

Dissecting gastrointestinal dysfunction and inflammation in a “pre-motor” rodent model of Parkinson’s disease

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Abstract

Research on Parkinson's disease (PD) has mainly focused on the degeneration of the dopaminergic neurons of nigro-striatal (NS) pathway; also, *post-mortem* studies have demonstrated that the noradrenergic and the serotonergic transmitter systems are also affected (Jellinger, 1999). Degeneration of these neuronal cell bodies is generally thought to start prior to the loss of dopaminergic neurons in the NS pathway and precedes the appearance of the motor symptoms that are the "hallmark" of PD.

Gastrointestinal (GI) motility is often disturbed in PD, manifesting chiefly as impaired gastric emptying and constipation. These GI dysfunction symptoms may be the result of a loss in noradrenergic and serotonergic innervation. GI deficits were evaluated using an organ bath technique. Groups treated with different combinations of neurotoxins (6-OHDA alone, 6-OHDA + pCA or 6-OHDA + DSP-4) presented significant differences in gut contractility compared to control groups. Since a substantial body of literature suggests the presence of an inflammatory process in parkinsonian state (Whitton, 2007), changes in pro-inflammatory cytokines in the gut were assessed using a cytokine microarray. It has been found in this work that groups with a combined dopaminergic and noradrenergic lesion have a significant increase in both expressions of IL-13 and VEGF. IL-6 also shows a decrease in treatment groups; however this decrease did not reach statistical significance.

The therapeutic value of Exendin-4 (EX-4) was evaluated. It has been previously demonstrated that EX-4, a glucagon-like peptide-1 receptor (GLP-1R) agonist, is neuroprotective in rodent models of PD (Harkavyi *et al.*, 2008). In this thesis it has been found that EX-4 was able to reverse a decrease in gut contractility obtained through intracerebral bilateral 6-OHDA injection. Although more studies are required, EX-4 could be used as a possible therapy for the GI symptoms prominent in the early stages of PD.

Keywords: Parkinson's Disease, EX-4, Inflammation, Gastrointestinal Dysfunction, Enteric Nervous System.

Resumo

A investigação sobre a doença de Parkinson (DP) é centrada, principalmente, na degeneração dos neurónios dopaminérgicos do eixo nigro-estriado; no entanto, estudos *post-mortem* demonstraram que os sistemas de neurotransmissores noradrenérgico e serotoninérgico são, também, afetados (Jellinger, 1999). A degeneração destes corpos celulares neuronais ocorre, geralmente, previamente à perda de neurónios dopaminérgicos do eixo nigro-estriado e precede o aparecimento dos sintomas motores, que são a "marca" da DP.

A motilidade gastrointestinal (GI) é, frequentemente, afetada na DP manifestando-se, principalmente, através de esvaziamento gástrico lento e obstipação. Estes sintomas de disfunção gastrointestinal podem ser o resultado de uma perda quer na inervação noradrenérgica, quer na inervação serotoninérgica. A contractilidade GI foi avaliada por meio de uma técnica de banho de órgãos. Os grupos tratados com diferentes combinações de neurotoxinas (6-OHDA sozinho, 6-OHDA + pCA ou 6-OHDA + DSP-4) apresentaram diferenças significativas na contractilidade intestinal em comparação com grupos controlo.

Uma vez que um corpo substancial de literatura sugere a presença de um processo inflamatório no estado parkinsoniano (Whitton., 2007), alterações na expressão de citocinas pró-inflamatórias no intestino foram avaliadas através de um *microarray*. Observou-se que, os grupos com uma lesão dopaminérgica combinada com uma lesão noradrenérgica têm um aumento significativo na expressão de IL-13 e VEGF. Apesar de não apresentar significância estatística, a expressão de IL-6 também diminui.

O valor terapêutico de Exendina-4 (EX-4) foi avaliado. Foi previamente demonstrado que EX-4, um agonista de GLP-1R's (do inglês: *glucagon-like peptide-1 receptor*), é neuroprotetor em modelos da PD (Harkavyi *et al.*, 2008). Neste trabalho, sugere-se que EX-4 foi capaz de reverter a diminuição da contractilidade do intestino a partir de injeções bilaterais intracerebrais de 6-OHDA. Com maior quantidade de estudos, a EX-4 poderá ser utilizada como um possível tratamento para os sintomas gastrointestinais proeminentes nas fases iniciais da DP.

Palavras-chave: Doença de Parkinson, EX-4, Inflamação, Disfunção Gastrointestinal, Sistema Nervoso Entérico.

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List of Abbreviations

5-HIAA - 5-Hydroxyindoleacetic acid

5-HT - 5-hydroxytryptamine, serotonin

5-HTP - 5-Hydroxytryptophan

6-OHDA - 6- Hydroxydopamine

AADC - Aromatic L-Amino Acid Decarboxylase

AC - Adenylate cyclase

Ach – Acetylcholine

AchC – Acetylcholine Chloride

AIF – Apoptosis-Inducing Factor

ANS - Autonomic Nervous System

AS – α – synuclein

ATP – Adenosine Triphosphate

ATP13A2 – ATPase Type 13A2

BBB- Blood Brain Barrier

cAMP - Cyclic adenosine monophosphate

c-cas-3 – cleaved caspase-3

CREB - cAMP Response Element Binding Protein

CNS – Central Nervous System

COMT - Catechol-*O*-methyl transferase

DA - Dopamine (3, 4-dihydroxyphenethylamine)

DAT - Dopamine transporter

DMV – Dorsal Motor Nucleus of the Vagus nerve

DPP-4 - Dipeptidyl Peptidase-4

DSP-4 - N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine

EC50 – Half maximal Effective Concentration

ENS – Enteric Nervous System

Epac2 - Exchange Proteins directly Activated by cAMP 2

EX-4 - Exendin-4

EX-(9, 39) – Exendin-(9, 39)

GDNF - Glial cell line-Derived Neurotrophic Factor

GI - Gastrointestinal

GLP-1- Glucagon-like peptide-1

GLP-1R - Glucagon-like peptide-1 receptor

GLP-2- Glucagon-like peptide-2

GRPP – Glicentin-Related Pancreatic Polypeptide

i.c. - Intracerebral (injection)

i.p. – Intraperitoneal

IFN- γ – Interferon-gamma

IL-6 – Interleukin-6

IL-1 β – Interleukin-1 β

IL-13 – Interleukin-13

IL-13R1 – Interleukin-13 Receptor 1

IP-1 – Intervening Peptide-1

IP3 – Inositol Triphosphate

IPAN – Intrinsic Primary Afferent Neuron

KO- Knockout

LC - Locus coeruleus

L-DOPA - L-dihydroxyphenylalanine

LRRK2 - Leucine-Rich Repeat Kinase 2

LPS - Lipopolysaccharide

MAPK - Mitogen Activated Protein Kinase

MAO - Monoamine oxidase

MPTP - 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine

NA - Noradrenaline, norepinephrine

NAT - Norepinephrine (noradrenaline) transporter

NF κ B - Nuclear Factor kappa B

NO – Nitric Oxide

OXM - Oxyntomodulin

PARK7 – Parkinson Disease 7

PBS - Phosphate buffered saline

pCA - Para-chloroamphetamine

PD - Parkinson's disease

PINK1 - PTEN-Induced Putative Kinase 1

PKA - Protein kinase A

PKC - Protein kinase C

PLC – Phospholipase C

ROS - Reactive Oxygen Species

SERT - Serotonin Transporter

SN – Substantia Nigra

SNpc - Substantia nigra pars compacta

TH - Tyrosine hydroxylase

TPH - Tryptophan hydroxylase

TPH1 - Tryptophan hydroxylase 1

TPH2 - Tryptophan hydroxylase 2

TNF- α – Tumor Necrosis Factor-alpha

VEGF – Vascular Endothelial Growth Factor

VIP – Vasoactive Intestinal Peptide

1. Introduction

1.1. History of PD

The pathology now referred to as Parkinson's disease (PD) has been in existence since medieval times and has afflicted all global populations. The ancient Indian medical system of Ayurveda described some symptomatic features of PD under the name *Kampavata* (Manyam and Sanchez-Ramos, 1999). Traditional therapies in the form of herbal preparations containing anticholinergics, levodopa, and monoamine oxidase inhibitors were used in the treatment of PD in India, China, and the Amazon region of South America (Gourie-Devi *et al.*, 1991). Galen of Pergamum (AD 138-201), a prominent Roman physician and philosopher, also described several features of PD and characterized it as the "shaking palsy". However, PD was not formally recognized and its symptoms were not documented until 1817 in *An Essay on the Shaking Palsy* by the British physician James Parkinson (Parkinson, 2002). PD was then known as *paralysis agitans*, the term "Parkinson's disease" being coined later by Jean-Martin Charcot. The underlying biochemical changes in the brain were identified in the 1950s largely due to the work of Swedish scientist Arvid Carlsson, who later went on to win a Nobel Prize in Physiology or Medicine for his research on dopamine (DA) (Carlsson *et al.*, 1957). PD is a progressive disorder and the motor symptoms manifest only when approximately 70% of the DA neurons in the substantia nigra have already degenerated (Fearnley and Lees, 1991). Although there is no current cure for the disease, there are a number of effective symptomatic therapies available (Poewe, 2006). The first specific treatment to be used for PD was L-dihydroxyphenylalanine (L-DOPA), which entered clinical practice in 1967 (Hornykiewicz, 2002). The first large study reporting the efficacy of this drug in patients with PD was published in 1968 (Cotzias, 1968).

1.2. General Introduction

The characteristic symptoms of PD are the result of a selective degeneration of dopaminergic neurons of the nigro - striatal axis (substance nigra - striatum) in the central nervous system (CNS), a highly reduced DA synthesis capacity and a consequent inability to activate DA receptors in the striatum (Whitton, 2007; Iarlori, 2009) .

Despite being strongly related to age PD approximately affects 3 % of the population aged 65 years and 4-5 % of the population aged over 85 years old, 5-10 % of patients are aged less than 40 years old (Iarlori, 2009) . The symptoms are visible only after a "silent" loss of approximately 80 % of dopaminergic neurons in the substantia nigra pars compacta (SNpc), possibly due to an ongoing apoptotic cascade, free radicals formation and neuroinflammation (Perry, 2012) (Figure 1.1).

