:10.1002/mds.21386

Li, Z. S., Pham, T. D., Tamir, H., Chen, J. J., & Gershon, M. D. (2004). Enteric dopaminergic neurons: definition, developmental lineage, and effects of extrinsic denervation. *The Journal of Neuroscience*, 24(6), 1330–1339.

Li, Z. S., Schmauss, C., Cuenca, A., Ratcliffe, E., & Gershon, M. D. (2006). Physiological modulation of intestinal motility by enteric dopaminergic neurons and the D2 receptor: analysis of dopamine receptor expression, location, development, and function in wild-type and knock-out mice. The Journal of Neuroscience : *The Official Journal of the Society for Neuroscience*, 26(10), 2798–807. doi:10.1523/JNEUROSCI.4720-05.2006

Linert, W., Herlinger, E., Jameson, R. F., Kienzl, E., Jellinger, K., & Youdim, M. B. H. (1996). Dopamine, 6-hydroxydopamine, iron, and dioxygen—their mutual interactions and possible implication in the development of Parkinson's disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1316(3), 160–168.

Litteljohn, D., Mangano, E., Clarke, M., Bobyn, J., Moloney, K., & Hayley, S. (2010). Inflammatory mechanisms of neurodegeneration in toxin-based models of Parkinson's disease. Parkinson's Disease, 2011, 713517. doi:10.4061/2011/713517

Liu, L., & Coupar, I. M. (1997). Characterisation of pre- and post-synaptic alpha-adrenoceptors in modulation of the rat ileum longitudinal and circular muscle activities. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 356, 248–256.

Liu, Y., Roghani, a, & Edwards, R. H. (1992). Gene transfer of a reserpine-sensitive mechanism of resistance to N-methyl-4-phenylpyridinium. *Proceedings of the National Academy of Sciences of the United States of America*, 89(October), 9074–9078. doi:10.1073/pnas.89.19.9074

Lucchelli, A., Boselli, C., Chiari, M. C., & Grana, E. (1986). Analysis of the relaxing effect of dopamine on the isolated rat jejunum. *Archives Internationales de Pharmacodynamie et de Therapie*, 279(2), 234–247.

Luk, K. C., Kehm, V., Carroll, J., Zhang, B., Brien, P. O., John, Q., & Lee, V. M. (2013). Pathological α-Synuclein Transmission Initiates Parkinson-like Neurodegeneration in Non-transgenic Mice, *Science*, 338(6109), 949–953. doi:10.1126/science.1227157.Pathological

Manning-Bog, A. B., McCormack, A. L., Li, J., Uversky, V. N., Fink, A. L., & Di Monte, D. a. (2002). The herbicide paraquat causes up-regulation and aggregation of ??-synuclein in mice: Paraquat and ??-synuclein. *Journal of Biological Chemistry*, 277, 1641–1644. doi:10.1074/jbc.C100560200

Mannisto, P., Tuomainen, P., & Tuominen, R. (1992). Different in vivo properties of three new inhibitors f catechol O-methyltransferase in the rat. *Br. J. Pharmacol*, (105), 569–574.

Markey, S. P., Johannessen, J. N., Chiueh, C. C., Burns, R. S., & Herkenham, M. a. (1984). Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism. *Nature*, 311, 464–467. doi:10.1038/311464a0

Matison R, Mayeux R, Rosen J, et al. (1982). "Tip-of-the-tongue" phenomenon in Parkinson disease. *Neurology*; 32: 567-570.

McCall, R. B., Lookingland, K. J., Bedard, P. J., & Huff, R. M. (2005). Sumanirole, a Highly Dopamine D2 -Selective Receptor Agonist : In Vitro and in Vivo Pharmacological Characterization and Efficacy in Animal Models of Parkinson ' s Disease. *The Journal of Pharmacology and Experimental Therapeutics*, 314(3), 1248–1256. doi:10.1124/jpet.105.084202.tions

McCormack, A. L., Thiruchelvam, M., Manning-Bog, A. B., Thiffault, C., Langston, J. W., Cory-Slechta, D. A., & Di Monte, D. A. (2002). Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurobiology of Disease*, 10(2), 119–127.

Meredith, G. E., Sonsalla, P. K., & Chesselet, M.-F. (2008). Animal models of Parkinson's disease progression. *Acta Neuropathologica*, 115, 385–398. doi:10.1007/s00401-008-0350-x

Missale, C., Nash, S. R., Robinson, S. W., Jaber, M., Caron, M. G., & Dopamine, M. G. C. (1998). Dopamine Receptors : From Structure to Function, 78(1), 189–225.