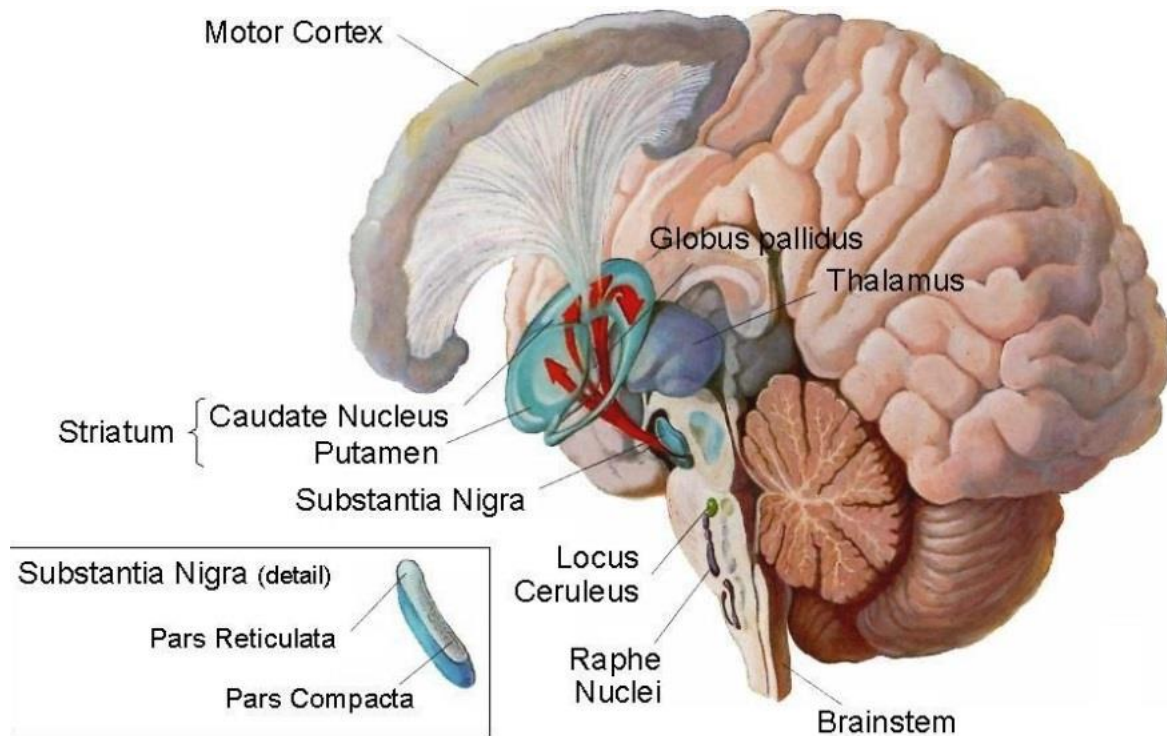


Figure 1.1 – Brain regions affected by Parkinson's disease. Adapted from Dauer and Przedborski, 2003.

However, due to the growing number of aging population - a result of increased quality of public health service - and, as the average life expectancy increases, it is expected that the percentage of patients with PD will increase. Epidemiological studies have shown that sporadic PD cases with late onset occurs in 95% of patients while the remaining 5 % are cases of familial PD with early onset (Tanner and Ben- Shlomo, 1999).

Although most cases of PD may not be associated with a specific cause, familial PD has been associated to mutations in several autosomal dominant and autosomal recessive genes encoding proteins such as parkin , PINK1 (PTEN-Induced Putative Kinase 1) or α - synuclein (Pils and Winklhofer, 2012) .

After four decades of scientific research, a therapeutic strategy that provides a cure or at least the possibility of preventing the progress of the disease remains elusive. Treatments are based on drugs that provide symptomatic relief and do not show efficacy in all patients. Additionally, the side effects of most drugs used in PD patients are associated with a high mortality rate, especially in cases of chronic use. Likewise, the treatment effectiveness inevitably decreases as cell death progresses. For this reason, there is a need and a lack of an innovative therapy that is both affordable and, most importantly, has the potential to prevent disease progression and mitigate symptoms (Harkavyi *et al.*, 2008; Hurtig, 1997).

1.3. Neuroanatomy in PD

The extended observation of Lewy bodies - the main location of α - synuclein (AS) - is a major feature of the pathology. AS is a small and relatively unstructured protein, with sticky elements that make it suitable to aggregate. Lewy bodies - eosinophilic inclusions with a diameter of approximately 8-30 μm and consisting of abnormal, insoluble aggregates of AS - are usually circular with a eosinophilic core and surrounded by a pale halo. Researchers believe that when a small cluster of AS is formed, it tends to attract proteins in the same neighborhood and eventually triggers the formation of long, insoluble fibrils which may be broken into fragments of shorter length. AS clusters of smaller size and formed near the site where the aggregation process starts are the most toxic forms of the protein and its toxicity will destroy neighboring neurons. If these structures are symptomatic or causative of PD is still unclear.

Braak and colleagues found that, in samples with mild pathology, which Braak called Stage 1, the Lewy bodies are typically confined to the olfactory bulb and the dorsal motor nucleus of the vagus nerve (DMV). In Stage 2, Lewy bodies continue to ascend into the brainstem, reaching the medulla oblongata and pontine tegmentum, parts of the brainstem that control swallowing, sleep, and other autonomic functions sometimes affected in PD. By Stage 3, pathology starts to show up in the amygdala (a mass of neurons involved in processing fear and other emotions, but also the sense of smell) and in the substantia nigra; this is the stage when the motor phase of the disorder begins. In Stage 4, pathology in areas affected in earlier stages worsens, and Lewy bodies progress to the forebrain and invade a portion of the cerebral cortex (the temporal mesocortex), whereas the neocortex, the part of the brain involved in higher functions, remains unaffected. In Stages 5 and 6, the pathology is full blown, appearing initially in the anterior association and prefrontal areas of the neocortex and then spreading to the posterior association areas, which are involved with memory and learning, and planning movement (Figure 1.2). Defects in these areas could explain many of the cognitive problems associated with advanced PD (Braak *et al.*, 2003a; Braak *et al.*, 2004).

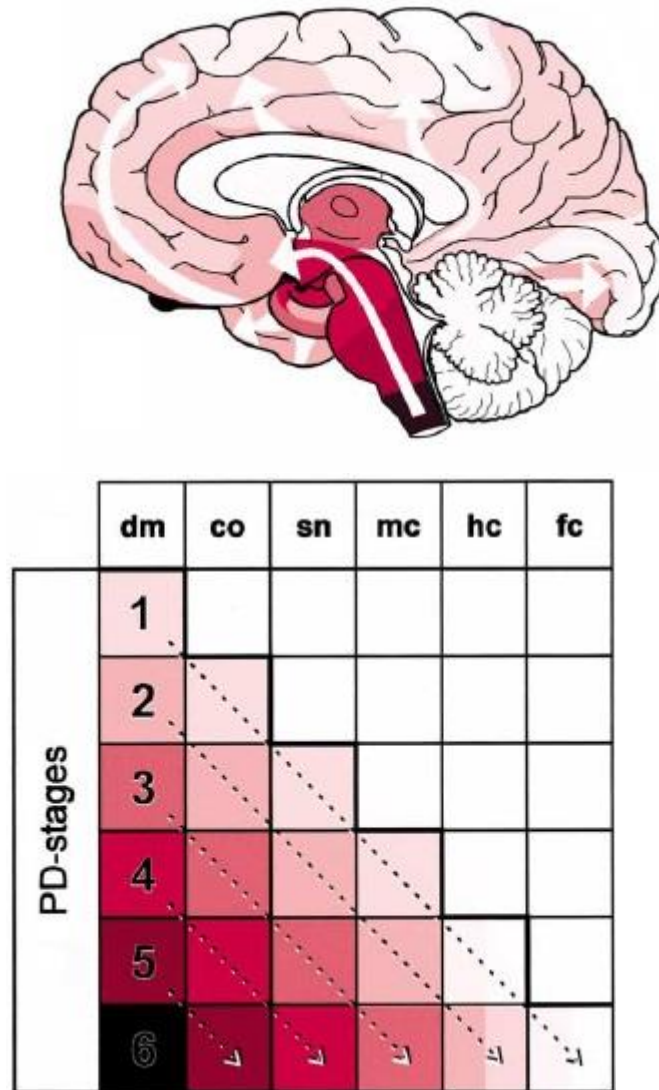


Figure 1.2 - Progression of PD-related intraneural pathology (Adapted from Braak *et al.*, 2003a). Upper – Diagram showing the ascending pathological process (white arrows). The increasing intensity of the colored areas indicates the growing severity of the pathology in vulnerable brain regions. Lower – Simplified diagram showing the topographic expansion of the lesions (from left to right: dm to fc) and, simultaneously, the growing severity on the part of the overall pathology (from top to bottom: stages 1–6). List of abbreviations: dm, dorsal motor nucleus of the vagal nerves; co, coeruleus complex; sn, substantia nigra; mc, mesocortex; hc, high order sensory association areas; fc, first order sensory association areas.

1.4. Motor and Non-motor Symptoms

The best motor symptoms associated with PD include tremors, rigidity in movement, language problems, cognitive problems, bradykinesia - slowness of coordinated voluntary movements - and

hypokinesia - loss of coordinated voluntary movements. Postural instability, which is characterized by loss of postural reflexes, leads to balance difficulties and a higher propensity for falls.

Other motor symptoms include dystonia - abnormal and painful muscle contractions - mostly on the feet but may include other skeletal muscles. Patients with PD have difficulty in swallowing; the facial muscles are also affected.

In the last decade, the so-called non-motor symptoms have attracted considerable scientific attention and, particularly, due to the appearance of new hypotheses concerning the beginning and progress of the disease (Woitalla and Goetze, 2011). The non-motor symptoms experienced by patients with PD have been increasingly recognized as part of the degenerative process associated with the pathology and contribute significantly to the morbidity and mortality related to the disease (Salat - Foix and Suchowersky, 2012).

Some of the non-motor symptoms include neuropsychiatric and cognitive disorders, anxiety, fatigue, apathy and dementia (visual and auditory hallucinations) and complications in the gastrointestinal (GI) system - constipation, fecal incontinence and abnormalities in intestinal transit. Although some of these symptoms are manifested before diagnosis of the disease (PD diagnosis requires identification of parkinsonian motor symptoms), its prevalence, severity and impact on quality of life of patients increases its progression.

A comprehensive survey of PD patients in the 1980's found that problems with saliva occurred in 70% of PD patients, dysphagia (trouble swallowing) in 52%, nausea in 24%, and constipation in 29%. In addition, defecatory dysfunction was reported by two thirds of PD patients – twice the control prevalence (Edwards *et al.*, 1994). Subsequent studies have confirmed the high frequency of GI abnormalities in PD. Abnormal gastric emptying has been described in 43%-88% of PD patients and can worsen as PD progresses (Goetze *et al.*, 2006; Goetze *et al.*, 2005). The incidence rate of constipation has been estimated to be anywhere from 29%-89% and problems with defecation are also much more common in PD (~60%) than in age-matched controls (Siddiqui *et al.*, 2002; Singer *et al.*, 1992). Total GI tract transit time is also significantly prolonged in PD (Davies *et al.*, 1996).

1.5. Physiology

1.5.1. Dopamine

DA was first discovered in 1952 by the Swedish scientist Arvid Carlsson. Arvid Carlsson was awarded the Nobel Prize for demonstrating that DA was a neurotransmitter in its own right and not just a precursor of noradrenaline and adrenaline. DA is the predominant neurotransmitter belonging to the group of catecholamines in the mammalian brain. The name “dopamine” is derived from its

precursor L- DOPA (Dihydroxy L- phenylalanine). DA belongs to the group of the catecholamine neurotransmitters – a family that possesses a catechol functional group in its structure.

Three of the major four dopaminergic pathways originate in the substantia nigra (SN) of the midbrain. The nigro - striatal axis is involved in the control of movement and is affected in PD and in other pathologies responsible for perturbations in the movement. The remaining two pathways originating from the SN, via the mesolimbic and mesocortical pathways, have a central role in the control of emotion and motivation. The fourth route of DA synthesis starts in the hypothalamus and is projected to the pituitary gland, which regulates the secretion of hormones.

The rate limiting step in the synthesis of DA is catalyzed by the enzyme tyrosine hydroxylase (TH), which is an oxidase that converts tyrosine to L- DOPA. TH is present in all cells with the ability of producing catecholamine neurotransmitters. L- DOPA is then decarboxylated by aromatic amino acid decarboxylase (AADC), forming DA and CO₂.

Neurons that use DA as their neurotransmitter only possess TH and AADC unlike, for example, noradrenergic neurons that also possess β -hydroxylase which in turn adds a hydroxyl group to DA to form noradrenaline. During development, the expression of genes encoding enzymes responsible for the synthesis of catecholamines can be regulated independently.