Mizuno, Y., Hattori, N., Kubo, S.-I., Sato, S., Nishioka, K., Hatano, T., … Mochizuki, H. (2008). Progress in the pathogenesis and genetics of Parkinson's disease. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 363(1500), 2215–27. doi:10.1098/rstb.2008.2273

Moreira, E. L. G., Rial, D., Aguiar, A. S., Figueiredo, C. P., Siqueira, J. M., Dalbó, S., Prediger, R. D. S. (2010). Proanthocyanidin-rich fraction from Croton celtidifolius Baill confers neuroprotection in the intranasal 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine rat model of Parkinson's disease. *Journal of Neural Transmission*, 117, 1337–1351. doi:10.1007/s00702-010-0464-x

Mounsey, R. B., & Teismann, P. (2010). Mitochondrial dysfunction in Parkinson's disease: pathogenesis and neuroprotection. Parkinson's Disease, 2011, 617472. doi:10.4061/2011/617472

Muramatsu, Y., Kurosaki, R., Watanabe, H., Michimata, M., Matsubara, M., Imai, Y., & Araki, T. (2003). Expression of S-100 protein is related to neuronal damage in MPTP-treated mice. *Glia*, 42(January), 307–313. doi:10.1002/glia.10225

Natale, G., Kastsiushenka, O., Fulceri, F., Ruggieri, S., Paparelli, A., & Fornai, F. (2010). MPTP-induced parkinsonism extends to a subclass of TH-positive neurons in the gut. *Brain Research*, 1355, 195–206. doi:10.1016/j.brainres.2010.07.076

Neve, K. a., Seamans, J. K., & Trantham-Davidson, H. (2004). Dopamine Receptor Signaling. *Journal of Receptor and Signal Transduction Research*, 24(3), 165–205. doi:10.1081/LRST-200029981

Newhouse, K., Hsuan, S. L., Chang, S. H., Cai, B., Wang, Y., & Xia, Z. (2004). Rotenone-induced apoptosis is mediated by p38 and JNK MAP kinases in human dopaminergic SH-SY5Y cells. *Toxicological Sciences*, 79, 137–146. doi:10.1093/toxsci/kfh089

Nicklas, W. J., Vyas, I., & Heikkila, R. E. (1985). Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *Life Sciences*, 36(c), 2503–2508. doi:10.1016/0024-3205(85)90146-8

Obeso, J. a, Rodriguez-Oroz, M. C., Goetz, C. G., Marin, C., Kordower, J. H., Rodriguez, M., … Halliday, G. (2010). Missing pieces in the Parkinson's disease puzzle. *Nature Medicine*, 16(6), 653–61. doi:10.1038/nm.2165

Olsson, C., & Holmgren, S. (2001). The control of gut motility. Comparative Biochemistry and Physiology. Part A, *Molecular & Integrative Physiology*, 128, 481–503. doi:10.1016/s1095-6433(00)00330-5

Palczewski, K., Kumasaka, T., Hori, T., Benhnke, C., Motoshima, H., Fox, B., … Miyano, M. (2000). Crystal Structure of Rhodopsin: A G Protein-Coupled Receptor. *Science*, 289(August), 739–745.

Palumbo, A., Napolitano, A., Barone, P., & d'Ischia, M. (1999). Nitrite-and peroxide-dependent oxidation pathways of dopamine: 6-nitrodopamine and 6-hydroxydopamine formation as potential contributory mechanisms of oxidative stress-and nitric oxide-induced neurotoxicity in neuronal degeneration. *Chemical Research in Toxicology*, 12(12), 1213–1222.

Petzinger, G. M., & Langston, J. W. (1998). The MPTP-lesioned non-human primate: A model for Parkinson's disease. *Advances in Neurodegenerative Disease*, 1, 113–148.

Pfeiffer, R. F. (2011). Gastrointestinal dysfunction in Parkinson's disease. *Parkinsonism & Related Disorders*, 17(1), 10–5. doi:10.1016/j.parkreldis.2010.08.003

Politis, M., Piccini, P., Pavese, N., Koh, S. B., & Brooks, D. J. (2008). Evidence of dopamine dysfunction in the hypothalamus of patients with Parkinson's disease: An in vivo11C-raclopride PET study. *Experimental Neurology*, 214(1), 112–116. doi:10.1016/j.expneurol.2008.07.021

Prediger, R. D. S., Aguiar, A. S., Rojas-Mayorquin, A. E., Figueiredo, C. P., Matheus, F. C., Ginestet, L., … Raisman-Vozari, R. (2010). Single intranasal administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in C57BL/6 mice models early preclinical phase of Parkinson's disease. *Neurotoxicity Research*, 17(2), 114–29. doi:10.1007/s12640-009-9087-0