There are several mechanisms for the inactivation of DA after release into the synaptic cleft . The main mechanism is the reuptake of DA in the synaptic cleft back into the presynaptic dopaminergic neuron through membrane transporters. Released DA into the synaptic cleft also undergoes enzymatic degradation by monoamine oxidase (MAO) – found on the outer membrane of mitochondria – and by catechol -O-methyl transferase (COMT).

There are, at least, five dopamine receptors - D1 to D5. The five receptors have similarities among them since all of them present metabotropic properties – meaning they are G-protein-linked. The D1 and D5 receptors stimulate the production of cyclic adenosine monophosphate (cAMP), while the D2, D3 and D4 receptors inhibit the production of cAMP (Kebabian and Calne, 1979).

D1 and D2 were the first to be discovered and are the most common in nigro-striatal axis. The D4 receptor is expressed in the hypothalamus and limbic areas associated with emotions.

1.5.2. Noradrenaline

Noradrenaline (NA) was isolated by Polish physiologist Napoleon Cybulski in 1895. NA shares the first two reactions in its biosynthetic pathway with DA in a series of enzymatic steps from the amino acid tyrosine. However, cells producing NA possess dopamine- β -hydroxylase enzyme, which adds a hydroxyl group to DA to form NA. Unlike other enzymes in the biosynthetic pathways of small molecules, DA - β -hydroxylase is a membrane associated protein strongly attached to the

inner surface of aminergic vesicles. Consequently, NA is synthesized within vesicles, and thus synthesized as a single transmitter.

In the CNS, NA is used as a neurotransmitter by neurons whose cell bodies are present in the locus coeruleus (LC). Although these noradrenergic neurons are present in small numbers, their projections are widespread across the cortex, cerebellum, striatum, hypothalamus, hippocampus and spinal cord. NA is usually excitatory in nature and has been implicated in governing arousal, motivation, emotional state and alertness.

NA exerts its effects via activation of noradrenergic G-protein coupled receptors, which are clustered into two main groups - α (α_1 and α_2) and β (β_1 , β_2 and β_3). Upon activation of α_1 -adrenergic receptors, a G-protein (G_q) activates phospholipase C (PLC), which causes an increase in inositol triphosphate (IP3) and Ca^{2+} . This triggers further effects, primarily through the activation of Protein Kinase C (PKC). The activation of α_2 -adrenergic receptors causes the inactivation of adenylate cyclase (AC), resulting in a decrease of the second messenger cAMP produced from adenosine triphosphate (ATP). The activation of β -adrenergic receptors causes an increase in the intracellular concentration of cAMP. The effector of cAMP is the protein kinase A (PKA), which will enhance protein phosphorylation.

There are several mechanisms of NA inactivation after its release in the synaptic cleft. The major mechanism is reuptake back into the presynaptic terminal via NA transporters (NAT) present in the membrane. Released NA is also subject to enzymatic cleavage by MAO and by COMT.

In addition to acting as neurotransmitter, NA also functions as a hormone. As a hormone, NA is released through the adrenal medulla, along with adrenaline in the blood in conditions of sympathetic nervous system activation (control under stress). This activation usually takes the form of the “fight or flight response”, hereafter NA has the ability to increase heart rate, blood pressure, glycogenolysis in the liver and adipose tissue lipolysis.

1.5.3. Serotonin

Vittorio Erspamer was the first scientist to isolate serotonin (5-HT), in 1935 from enterochromaffin cells - a type of endocrine cells in the epithelium of the digestive tract and respiratory system. These cells contain approximately 90 % of the 5-HT stored in the body.

Two enzymes are necessary to synthesize serotonin from tryptophan - tryptophan hydroxylase (TPH) and 5-hydroxytryptophan (5-HTP) decarboxylase. The rate limiting step of the reaction is catalyzed by the first enzyme in the biosynthetic pathway - TPH. In the CNS, the neuronal cell bodies of serotonergic neurons are found in and around the nucleus of Raphe and in the brain stem, which are

involved in regulating the level of attention and other cognitive functions. The projections of these neurons are widely distributed in various areas of the brain and spinal cord.

In the peripheral nervous system, 5-HT functions to regulate GI motility, blood clotting, vasoconstriction, and cell growth.

There are seven families of 5-HT receptors (5-HT₁ - 5HT₇) responsible for both inhibitory and excitatory signals. With the exception of 5-HT₃ receptor - an ion channel regulated by binding of its ligand -, the other 5-HT receptors are coupled to G proteins, thereby activating an intracellular second messenger cascade. 5-HT receptors modulate the release of many neurotransmitters, including glutamate, GABA, DA, adrenaline / NA, and acetylcholine (Ach).

There are several mechanisms of 5-HT inactivation after its release in the synaptic cleft. The major mechanism is reuptake back into the presynaptic terminal through 5-HT transporter (SERT) present in the membrane. The released 5-HT is also subject to enzymatic cleavage and is converted to 5-hydroxyindoleacetic acid (5-HIAA) by MAO.

The 5-HT receptors influence various biological and neurological processes such as aggressive behavior, anxiety, appetite, cognition, learning, memory, sleep and thermoregulation. The 5-HT receptors are subject to a wide variety of drugs and illegal drugs such as antidepressants, antipsychotics, hallucinogens and amphetamines.

1.6. Gastrointestinal Dysfunction in PD

The most prominent clinical features associated with PD are the motor symptoms, mainly associated with the loss of dopaminergic neurons in the SNpc. Symptoms associated with GI dysfunction had previously attracted a minority of attention but now a new focus of interest and an increasing number of references are addressing the GI system in the pathogenesis of PD. The GI tract has a special importance because it can be the starting point of the disease (Woitalla and Goetze, 2011).

The GI tract is the largest interface between neural tissue and the environment and has a sufficiently high number of neuronal cells to be known as the "second brain". The lumen of the GI tract holds the largest and most diverse resident microbial community in the human body, with the ability to induce inflammation and pro-oxidative pathways. In the various regions of the gastrointestinal (GI) tract, muscle layers of the gut wall and their innervation are adapted and organized to subserve the motor functions of that region. Along the GI tract, the gut interacts with the CNS through autonomic neurons. Various types of GI dysmotility have been documented repeatedly in PD, mainly delayed gastric emptying and obstipation, and most likely reflect dysfunction at one or more levels of the brain-gut axis (Kellow *et al.*, 1999).

Korczyn described that the gastrointestinal symptoms precede motor symptoms in Parkinson patients (Korczyn and Gurevich, 2010). The DMV is always involved in the pathology of the disease in Stage 1 (Braak *et al.*, 2004). Once the vagus nerve connects the brain to the enteric nervous system (ENS), the authors proposed that the Lewy bodies could form in the gut and move along the vagus nerve in an upstream, or retrograde, direction toward the brain.

1.6.1. Neuroanatomy of GI Dysfunction in PD

The basis of an appropriate GI transport is a coordinated contraction of smooth muscle cells (most of the GI tract is composed of smooth muscle cells). The contractions and local reflections are coordinated by the ENS located in the intestinal wall. The ENS besides being responsible for bowel movements also integrates impulses from the CNS (through extrinsic innervation from the vagal nerve).

The ENS is a subdivision of the autonomic nervous system (ANS). The ENS comprises two neuronal plexus - the submucosal (or Meissner's) plexus, which controls mucosal secretion and blood flow, and the myenteric (or Auerbach's) plexus, which controls peristaltic movements. From a functional point of view the Auerbach's plexus operates as a multisynaptic reflex, in which local factors, such as wall distention or the chemical composition of intraluminal contents, are sensed by the so-called intrinsic primary afferent neurons (IPANs). IPANs subsequently transmit this information to specific neurons capable of triggering motor, secretomotor or vasomotor responses that ultimately reach the smooth muscle cells (Figure 1.3) (Salat-Foix and Suchowersky, 2012).

The ENS has approximately one hundred million neurons (one thousandth of the number of neurons in the brain) and a number of neurotransmitters, including DA and Ach. The main excitatory neurotransmitter in the ENS and in the parasympathetic nerves is Ach, and in the sympathetic nerves the main inhibitory neurotransmitter is NE. In all the other systems nitric oxide (NO), vasoactive intestinal peptide (VIP) and ATP are the main inhibitory neurotransmitters (Figure 1.3). Despite the ability of the ENS to run autonomously, it interacts closely with the CNS. In the stomach, the myenteric plexus connects directly to the vagus nerve, providing a direct link between the CNS and stomach.

The mobility in the small intestine as well as in all parts of the GI system is modulated predominantly by excitatory or inhibitory signals from the ENS. However, GI mobility is also regulated by signals originating in the CNS. GI hormones (gastrin, GLP1, GLP2) also appear to affect the GI mobility in some way.

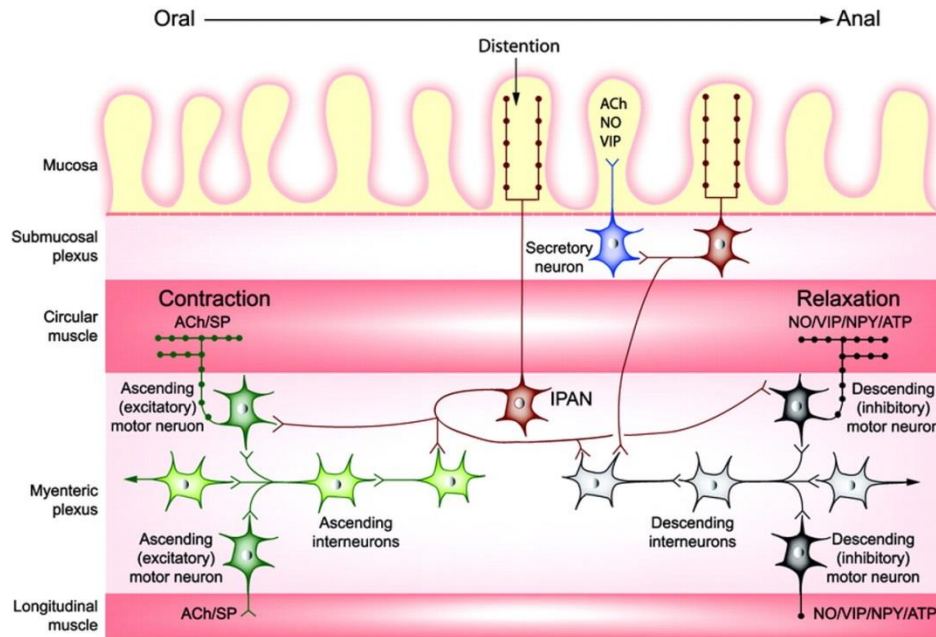


Figure 1.3 - Schematic representation of some of the connections involved in local enteric reflexes. (Adapted from Benarroch, 2007). List of abbreviations: IPAN, intrinsic primary afferent neurons; Ach, acetylcholine; NO, nitric oxide; VIP, vasoactive intestinal peptide; SP, substance P; NPY, neuropeptide Y; ATP, adenosine triphosphate.

Phosphorylated AS in Lewy bodies and Lewy neurites - one of the pathological hallmarks of idiopathic PD - are present in the myenteric plexus of patients with PD and the first observation of AS in the ENS coincides with the appearance of the same in the DMV. These inclusions have been detected in patients with advanced PD, but also in non-symptomatic patients with lesions limited to the brain stem, thus supporting the hypothesis that changes in the GI tract may be an initial characteristic of pathology. Although animal models have inherent limitations and the findings related to the accumulation of AS and its dissemination are not wholly consistent, there is a growing body of evidence to suggest that the GI system may be critical to the pathogenesis of PD.

Drolet *et al.* exposed healthy rats to low doses of rotenone for six weeks, a mitochondrial complex I inhibitor. Animals lost some of their ability to coordinate digestion and showed evidence of Lewy bodies in enteric neurons (Drolet *et al.*, 2009).

1.6.2. Gastrointestinal Complications

GI disorders are common in all stages of PD patients. Approximately 30% of PD patients report structural and functional abnormalities in the GI tract, and practically, vulnerabilities have been observed throughout the entire GI system (Table 1.1).

Swallowing difficulties and constipation symptoms were included in the first description of PD in 1817 (Parkinson, 2002). Constipation is the most traditional symptom of GI dysfunction in PD patients. Constipation was observed in 90% of patients and it has been described as a feature of the pre-motor stages of the disease.

The stomach is one of the first places where deposition of AS is observed in parkinsonian patients, suggesting that the stomach, although not receiving as much attention as abnormalities of gut motility, is also involved in the pathogenesis of PD.

Gastrointestinal disorders affect the quality of life of many patients and an altered gastric function may cause inadequate absorption of drugs, exacerbating the classic motor symptoms of PD. The worsening of GI dysfunction in PD is repeatedly associated with the progression of PD state.

Table 1.1 – GI symptoms associated with PD (adapted from Marrinan *et al.*, 2013)

Level of the GI tract	Site of dysfunction	Problem
Upper GI tract	Mouth	Sialorrhea
		Dental deterioration
	Pharynx	Oropharyngeal dysphagia
	Esophagus	Esophageal dysphagia
	Stomach	Delayed gastric emptying
		Gastroesophageal reflux
Lower GI tract	Small intestine	Dilatation
	Large intestine	Constipation
		Dysmotility
		Volvulus
		Megacolon
		Perforation
	Rectum	Defecatory difficulties

Except for dysphagia, which may lead to weight loss and respiratory tract infections (aspiration pneumonia is a leading cause of death among PD patients), GI symptoms are mainly a source of discomfort and social embarrassment, and their overall impact on the patient's life expectancy is considered marginal.

1.6.3. Catecholamines in the ENS

Catecholamines modulate GI motility. Nerve endings of the sympathetic nervous system release NA, which inhibits the release of ACh from parasympathetic motor neurons (by α_2 -adrenoceptors) and decreases the contraction of intestinal smooth muscle cells. Moreover, the release

of ACh from parasympathetic nerve terminals enhances the contraction of smooth muscle through activation of M receptors.

DA was recently hailed as an enteric neurotransmitter as DA, TH and DA transporter (DAT) are co-localized in neurons of the ENS.

DA acts as a negative regulator of motility in the GI tract to inhibit the release of ACh from cholinergic neurons expressing dopamine D2 receptor.

Tian *et al.* recently demonstrated an increased expression of DA, TH and DAT in the GI tract of mice subjected to a bilateral injury induced by a bilateral injection of 6- hydroxydopamine (6-OHDA), a neurotoxin with selective toxicity to dopaminergic neurons. Given the inhibitory nature of DA in ENS, an overexpression of this neurotransmitter would reduce the mobility and contractions of the GI tract, especially gastric emptying (Tian *et al.*, 2008).

In fact, in patients with PD, administration of the D2 antagonist, domperidone receptor, accelerates gastric emptying delayed by administration of therapy with L- DOPA (Tonini *et al.*, 2004).

1.6.4. Serotonin in the ENS

For decades, it has been extensively described in the literature the role of 5-HT in regulating GI function. The majority of 5-HT is synthesized and stored in the intestine and different types of serotonergic receptors are found in the intestinal wall. However, despite many evidences, the actual functions of this neurotransmitter in the GI tract have proven difficult to identify. The reasons for this gap are related to the existence of 5-HT prevenient from neurons in the CNS and 5-HT prevenient from neurons in the ENS and to the widespread and overlapping distribution of specific subtypes of serotonergic receptors.

In 2011, Li *et al.* (Li *et al.*, 2011) used knockouts (KO) of tryptophan hydroxylase 1 (TPH1), the rate-limiting enzyme in the synthesis of enteric 5-HT, and KO of tryptophan hydroxylase 2 (TPH2), the rate-limiting enzyme in the synthesis of neural 5-HT, to selectively delete the production of 5-HT from the two possible sources. The TPH1 KO did not differ from controls in any studied function - GI emptying, intestinal transit and colonic motility. By contrast, TPH2 KO showed major changes in each of the examined functions; KO mice for both enzymes were indistinguishable from TPH2 KO animals. The presence of a mediator to replace 5-HT in KO TPH1 is plausible. However, without a likely candidate for a compensatory mediator, the most obvious conclusion is that 5-HT in the intestinal mucosa has a very minor role in the regulation of GI motility in the mouse whereas, neural 5-HT may have a more substantial role than previously thought.

Perhaps the 5-HT from the intestinal mucosa plays a significant role only after some pathophysiological insult, for example, inflammation. Moreover, neuronal 5-HT is clearly necessary

for normal functioning of the GI tract. The role of enteric 5-HT remains unclear and requires further study, especially, being the source of all circulating 5-HT.

1.6.5. Diagnostic and Therapy

The identification of gastrointestinal symptoms in parkinsonian patients is relatively direct and straightforward through appropriate questions directly to the patient. GI symptoms may be related to the administration of antiparkinsonian drug therapies, either as a side effect, or as a manifestation of improper treatment.

The management of these symptoms in PD patients should be performed identically to the management of the same in the general population - anticholinergics and botulinum toxin to prevent the frequency of drooling, proper diet and laxatives if constipation. The treatment of dysphagia within the PD patients is usually ineffective (Menezes and Melo, 2009). The adjustment of dopaminergic treatment is rarely effective and usually maintaining a proper diet with intake of homogeneous food with thick texture is the best solution. Patients are advised to eat small portions and chew their food several times before swallowing (Baijens and Speyer, 2009). If the above measures prove ineffective, feeding through a gastric tube may be necessary.

The management of constipation in PD is carried out along the general lines that are observed in any other setting. Nonpharmacological measures, such as increasing dietary fiber and fluid intake, and avoiding sedentarism, are recommended, although their effectiveness has not been proven in well-designed clinical trials. Pharmacologic treatment, if needed, can be attempted with various laxatives (Salat-Foix and Suchowersky, 2012).

1.7. Neuroinflammation

Several hypotheses have been postulated regarding the possible causes of neuronal neurodegeneration observed in PD patients. These include genetic factors, environmental toxins, mitochondrial dysfunction and cell death mediated by free radicals (Schapira, 1994; Rosenberg, 2002). Although there is a smaller body of evidence to suggest that neuroinflammation is the main stimulus to the onset of neurodegeneration, preclinical and epidemiological data suggest that chronic, slow and steady, neuroinflammation may be a reason for neuronal dysfunction during the asymptomatic phase of PD (Lee *et al.*, 2009).

Neuroinflammation induced by exposure to either infectious agents or toxic agents with proinflammatory characteristics is currently recognized as a major contributor to the pathogenesis of PD (Whitton, 2007).

The traditional features of neuroinflammation include the presence of activated microglia cells and reactive astrocytes, direct participation of the adaptive immune system and overproduction of cytokines, chemokines, prostaglandins and reactive oxygen species (ROS 's), which have the ability, in certain cases, to penetrate the blood-brain barrier (BBB) (Ransohoff and Perry , 2009).

Over a long period of time, the CNS has been considered a prime location of the immunological point of view because of an absent classic immune response (Di Filippo *et al.*, 2008). Thus, it is also thought that the brain would not be greatly affected by systemic immune or inflammatory reactions (Lucas *et al.*, 2006).

It is now widely accepted that there is an interaction between the nervous and immune systems involving a two-way cross-talk. Indeed, the CNS is stocked with a classical active immune surveillance and can be the starting point of an inflammatory responses caused by different types of lesions (Di Filippo *et al.*, 2008).

The secreted inflammatory mediators are responsible for the expression of cytokines and adhesion molecules, activation of the glial cells and stimulating astrogliosis (Figure 1.4). Therefore, neuroinflammation is seen as a complex cellular and molecular response of neuronal cells to harmful stimuli such as injury, stress or sepsis (Semmler *et al.*, 2008; Whitney *et al.*, 2009). The main objectives of neuroinflammation are defending against these damaging insults, removal of damaged neurons and maintain homeostasis of the CNS.

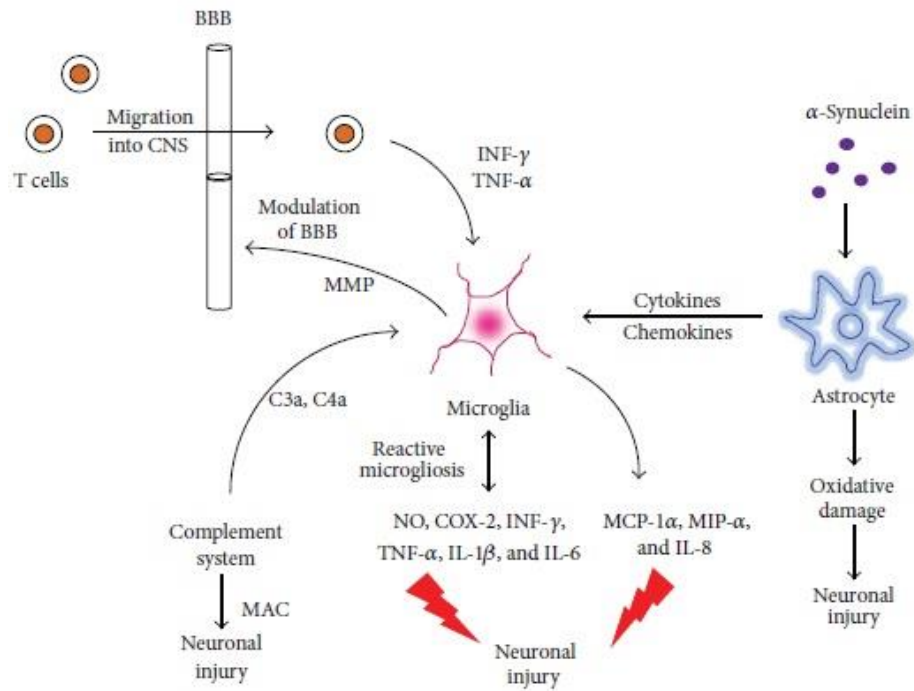


Figure 1.4 - A simplified schematic of the interaction between microglia and astrocytes (Adapted from More *et al.*, 2013). Abbreviations: NO: nitric oxide, COX-2: cyclooxygenase, INF-γ: interferon-γ, TNF-α: tumor necrosis factor α, IL-1β: interleukin-1β, IL-6: interleukin-6, MCP-1α: monocyte chemoattractant protein-1, MIP-α: macrophage inflammatory protein, IL-8: interleukin-8, MAC: membrane attack complex, α-syn: α-synuclein, MMP: matrix metalloprotein, BBB: blood brain barrier, C3a: complement component 3a, and C4a: complement component 4a.

However, when neuroinflammation is not controlled, its beneficial effects are subdued and may contribute to progression of the damage. In this case, the recruited cells release more inflammatory mediators, and establish a positive feedback leading to neuronal damage with changes in neurogenesis (Swarup *et al.*, 2008, Whitney *et al.*, 2009). Additionally, oligodendrocytes are extremely vulnerable to inflammatory molecules, which can result in damage to white matter in the appearance of neuromotor, cognitive and behavioral limitations in neurodegenerative disorders such as PD or Alzheimer's Disease.