Prediger, R. D. S., Batista, L. C., Medeiros, R., Pandolfo, P., Florio, J. C., & Takahashi, R. N. (2006). The risk is in the air: Intranasal administration of MPTP to rats reproducing clinical features of Parkinson's disease. *Experimental Neurology*, 202(2), 391–403. doi:10.1016/j.expneurol.2006.07.001

Prediger, R. D. S., Rojas-Mayorquin, A. E., Aguiar, A. S., Chevarin, C., Mongeau, R., Hamon, M., … Raisman-Vozari, R. (2011). Mice with genetic deletion of the heparin-binding growth factor midkine exhibit early preclinical features of Parkinson's disease. *Journal of Neural Transmission* (Vienna, Austria : 1996), 118(8), 1215–25. doi:10.1007/s00702-010-0568-3

Przedborski, S., Chen, Q., Vila, M., Giasson, B. I., Souza, Â. M., Djaldatti, R., … Ischiropoulos, H. (2001). Oxidative post-translational modifications of α-synuclein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease. *J. Neurochem.*, 76, 637–640.

Przedborski, S., Jackson-lewis, V., Djaldetti, R., Liberatore, G., Vila, M., Vukosavic, S., & Almer, G. (2000). The parkinsonian toxin MPTP : action and mechanism, 16, 135–142.

Przedborski, S., Przedborski, S., Naini, A. B., Naini, A. B., Akram, M., & Akram, M. (2001). The parkinson toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydopyridine (MPTP): a technical review of its utility and safety. *Journal of Neurochemistry*, 1265–1274.

Qualman, S. J., Haupt, H. M., Yang, P., & Hamilton, S. R. (1984). Esophageal Lewy bodies associated with ganglion cell loss in achalasia. *Gastroenterology*, 87(4), 848–856.

Ramsay, R. R., & Singer, T. P. (1986). Energy-dependent uptake of N-methyl-4-phenylpyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, by mitochondria. *Journal of Biological Chemistry*, 261, 7585–7587.

Reith, E. a. (2001). Amine Transporters. *Encyclopedia of Life Sciences*, 1–7. doi:10.1038/npg.els.0004091

Riley, D., Lang, a E., Blair, R. D., Birnbaum, a, & Reid, B. (1989). Frozen shoulder and other shoulder disturbances in Parkinson's disease. *Journal of Neurology, Neurosurgery & Psychiatry*, 52(1), 63–66. doi:10.1136/jnnp.52.1.63

Rojo, A. I., Montero, C., Salazar, M., Close, R. M., Fernández-Ruiz, J., Sánchez-González, M. a., … Cuadrado, A. (2006). Persistent penetration of MPTP through the nasal route induces Parkinson's disease in mice. *European Journal of Neuroscience*, 24(March 1988), 1874–1884. doi:10.1111/j.1460-9568.2006.05060.x

Ross, G. W., Petrovitch, H., Abbott, R. D., Nelson, J., Markesbery, W., Davis, D., … White, L. R. (2004). Parkinsonian signs and substantia nigra neuron density in decendents elders without PD. *Annals of Neurology*, 56(4), 532–9. doi:10.1002/ana.20226

Saiardi, A., Bozzi, Y., Baik, J. H., & Borrelli, E. (1997). Antiproliferative role of dopamine: Loss of D2 receptors causes hormonal dysfunction and pituitary hyperplasia. *Neuron*, 19, 115–126. doi:10.1016/S0896-6273(00)80352-9

Samii A, Nutt JG and Ransom BR. (2004). Parkinson's disease. *Lancet*; 363: 1783-1793.

Samuels, E. R., & Szabadi, E. (2008a). Functional neuroanatomy of the noradrenergic locus coeruleus: its roles in the regulation of arousal and autonomic function part I: principles of functional organisation. *Current Neuropharmacology*, 6, 235–253. doi:10.2174/157015908785777229

Samuels, E. R., & Szabadi, E. (2008b). Functional neuroanatomy of the noradrenergic locus coeruleus: its roles in the regulation of arousal and autonomic function part II: physiological and pharmacological manipulations and pathological alterations of locus coeruleus activity in humans. *Current Neuropharmacology*, 6, 254–285. doi:10.2174/157015908785777193

Santos SAPRS (2005) Efeitos adversos dos triptanos: suas bases farmacológicas. Coimbra: Universidade de Coimbra.

Sathe, K., Maetzler, W., Lang, J. D., Mounsey, R. B., Fleckenstein, C., Martin, H. L., … Teismann, P. (2012). S100B is increased in Parkinson's disease and ablation protects against MPTP-induced toxicity through the RAGE and TNF-?? pathway. *Brain*, 135(NOVEMBER 2012), 3336–3347. doi:10.1093/brain/aws250

Sauer, H., & Oertel, W. H. (1994). Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat. *Neuroscience*, 59(2), 401–415.

Schapira, A. H., & Jenner, P. (2011). Etiology and pathogenesis of Parkinson's disease. *Movement Disorders : Official Journal of the Movement Disorder Society*, 26(6), 1049–55. doi:10.1002/mds.23732

Scheibner, J., Trendelenburg, A., Hein, L., Starke, K., & Blandizzi, C. (2002). a 2 - Adrenoceptors in the enteric nervous system : a study in a 2A -adrenoceptor-de ® cient mice. *British Journal of Pharmacology*, 697–704.

Setler, P. E., Sarau, H. M., Zirkle, C. L., & Saunders, H. L. (1978). Carenzi et al . (1975). Charles River male rats weighing 225--300 g were killed by cervical dislocation followed by decapitation and the caudate nuclei rapidly dissected on ice . The caudates were gently homogenized at 0 ° C by hand using a Teflon-glass. *European Journal of Pharmacology*, 50, 419–430.

Shahed, J., & Jankovic, J. (2007). Exploring the relationship between essential tremor and Parkinson's disease. *Parkinsonism & Related Disorders*, 13(2), 67–76. doi:10.1016/j.parkreldis.2006.05.033

Sherer, T. B., Kim, J.-H., Betarbet, R., & Greenamyre, J. T. (2003). Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and α-synuclein aggregation. *Experimental Neurology*, 179(1), 9–16.

Sherwood, L. (2010). Human Physiology: From Cells to Systems. *Centage Learning*, 7th editio, 766. doi:9781111577438

Shimizu, K., Matsubara, K., Ohtaki, K. I., & Shiono, H. (2003). Paraquat leads to dopaminergic neural vulnerability in organotypic midbrain culture. N*euroscience Research*, 46, 523–532. doi:10.1016/S0168-0102(03)00163-9

Shimizu, K., Matsubara, K., Ohtaki, K., Fujimaru, S., Saito, O., & Shiono, H. (2003). Paraquat induces long-lasting dopamine overflow through the excitotoxic pathway in the striatum of freely moving rats. *Brain Research*, 976(2), 243–252.

Shimizu, K., Ohtaki, K., Matsubara, K., Aoyama, K., Uezono, T., Saito, O., … Shiono, H. (2001). Carrier-mediated processes in blood-brain barrier penetration and neural uptake of paraquat. Brain Research, 906, 135–142. doi:10.1016/S0006-8993(01)02577-X

Shulman, L. M., Singer, C., Bean, J. a, & Weiner, W. J. (1996). Internal tremor in patients with Parkinson's disease. *Movement Disorders : Official Journal of the Movement Disorder Society*, 11(1), 3–7. doi:10.1002/mds.870110103

Siegel G. J.,Agranoff B. W., Albers R. W., Fisher S. K., Uhler M. D., (1999), *Basic Neurochemistry – Molecular, Celular and Medical Aspects*, 6th edition;

Singaram, C., Ashraf, W., Gaumnitz, E., Torbey, C., Sengupta, A., Pfeiffer, R., & Quigley, E. (1995). Dopaminergic defect of enteric nervous system in PD patients whith chronic constipation. *The Lancet Neurology*, 346, 861–864.

Singh, R., Pentland, B., Hunter, J., & Provan, F. (2007). Parkinson's disease and driving ability. *Journal of Neurology, Neurosurgery, and Psychiatry*, 78, 363–366. doi:10.1136/jnnp.2006.103440

Slivka, A., & Cohen, G. (1985). Hydroxyl radical attack on dopamine. *Journal of Biological Chemistry*, 260(29), 15466–15472.

Smeets, W. J. a J., & González, A. (2000). Catecholamine systems in the brain of vertebrates: New perspectives through a comparative approach. *Brain Research Reviews*, 33, 308–379. doi:10.1016/S0165-0173(00)00034-5

Smeyne, R. J., & Jackson-Lewis, V. (2005). The MPTP model of Parkinson's disease. *Molecular Brain Research*, 134, 57–66. doi:10.1016/j.molbrainres.2004.09.017

Stanford, S. C. (2001). Adrenaline and Noradrenaline: Introduction. In ELS. *John Wiley & Sons, Ltd*. doi:10.1038/npg.els.0004097

Strange, P. G., & Neve, K. (2013). The Dopamine Receptors. *Tocris Cookson, Ltd*. doi:10.1007/978-1-4757-2635-0

Sulzer, D. (2007). Multiple hit hypotheses for dopamine neuron loss in Parkinson's disease. *Trends in Neurosciences*, 30(5), 244–50. doi:10.1016/j.tins.2007.03.009

Takahashi, N., Miner, L. L., Sora, I., Ujike, H., Revay, R. S., Kostic, V., … Uhl, G. R. (1997). VMAT2 knockout mice: heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. *Proceedings of the National Academy of Sciences*, 94(18), 9938–9943.