1.7.1. Cytokines as inflammatory mediators

Cytokines are small signaling molecules - peptides, proteins or glycoproteins - involved in cell-to-cell communication, immune responses and movement of cells towards sites of inflammation, infection and trauma. Previously, cytokines were thought to only act in the peripheral system. Nowadays, an increasing number of observations reveal that these molecules exert different actions in the CNS and they are involved in neuronal development. Proinflammatory cytokines such as Tumor

Necrosis Factor- α (TNF- α) and interleukin-1 β (IL- β) are synthesized by neural cells and participate in standard intercellular communication, assuming an important role in maintaining cellular homeostasis (Giulian *et al.*, 1988, Zhao and Schwartz, 1998).

Typically, the levels of these cytokines are decreased (in order of picomolar), but their expression increases rapidly in the event of neuroinflammation and this increase may be 1000 fold. When this increased expression is unsustainable, proinflammatory cytokines can become pathological, deregulating cytokine release and causing the death of neurons and oligodendrocytes (Donnelly and Popovich, 2008).

Yet, the detrimental effect of cytokines may rely on the type and level of cytokines produced. In other words, the low physiological rates of cytokine expression may be important for the cross-talk between the neural cells during development, but the overexpression observed during the neuroinflammatory stage may compromise neuronal survival and plasticity.

Therefore, regulating the production of neuroinflammatory mediators or their action on their receptors would be an effective approach to mitigate the inflammatory processes in PD. This knowledge may be helpful in developing pharmacologic strategies for treating neuroinflammation in PD (Whitton, 2007).

1.8. Causative Factors

Most cases of PD are not inherited and although aging is a clear risk factor, the disease affects only a fraction of the elderly population. This indicates the involvement of external factors - genetic and environmental - as causatives of the disease.

1.8.1. Genetic Factors

PD is a multifactorial disease in which 5% of cases are hereditary in nature, caused by mutations in autosomal dominant and recessive genes. Autosomal dominant mutations in genes encoding proteins such as α -synuclein and Leucine-Rich Repeat Kinase 2 (LRRK2), and autosomal recessive mutations in genes encoding parkin, PTEN-Induced Putative Kinase 1 (PINK1), ATPase Type 13A2 (ATP13A2) and Parkinson Disease 7 (PARK7) are involved in the degeneration associated the pathogenesis of PD.

Mutations in the proteins encoded by these genes have resulted in the formation of aggregates of misfolded proteins, changes in the ubiquitination pathway and changes and defects in the mitochondrial respiratory chain.

1.8.2. Environmental Factors

Sporadic PD (95% of cases have a sporadic nature) is caused by environmental factors and toxins. Herbicides such as paraquat and rotenone, or the toxin MPTP (1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine) appear to induce parkinsonism in humans and animal models. Either MPTP or rotenone are inhibitors of complex I of the mitochondrial respiratory chain, which will cause a decrease in ATP production and an increase in the synthesis of ROS's. Activation of apoptotic cascades (members of the Bcl-2 and caspase family) will trigger the death of neurons in the nigro-striatal axis.

These findings were observed when analyzing post mortem brain tissue of patients with PD who presented elevated levels of lipid peroxidation markers in the CNS, suggesting high levels of oxidative stress (Andersen, 2004).

1.9. Current Treatments for Motor Symptoms

1.9.1. Pharmacological Treatments

Currently, there are several sites of action for pharmacological therapies related to PD, however, existing drug therapies on the market have the potential to only attenuate or delay the progression of symptoms and do not prevent or reverse the motor and non-motor symptoms of the disease.

Administration of Levodopa will increase DA levels.

In PD, DA depletion is accompanied by an increased activity of the striatal cholinergic system, subsequent rearrangement of the striatal circuitry, and appearance of the motor symptoms. Ach inhibitors block the action of this neurotransmitter in the striatum, attenuating the stiffness and gear-wheel movements, typical of patients with PD. Antagonists of muscarinic cholinergic receptors were the first drugs approved and introduced in the market for the treatment of PD (Hobson *et al.*, 2002), being the first demonstration of the functional antagonism between DA and Ach - activation of inhibitory dopamine D2 receptors reduces the release of Ach (Polymeropoulos *et al.* 1997). Although this class of drugs has shown beneficial effects, it is also associated with adverse neuropsychiatric effects (Katzenschlager *et al.*, 2003).

The use of Amantadine stimulates the release of DA in the synaptic cleft and inhibits the reuptake of the released DA in the synaptic cleft back into the presynaptic neuron, increasing the period of time during which DA remains bound to its receptors.

The use of MAO inhibitors - present in the outer mitochondrial membrane which plays a major role in oxidative deamination of monoamines, such as DA - is another recurring

pharmacotherapeutic strategy in the treatment of PD. There are two isoforms of the enzyme, MAO-A and MAO-B. The B-isoform is present predominantly in the brain and is found in astrocytes but not neuronal cells (Westlund *et al.*, 1985). The most well-known MAO inhibitor in the treatment of PD is selegiline, which was introduced in 1989, followed by rasagiline - a selective compound for MAO-B. The administration of selegiline will prevent degradation of DA, which will be available for release into the synaptic cleft (Deane *et al.*, 2004).

Another pharmacotherapy strategy for the treatment of PD is the administration of COMT inhibitors. COMT is an intracellular enzyme located in postsynaptic neurons whose substrate is any compound having a catechol structure, including L-DOPA, DA and NA (Deane *et al.* 2004). COMT inhibitors act peripherally to prevent L-DOPA and DA degradation. COMT inhibitors cause a prolonged stay of DA in the synaptic cleft and enhance its effects.

There are two drugs in this class which have been approved for PD treatment. Tolcapone, introduced in 1997, followed by entacapone, in 1999. Both drugs increase the bioavailability of L-DOPA, but also causes a large number of side effects, as diarrhea, hypotension, acute liver failure (Rinne *et al.*, 1998; Obeso *et al.*, 2000).

One of the most common therapeutic strategies is to stimulate DA receptors in the striatum directly through the administration of DA receptors agonists. Several DA are available clinically, including rotigotine, pramipexole and piribedil. Some of the side effects observed in patients under administration of DA agonists include excessive sleepiness and sleep attacks (Richard and Kurlan, 1997), pathological gambling, and related impulse control disorders where there is an inability to resist an impulse desire despite negative consequences.

1.9.2. Experimental Treatments

The possibility of developing cell transplantation therapies for PD is based on the premise that it is the degeneration of nigro-striatal axis the main cause of the onset of symptoms associated with the disorder.

Neurotransplantation is a recently studied experimental strategy for the treatment of PD involving the implantation of cells producing AD in the brain of patients. However, information regarding the effectiveness of this treatment is limited and is not, to date, an accepted treatment throughout the medical community. Promising results were obtained in studies involving rats undergoing treatment with 6-OHDA, where improvements were observed in the progression of motor symptoms (Hurtig *et al.*, 2000).

Chemical infusion directly into the basal ganglia was also performed in some patients with PD. This technique aims an infusion of growth factors into the brain, preventing cell death and

simultaneously stimulating the growth of DA-producing neurons. This procedure is still in clinical trials and 10 to 20 years will be needed until its effects are visible.

For many years research has been done in order to deepen the possibility of replacing damaged DA producing cells by brain tissue from human fetuses, hoping that fetal tissue has the ability to produce DA and possibly fix the problems caused by the failure of the same. To date, results have proved ambiguous and treatment is only experimental. Scientists believe that this type of treatment will probably remain experimental in the next 5-10 years.

Stem cells, especially human embryonic stem cells have the ability to differentiate into dopaminergic neurons , although behaviors and cell survival are limited and the potential risk of teratoma formation is high (Goya *et al.*, 2008).

1.10. Current Treatments for GI Dysfunction

Although GI symptoms significantly impair quality of life for PD patients, their treatment is made more difficult by ignorance of the complex enteric regulating mechanisms and lack of medical treatment options. Established treatment options for GI dysfunction differ according to the individual clinical picture and the symptoms to be treated.

Despite growing knowledge of the factors influencing GI motility, therapeutic options are still limited, and only a small number of studies are being performed to examine drug effects on GI function in humans. Limitations of medical knowledge are even more striking, especially concerning studies on GI dysmotility as a condition in PD (Woitalla and Goetze, 2011).

1.10.1. Dopamine Receptors

The clinical effects of levodopa on gastric emptying are controversial. By stimulating dopaminergic chemoreceptors, levodopa causes nausea. In its peripheral effects, levodopa normalizes partially myoelectric activity in the stomach, probably due to its dopaminergic activity in the stomach itself (Lu *et al.*, 2004). Other reports describe the inhibition of ileum coordination, an effect that can be antagonized by blocking peripheral DA receptors (Schuurkes and Van Neuten., 1984).

Domperidone, a potent, mainly peripheral-acting DA antagonist relieves symptoms of nausea, vomiting, anorexia, abdominal bloating and regurgitation but not constipation in PD patients (Soikan *et al.*, 1997). Domperidone increases the amplitude of oesophageal motor function and accelerates gastric emptying (Weihsrauch and Ehl, 1981). Considering the effects of domperidone on a cellular level, its action is probably due to blocking D2 receptors (Reddymasu *et al.*, 2007).

1.10.2. 5-HT₄ Receptor Agonists

Of the 5-HT₄ receptor agonists, cisapride has the widest use. Studies in PD patients have been conducted with cisapride, showing beneficial effects on motor fluctuations (Djaldetti *et al.*, 1995). Cisapride is a peripherally acting substance with positive effects on GI function in PD (Jost, 1997), improving levodopa absorption and motor fluctuations in PD patients (Djaldetti *et al.*, 1995). Tegaserod, a 5-HT₄ agonist was approved for constipation. Studies in PD revealed a tendency for improvement of abdominal discomfort and symptoms (Sullivan *et al.*, 2006). Prucalopride is a 5-HT₄ agonist approved for colonic constipation. The stimulating effects of prucalopride in gastric and colonic transit have been demonstrated (Bouras *et al.*, 2001).

1.11. Glucagon Family and Exenatide

1.11.1. Introduction to the GLP-1 family peptides

Glucagon-Like Peptide 1 (GLP-1) is a newly discovered molecule, derived from the L cells of the intestine. This peptide has insulintropic properties and this is the predominant feature that generated interest in its research. GLP-1 has been described for the first time in 1985 (Schmidt *et al.*, 1985) after cloning the gene encoding proglucagon. GLP-1 is largely responsible for increasing the amount of insulin released by pancreatic β cells after ingestion of nutrients, and for decreasing the secretion of glycogen. GLP-1 acts on a transmembrane G-coupled receptor, the Glucagon-Like Peptide 1 Receptor (GLP-1R) thus stimulating AC and cAMP formation, with downstream effects (Yada *et al.*, 1993) in gene expression.

In addition to its insulintropic properties, GLP-1 is involved in neogenesis, differentiation and proliferation of β cells in islets of Langerhans of the pancreas, in the activation of the hypothalamic-pituitary-adrenal axis and neuroprotection and response to stress in the CNS. GLP-1 functions are summarized in Figure 1.5.