Talpade, D. J., Greene, J. G., Higgins, D. S., & Greenamyre, J. T. (2000). In Vivo Labeling of Mitochondrial Complex I (NADH: UbiquinoneOxidoreductase) in Rat Brain Using [3H] Dihydrorotenone. *Journal of Neurochemistry*, 75(6), 2611–2621.

Thiruchelvam, M., McCormack, A., Richfield, E. K., Baggs, R. B., Tank, a. W., Di Monte, D. a., & Cory-Slechta, D. a. (2003). Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. *European Journal of Neuroscience*, 18, 589–600. doi:10.1046/j.1460-9568.2003.02781.x

Tipton, K. F., & Singer, T. P. (1993). Advances in our understanding of the mechanisms of the neurotoxicity of MPTP and related compounds. *Journal of Neurochemistry*, 61, 1191–1206. doi:10.1111/j.1471-4159.1993.tb13610.x

Ungerstedt, U., & Arbuthnott, G. W. (1970). Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Research*, 24(3), 485–493.

Vallone, D., Picetti, R., & Borrelli, E. (2000). Structure and function of dopamine receptors. *Neuroscience and Biobehavioral Reviews*, 24(99), 125–132. doi:10.1016/S0149-7634(99)00063-9

Vander A, Sherman J, Luciano D, Widmaier E, Raff H, S. H. (2001). Human Physiology: The Mechanism of Body Function, 8th Edition. *McGraw-Hill* (Vol. 40, p. 4). doi:10.1017/S0022149X00028327

Vila, M., & Przedborski, S. (2003). Targeting programmed cell death in neurodegenerative diseases. *Nature Reviews. Neuroscience*, 4(May), 365–375. doi:10.1038/nrn1100

Wakabayashi, K., Takahashi, H., Ohama, E., & Ikuta, F. (1990). Parkinson's disease: an immunohistochemical study of Lewy body-containing neurons in the enteric nervous system. *Acta Neuropathologica*, 79, 581–583. doi:10.1007/BF00294234

Walker, J. K., Gainetdinov, R. R., Mangel, a W., Caron, M. G., & Shetzline, M. a. (2000). Mice lacking the dopamine transporter display altered regulation of distal colonic motility. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 279, G311–G318.

Wang, G. J. (2011). Imaging of Brain Dopamine Pathways, *Imaging* 3(1), 8–18. doi:10.1097/ADM.0b013e31819a86f7.

Webb, R. C., Park, S., Gifford, J. R., Andtbacka, R. H. I., Trinity, J. D., John, R., … Macdonald, J. a. (2003). Relaxation Smooth Muscle Contraction and Relaxation. *Advan in Physiol Edu*, (27), 201–206.

Widdowson, P. S., Farnworth, M. J., Simpson, M. G., & Lock, E. A. (1996). Influence of age on the passage of paraquat through the blood-brain barrier in rats: a distribution and pathological examination. *Human & Experimental Toxicology*, 15(3), 231–236.

Williams, D. R., Watt, H. C., & Lees, a J. (2006). Predictors of falls and fractures in bradykinetic rigid syndromes: a retrospective study. *Journal of Neurology, Neurosurgery, and Psychiatry*, 77(4), 468–73. doi:10.1136/jnnp.2005.074070

Xu, J., Kao, S.-Y., Lee, F. J. S., Song, W., Jin, L.-W., & Yankner, B. a. (2002). Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nature Medicine*, 8(6), 600–606. doi:10.1038/nm0602-600

Zhang, J., Xiong, B., Zhen, X., & Zhang, A. (2009). Dopamine D1 Receptor Ligands: where are we now and where are we going. *Medicinal Research Reviews*, 29(2), 272–294. doi:10.1002/med

Zizzo, M. G., Mulè, F., Mastropaolo, M., & Serio, R. (2010). D1 receptors play a major role in the dopamine modulation of mouse ileum contractility. *Pharmacological Research : The Official Journal of the Italian Pharmacological Society*, 61(5), 371–8. doi:10.1016/j.phrs.2010.01.015