Processing of the proglucagon gene gives rise to 29 amino acid glucagon itself and a number of biologically active peptides including glicentin, oxyntomodulin (OXM), GLP-1 and glucagon-like peptide 2 (GLP-2). Both OXM and glicentin contain the whole 29 amino acid sequence of glucagon and a C-terminal 8-amino acid extension called intervening peptide-1 (IP-1). Compared to OXM (37 amino acids), glicentin (69 amino acids) also contains the N-terminal extension called glicentin-related pancreatic polypeptide (GRPP). OXM has recently been found to suppress appetite and a recent clinical study found that it could be used as a treatment for obesity (Wynne *et al.*, 2006b). The mechanism of action of OXM is poorly understood. It has been shown to bind to both the glucagon-

like peptide 1 receptor (GLP-1R) and the glucagon receptor, but it is likely that its effects are mediated by a novel receptor (Wynne and Bloom, 2006a). Effects of glicentin are not well understood, but it is thought to be implicated in the growth of intestinal mucosa by mechanisms involving the GLP-1R (Ayachi *et al.*, 2005). On the other hand, a lot more is known about functions of GLP-1 and its mechanisms of action

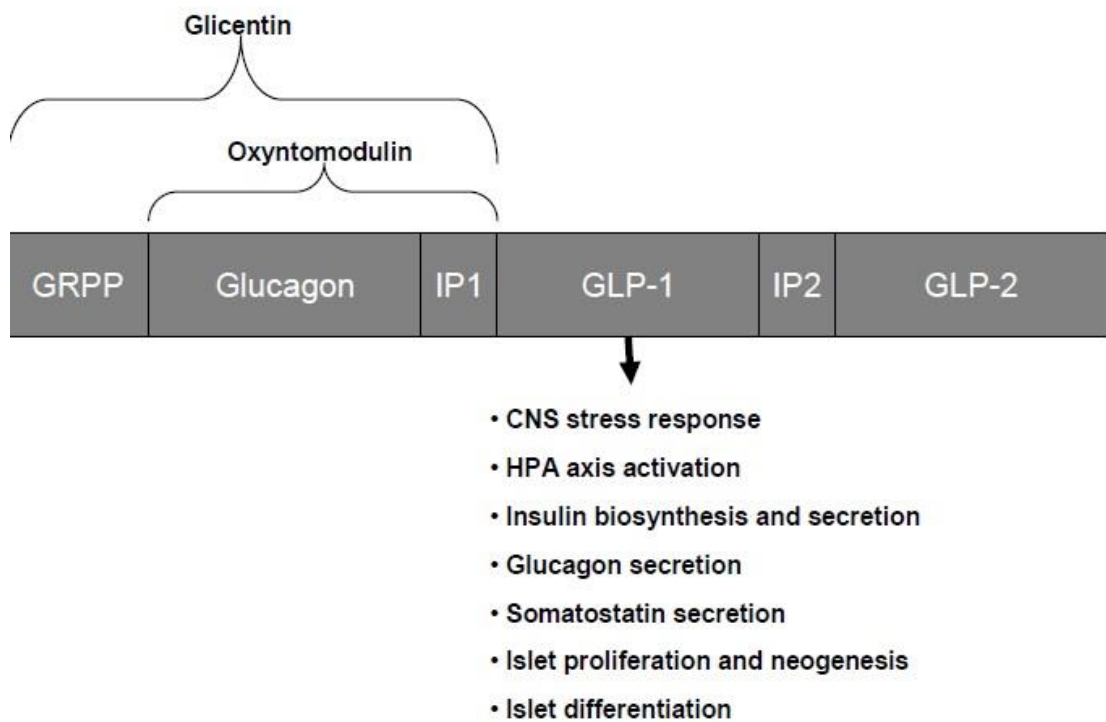


Figure 1.5 – Structure of proglucagon gene fragment contains sequences coding for several biologically active peptides: Glicentin Related Pancreatic Polypeptide (GRPP), glucagon, Intervening Peptide 1 (IP1), Glucagon-Like Peptide 1 (GLP1), Intervening Peptide 2 (IP2) and Glucagon-Like Peptide 2 (GLP-2). Figure also summarizes the known functions of GLP-1 (Adapted from Rampersaud, 2010).

1.11.2. Glucagon-Like Peptide 1 Receptor

GLP-1R is a classic seven transmembrane domain G-protein coupled receptor. The cDNA s of GLP-1R of rat and human have been cloned and sequenced, for the first time in the 90's . The rat and the human GLP-1Rs show 95 % homology in amino acid sequence, differing only at position 42 (Tibaduiza *et al.* 2001). The human GLP-1R gene was mapped on the long arm of chromosome 6, band p21.1 and encodes a protein of 64 kDa (Stoffel *et al.* 1993).

GLP-1R has been identified and expressed in a wide range of tissues and cells including α , β and δ cells of the islets of Langerhans, intestines, lungs, heart, kidneys and various CNS regions,

including the hypothalamus, thalamus and brainstem. Additionally, although at a lower density of neuronal binding sites were identified in the striatum, hippocampus and cerebral cortex (Calvo *et al.*, 1995; Goke *et al.*, 1995).

The extracellular N-terminal region of GLP-1Rs is essential for binding to GLP-1 while the different domains in the third intracellular loop are critical for coupling to specific G-proteins. GLP-1Rs can interact with *Gas*, *Gaq*, *Gao* and *Gai* proteins leading to an increase in AC activation. Activation of AC gives rise to increased production of cAMP. GLP-1Rs are also responsible for activating signal transduction pathways of PKA, PKC and MAPK (Mitogen Activated Protein Kinase) (Drucker *et al.*, 1987).

A Gq subunit was shown to activate PLC pathway, leading to PKC activity and an increase in intracellular Ca^{2+} (Wan *et al.*, 2004). It is believed that PKC is responsible for activating Nuclear Factor kappa B (NF- κ B), known for its anti-apoptotic properties (Fowler *et al.*, 1996).

1.11.3. Exenatide

The list of therapies implemented in patients with PD is based only on relief and symptom control in the various stages of the disease. No universally accepted approach to modify disease progression, despite the large financial investment in clinical trials with the use of drugs with neuroprotective potential effect exists.

There is, however a recent interest in the use of GLP-1R agonists as therapeutic agents in neurodegenerative diseases. The first to be discovered, exendin-4 (EX-4) which is a naturally occurring form of exenatide, was originally isolated from the saliva of the lizard *Heloderma suspectum* (Eng *et al.*, 1992), also known as the Gila monster. This peptide was named exendin by Eng and Raufman since it was isolated from an exocrine gland (Eng *et al.*, 1990; Eng *et al.*, 1992). The synthetic form of EX4, exenatide (Byetta®) is the first GLP-1 mimetic to receive FDA approval in 2005 (Gedulin *et al.*, 2005).

EX-4 has 53 % homology with mammalian GLP-1 and exerts its effect by activating GLP-1R's (Chen and Drucker, 1997). In mammals, GLP-1 is degraded by dipeptidyl peptidase -IV (DPP-4) and its plasma half-life is approximately 90 seconds (Kolterman *et al.*, 2003). However, EX-4 appears to be more resistant to inactivation by the same enzyme and has a plasma half-life substantially longer. EX-4 has beneficial effects on glucose homeostasis through stimulation of insulin release in a glucose dependent manner. EX-4 has been shown to increase β -islet mass by promoting its proliferation and neogenesis from precursor cells in both *in vitro* and *in vivo* (Tourrel *et al.*, 2002).

1.11.4. Neuroprotective Effects

The first evidence that EX-4 might have neuroprotective properties appeared in 2002. Both EX - 4 and its endogenous peptide analogue GLP- 1 were shown to be neuroprotective, behaving in a similar manner to nerve growth factor.

In 2008, Harkavyi *et al.* demonstrated that EX -4 reversed the loss of extracellular DA in the striatum, using two animal models of PD, 6-OHDA and LPS (lipopolysaccharide) (Harkavyi *et al.*, 2008). These neuroprotective effects are mediated by GLP- 1R since incubation of cells with a GLP-1R antagonist reversed all the protective actions stimulated by GLP-1R. This evidence suggests that pharmacological manipulation of GLP- 1R would have substantial therapeutic utility in PD.

In contrast to other peptide agents that have demonstrated neuroprotective properties in preclinical models of PD, EX-4 crosses freely the BBB contributing to the evaluation of clinical efficacy of EX - 4 in patients with PD.

In 2009, Li and co-workers showed that treatment with EX -4 protected dopaminergic neurons from degeneration, preserving DA levels and improving motor function in an animal model of PD induced by MPTP (Li *et al.*, 2009). The results from this study offer further support to the idea that EX-4 works by activating GLP-1Rs by testing the drug in GLP-1R KO (Li *et al.*, 2009; Vaillancourt *et al.*, 2009)

Finally, in 2013, Aviles-Olmos developed a clinical trial with a population of 45 patients with PD, where individuals treated with EX-4 injections for a period of one year showed an improvement of 2.7 points in the index MDS - UPDRS (Movement Disorder Society - Unified Parkinson's Disease Rating Scale) compared with a decline of 2.2 points in patients in the control group (Aviles-Olmos *et al.*, 2013).

The mechanism of action of the compound in PD pathogenesis remains unclear but there is considerable evidence that influences the formation, maintenance and mode of action of mitochondria. It is possible that EX-4 promotes neurogenesis, influences the development or increases mitochondrial survival. The authors demonstrated that EX-4 reduces oxidative stress, thus contributing to the anti-apoptotic action of the drug (Aviles-Olmos *et al.*, 2013).

1.12. Aims of the project

PD is widely distributed to neuropathy of dopaminergic neurons in the nigrostriatal pathway (Hirsch *et al.*, 1988). Degeneration of noradrenergic and serotonergic neurons in the brain and non-motor symptoms, including GI dysfunction, may actually precede the DA lesion but has received comparatively little attention (Braak *et al.*, 2006; Jellinger, 1999). It is hypothesized that damage in GI motility is present already in the early phase of the disease and greatly deteriorate the patient's quality of life (Jost, 2009). The aim of this project was to study GI motility dysfunction and inflammation in a premotor neurochemical rodent model of PD that will allow a better understanding of non-motor symptoms with the future goal of improved treatment.

Recently, increasing evidence from human and animal studies has suggested that neuroinflammation is a cause or rather a consequence of neurodegeneration. Several findings have indicated that immune mechanisms are involved in the pathogenesis of PD. Neurons, including enteric neurons, as a result of lack of ability to divide and little ability to recover from injury, are extremely vulnerable to a destructive immune and inflammatory response. Pro-inflammatory cytokines are an essential component of the inflammatory processes and are closely related to the degeneration of neurons. (Iarlori, 2009).

In this project it was utilized the selective toxins *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) and parachloroamphetamine (pCA) to create partial lesions of the noradrenergic and serotonergic systems, respectively. DSP-4 has long been used to create lesions of NA afferents stemming from the LC (Archer and Fredriksson, 2006; Ross and Reis, 1974; Ross, 1976; Srinivasan and Schmidt, 2003). pCA has been used extensively to target serotonergic afferents and reduce overall 5-HT and 5-Hydroxyindoleacetic acid (5-HIAA) content (Kornum *et al.*, 2006; Leonard, 1976). These lesions were then followed by a bilateral intracerebral (i.c.) injection of 6-OHDA to create a partial dopaminergic deficiency and mimic the premotor parkinsonian condition (Branchi *et al.*, 2008; Lee *et al.*, 1996; Sauer and Oertel, 1994). This sequential administration of the toxins was performed to duplicate the supposed progression of the clinical pathology. Ileum contractions were assessed using an organ bath and then dose-response curves were established. Samples were analyzed for the simultaneous detection of TNF- α , Vascular Endothelial Growth Factor (VEGF), Interferon-gamma (IFN- γ), IL-1 β , Interleukin-6 (IL-6) and Interleukin-13 (IL-13) using a rat cytokine array and performed in duplicate.

Recent experiments strongly suggest that stimulation of GLP-1 receptors by the peptide EX-4, is neuroprotective in several systems, even as a late-stage intervention in rodent models of PD (Bertilsson *et al.*, 2008; Harkavyi *et al.*, 2008; Kim *et al.*, 2009). It was thought to evaluate the therapeutic value of EX-4 regarding GI motility in the "pre-motor" rodent model used in this project. In the performed experiments, EX-4 was only used in tissue from 6-OHDA treated animals due to the

extended body of literature regarding this GLP-1R agonist and rodent models of Parkinsonism that focus mainly in the degeneration of dopaminergic neurons. In 2008, Harkavyi and co-workers used a variety of measures, neurochemical and histological which indicate a clear protective role for EX-4 against 6-OHDA mediated nigrostriatal lesions. When EX-4 is given after 6-OHDA, a reversal of TH+ immunoreactivity loss occurs (Harkavyi *et al.*, 2008). This might suggest that EX-4 is able to rescue dopaminergic neurons once damage is established.

Also Li and colleagues showed that EX-4 afforded complete protection against dopaminergic neuron damage and motor impairment promoted by MPTP. Mice given EX-4 before MPTP showed no differences from controls in TH+ immunoreactivity and DA levels (Li *et al.*, 2008).

This principle (if reproduced in the human GI tract) would have obvious clinical significance since GI symptoms are often underdiagnosed. A delay in diagnosis may reflect a tendency of clinicians, patients, and family members to focus on the more apparent motor features of PD, which are visible only once neuronal loss reaches 70-80%.

2. Materials and Methods

2.1. Reagents

Table 2.1 - List of reagents and supplementary materials used in the course of work.

Reagent	Manufacturer
Air containing 95% O ₂ / 5% CO ₂ Medical Gas Mixture	BOC Medical
Acetylcholine Chloride	Sigma Aldrich
Calcium Chloride, minimum 93% granular, anhydrous	Sigma Aldrich
D-Glucose	BDH Chemicals Ltd
DSP-4	Sigma Aldrich
IsoFlo 100% w/w Inhalation Vapour, Liquid	Abbott
L-Ascorbic Acid	Sigma Aldrich
Magnesium Sulfate Heptahydrate	Fluka BioChemika
pCA	Sigma Aldrich
Phosphate Buffer Saline	Sigma Aldrich
Potassium Chloride	BDH AnalaR
Potassium Dihydrogen Orthophosphate	BDH AnalaR
Sodium Chloride	BDH AnalaR
Sodium Hydrogen Carbonate	BDH AnalaR
6-OHDA	Sigma Aldrich

2.2. Equipment

Table 2.2 - List of equipment used in the course of work.

Equipment	Manufacturer
Autovortex Mixer SA2	Stuart Scientific
Centrifuge Biofuge Pico	Heraeus Instruments
Microplate Mixer	SciQuip
Needles 0,5 mm x 16 mm	Microlance
Non-Sterile Carbon Steel Surgical Blade 10, 11 and 22 mm	Swann-Morton
Organ Bath	Harvard Apparatus
Pipettes 10, 20, 100, 200, 1000 μ L	Gilson
Plate Reader	Versamax
Potter-Elvehjem Homogenizer MPD 6488	Challenge
Scale	Sartorius
Stereotaxic frame	David Kopf Instruments
Magnetic Stirrer	HANNA Instruments
Student Oscillograph	Harvard
Syringe 10 μ L	Hamilton Company
Transduction Amplifier	Harvard

2.3. Solutions

Table 2.3 – Composition of Krebs Solution used in the course of work. This solution is prepared immediately before use and stored at 4° C.

Krebs Solution (2.5 L) pH 7.2	
Reagent	Concentration (mM)
NaCl	136.9
NaHCO ₃	11.9
D-Glucose	5.55
KCl	2.68
MgSO ₄ .7H ₂ O	1.05
KH ₂ PO ₄	0.42
CaCl ₂	1.8

Make up with nanopure water to a volume of 2.5 L.

Table 2.4 – Composition of PBS Buffer Solution used in the course of work. This solution is stored at room temperature.

PBS Buffer Solution 10x	
Reagent	Concentration (mM)
NaCl	137
KCl	2.7
Na ₂ HPO ₄	10
KH ₂ PO ₄	1.8

2.4. Methods

2.4.1 Animals and Husbandry

Albino Wistar rats (180-250 g) were purchased from Harlan Laboratories, Inc., UK and group-housed (n = 4 per cage) in the Biological Services Unit (BSU) of the university. The BSU maintained conditions of constant humidity (40-60%), temperature (18-22°C), and a 12 hr light-dark cycle (light presented from 07:00-19:00 hrs daily) in accordance with Home Office regulations. Access to food (standard rodent diet) and water was *ad libitum*. Animals used were subjected to a 7-day habituation and handling period prior to experimental usage. All experimental procedures were conducted in strict adherence to the terms of the 1986 Animals (Scientific Procedures) Act.

2.4.2 Stereotaxic surgery

Animals were secured using blunt ear bars to a stereotaxic frame (David Kopf, U.S.A.) and anaesthetized using isoflurane – 5% in O₂ for induction and 2,5% CO₂ for maintenance – delivered through an anesthetic nose mask (Abbot Laboratories Ltd. Kent). A sterile blade was used to expose the surface of the skull. The bregma was located and referenced with a black fine tip marker. Stereotaxic coordinates from the atlas of Paxinos and Watson (1982) were used to locate the ventrolateral area of the dorsal striatum (from bregma in mm; AP 1.1 mm, ML 3.2 mm and DV -7.2 mm). A dental drill fitted with a tungsten carbide burr tip (2 mm) was used to drill an insertion point through the skull exposing the dura. A 10 µL Hamilton syringe (Hamilton Company, U.S.) was used to administer the intracerebral (i.c) injections.

2.4.3. 6-OHDA Injection

6-OHDA was dissolved 5mg/ml in saline containing 0.2% ascorbic acid. 15 µg of 6-OHDA or vehicle (saline) were injected into the left and right striatum of each rat to induce partial destruction of the nigro-striatal dopaminergic system. After the injection was complete, the needle was kept in place for 2 minutes to ensure adequate local diffusion of 6-OHDA. The needle was then slowly retracted to prevent any unwanted back flow. The site of incision was closed using Michel clips and immediately following surgery the animal was placed on a cage and wrapped in a paper.

2.4.4 DSP-4 Intraperitoneal Injection

DSP-4, is a neurotoxin with selective and long-lasting effects on both central and peripheral NA nerve terminals of mammalian brains (Ross and Reis, 1974). It was injected at a dose of 25mg/kg via intraperitoneal (i.p.) injection four days prior to 6-OHDA i.c. injection – the chosen dose was selected to induce partial degeneration of the NA neurons in the LC. DSP-4 was dissolved 10 mg/mL in H₂O.

2.4.5. pCA Intraperitoneal Injection

pCA is an amphetamine derivative and monoamine releaser. pCA acts as a serotonergic neurotoxin on prolonged administration or at high dosage due to the unrestrained release of serotonin from axon terminals by a nonexocytotic mechanism and blocks the reuptake of serotonin. pCA also inhibits tryptophan hydroxylase activity. It was injected at a dose of 6 mg/kg via i.p. injection four days prior to 6-OHDA i.c. injection – the chosen dose was selected to induce partial degeneration of the serotonergic nerve terminals and cell bodies. pCA was dissolved 5 mg/ml in H₂O.

2.5. Experimental Protocol and Animal Usage

Each experimental group consisted of 3 rats. DSP-4 and/or pCA or vehicle insult was induced 4 days before exposure to either 6-OHDA or vehicle lesion. This methodology was selected to mimic the sequential condition of neurodegeneration present in PD patients. In other words, a serotonergic or a noradrenergic deficit was induced prior to a dopaminergic insult. At the conclusion of the treatment period, the animals were sacrificed and their ileums were removed to perform subsequent organ bath measurements and cytokine array analysis. Figure 2.4 graphically illustrates the experimental protocol employed in this study.

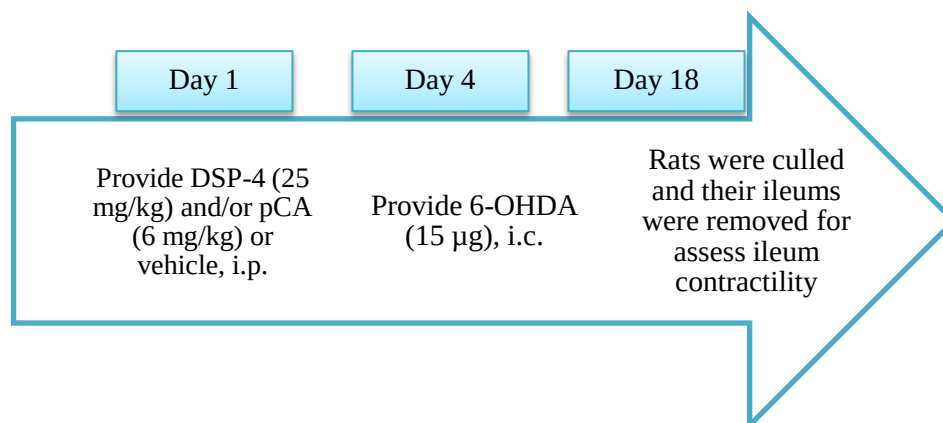


Figure 2.1 – Experimental protocol

2.6. Removing the gut

An upward direction cut was made through the abdominal wall of the rat using pointed scissors. When the distal small intestine was loose from the body wall, it was moistened and submerged in ice-cold Krebs solution. Luminal contents, adhering fat and connective tissues were gently flushed with Krebs solution with little pressure by using a syringe. Small intestine, specifically the ileum was cut longitudinally in several sections (1,5-2 cm) and the sections were kept in Krebs solution before being mounted on the transducers in a 25 mL organ bath containing Krebs solution at 37° C, continuously oxygenated with 95% O₂/5% CO₂. For assay purposes, ileum was chosen, because is able to produce a steady base-line for studying the effects of drugs on movements. Pieces of ileum will continue to give responses for many hours if kept in a suitable salt solution. Up to three segments of full-thickness strips, were used from each animal. Each preparation was used for a single experiment only.

2.7. Mounting the gut in the organ bath

The preparation used to study how the size of the response varies with the concentration of drug applied to it is a segment of rat ileum. As said before, a 1,5- 2 cm length of ileum was taken and attached to an organ bath using needle and cotton. The piece of ileum was mounted vertically - the lower end of the ileum was tied to a silk thread hook and the hook was mounted on the ring stand so the ileum is in the organ bath (25 mL) filled with Krebs solution (37°C ±0,5°C) aerated continuously with oxygen (95% O₂, 5% CO₂) to ensure the viability of the tissue throughout the experiment. The upper end of the ileum was connected to an isotonic transducer (Harvard Transducer, UK). The force transducer was carefully raised until the gut is under slight tension. The gut should be completely submerged in Krebs solution. Smooth muscle contraction of the ileum was measured using a Harvard isotonic transducer under 1g weight. Ileum contractions were displayed and recorded on Universal Harvard Oscillograph, (UK). The specimens were allowed to equilibrate for 45 min – 1 h before the addition of the different drugs.

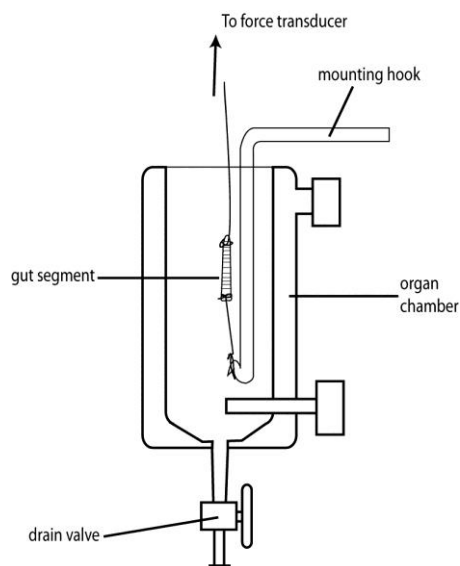


Figure 2.2 – Scheme of a gut segment mounted in the organ chamber for recording.

2.8. Effect of acetylcholine chloride (AChC) concentration in gut contractility

Contraction was induced by acetylcholine chloride (AChC, stock solution 10 μ M) and then dose response curves were established. An initial volume of AChC was added to the bath to induce contraction of the ileum. AChC was in contact with the tissue for 30 seconds before it was washed off with fresh Krebs solution before the addition of another volume of AChC solution (drugs such AChC produce a very rapid response and can be washed out after 30 seconds). Occasionally, it was necessary to wash the preparation a second time especially if large effects were produced with the previous dose, to exclude the risk of the tissue not having had enough time to recover completely. Effect of AChC was examined on contraction using two fold concentration increments until there was no further increase in the magnitude of the response and the next trial was not performed until the contractions of the segment of rat ileum were restored to a steady state.

2.9. Effect of Exendin-4 (EX-4) in gut contractility

Effect of Exendin-4 (EX-4) (1 μ g/mL) was examined on contraction induced by AChC and matched controls were evaluated. The concentration of AChC added to the specimen was doubled each time until there was no further increase in the magnitude of the response. 320 μ L of the 1 μ g/mL EX-4 stock solution were added into the bath solution when the contraction of intestinal smooth muscle induced by AChC reached the peak. After the addition of EX-4 a five minutes break was made before the addition of AChC to test whether EX-4 was able to stimulate the magnitude of the response. After

the addition of AchC the contraction curve was recorded within 30 seconds. All the drugs were then removed from the bath and fresh Krebs solution was added, and the next trial was not performed until the contractions of the segment of rat ileum were restored to a steady state. The injections were performed according the following scheme:

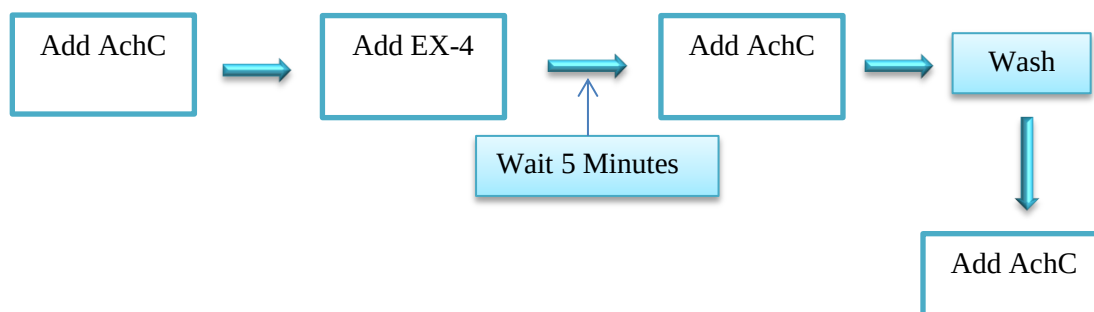


Figure 2.3 – Diagram representing the addition of EX-4 to the organ bath.

2.10. Effect of Exendin-(9,39) in gut contractility

Exendin-(9,39) (EX-9,39) (1 $\mu\text{g/mL}$) was added to the bath and the effect of this peptide on the segment of rat ileum was evaluated. EX-4 was first added into the bath solution. When the contraction of intestinal smooth muscle induced by EX-4 reached the peak, 320 μL of the 1 $\mu\text{g/mL}$ EX-(9,39) stock solution were added into the bath solution. After the addition of EX-(9,39) a five minutes break was made before the addition of EX-4 to test whether EX-(9,39) was able to inhibit the magnitude of the response. After the addition of EX-4 the contraction curve was recorded within 30 seconds. The concentration of EX-4 added to the specimen was doubled each time. EX-4 was then removed from the bath and fresh Krebs solution was added. The next trial was not performed until the contractions of the segment of rat ileum were restored to a steady state.

The injections were performed according the following scheme:

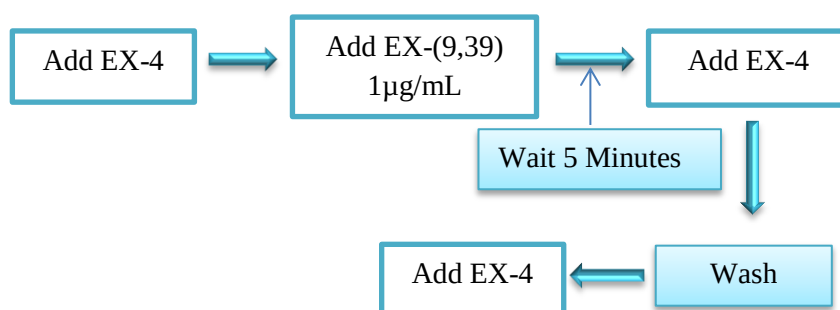


Figure 2.4 – Diagram representing the addition of EX-(9, 39) to the organ bath.

2.11. Tissue dissection and homogenization

A piece of ileum with approximately 1 cm length was dissected and put it in 1mL PBS solution with protease inhibitors (10µg/mL aprotinin, 10µg/mL leupeptin, 10µg/mL pepstatin) and 75% Triton X-100 in the Potter-Elvehjem homogenizer machine's glass beaker. Then, the tissue was homogenized with the apparatus set at 500-1500 rpm, allowing 5-10 seconds per stroke until the piece of tissue becomes a liquid as homogeneous as possible. Between homogenization of each sample, the Potter-Elvehjem homogenizer machine's glass beaker was washed three repeated times with PBS.

Samples were aliquoted and snap frozen in liquid nitrogen and thawed in a water bath at 37,5 °C. Freeze-thaw cycle was repeated three times in order to disrupt cell membrane. Samples were left on a rotator at 4°C for 1 hour and centrifuged at 10 000 x g at room temperature for 15 minutes to pellet cell debris. Supernatant was transferred to a fresh eppendorf tube without disturbing the pellet. Once tissue lysate is completed, all samples were stored at -70 °C until use.

Biorad protein assays were performed to determine total protein concentrations in each sample.

2.12. Bradford analysis for a 96 well-plate assay

A Bradford analysis was performed to know how much of the sample would be used in the Rat Cytokine Array. The Biorad Reagent was diluted in H₂O (1:5). A solution of 1 mg/mL BSA in H₂O and a solution of 2 mg/mL BSA in H₂O were prepared to make the standards. All samples and all standards were pipetted in duplicate directly on the 96-well plate assay, according to the following table.

Table 2.5 – Bradford assay to quantify protein amount.

BSA (mg)	BSA (μL)	H ₂ O (μL)	PBS (μL)	Biorad Reagent (μL)
0	0	10	2	200
1	1	9	2	200
2	2	8	2	200
3	3	7	2	200
4	4	6	2	200
5	5	5	2	200
10	10	0	2	200
20	10 *	0	2	200
Sample (μL)	BSA (μL)	H ₂ O (μL)	PBS (μL)	Biorad Reagent (μL)
2	0	10	0	200

*BSA 2 mg/mL

All samples were let to incubate at room temperature for 5 minutes. Absorbance was measured at 595 nm.

2.13. Rat Cytokine Array

Commercially available Rat Cytokine Array Panel A (Catalog Number ARY008 R & D Systems, Minneapolis, MN, USA) was used to quantify proteome profiler with high sensitivity (Appendix 1). Sample amount chosen for tissue lysate was 400 μg. After blocking with Array Buffer 6 (R & D Systems, Minneapolis, MN, USA) for 1 hour on platform shaker, samples were incubated in a mixture containing biotinylated antibodies, overnight at 2°C. After washing with Washing Buffer (R & D Systems, Minneapolis, MN, USA), samples were incubated for 30 minutes in streptavidin-HRP (diluted 1:2000) and after washing with Washing Buffer, samples were developed with Chemi Reagent Mix (R & D Systems, Minneapolis, MN, USA), using GeneScan® detection software.

2.13. Measurements and Statistical Analysis

All data are expressed as mean and error bars represent the standard error of the mean (SEM). Results were analysed using non-linear regression (curve fit) with an extra sum-of squares F test comparison method for $\log EC_{50}$ using GraphPad Prism® software. GraphPad Prism® software was instructed to compare the fitted midpoints ($\log EC_{50}$) of the two curves statistically, i.e., whether the curves statistically different with respect to the specified parameter, in this case $\log EC_{50}$.

Pixel densities on developed rat cytokine array membranes were measured using GeneScan® software and were analysed using ImageJ® software.

3. Results

3.1. Introduction and Experimental Details

In this chapter, the aim was to use a premotor rodent model of PD with selective noradrenergic or serotonergic lesions. 25 mg/kg of DSP-4 or 6 mg/kg of pCA were utilized to create a partial lesion of the noradrenergic or serotonergic system, respectively (dosage optimized by previous work). Administration of both drugs was done four days prior to the administration of 6-OHDA. This dosing regimen was chosen to mimic the Braak staging scheme of PD progression (Braak *et al.*, 2003a). In this scheme, noradrenergic and serotonergic deficits appear prior to dopaminergic degeneration (in this case, induced by the injection of 6-OHDA). The 15 mg/kg dosage of 6-OHDA was injected bilaterally into the striatum to induce partial dopaminergic lesion (dosage optimized by previous work) (Deumens *et al.*, 2002). The bilateral model of DA lesion was selected because this specific method more faithfully resembles a clinical parkinsonian condition experienced by PD patients and the progression of lesion is more gradual. In addition, bilateral systemics deficits of monoamines would produce a more valid model of the non-motor symptoms that PD patients experience. EX-4 has previously been shown to be highly neuroprotective in rodent models of PD (Harkavyi *et al.*, 2008). In this thesis, it was explored whether the therapeutic value of EX-4 could be applied to one of the most prominent non-motor symptoms experienced by PD patients, GI dysfunction, and promote the recovery of GI contractility.

The organ bath technique was utilized to measure smooth muscle contraction of the rat ileum. Contraction was induced by AchC (AchC, stock solution 10 μ M) and the effect of Ach was examined on contraction using two fold concentration increments in order to provide a range over which it can be tested the ileum's response. Using Ach *in vitro* on illial tissue to induce contraction serves as a model where excessive ENS activation would be responsible for GI motility (Abuirmeileh *et al.*, 2014). By the time the muscle has reached maximal contraction, the added AchC has essentially equilibrated among the accessible tissue compartments. The EC50 (drug concentration that provokes a 50% of maximum response) was calculated in order to assess *in vitro* contractility. To assess whether EX-4 exerts a neuroprotective function in the rat GI tract after an induced parkinsonian state with 6-OHDA only, EX-4 was injected into the organ bath and the effect of the drug was examined on contraction.

Cytokines are now increasingly recognized as a major contributor to the pathogenesis of PD, where they control multiple aspects of the inflammatory process. In particular, the imbalance between pro-inflammatory and anti-inflammatory cytokines leads to disease perpetuation and tissue destruction. Few data concerning the effects of cytokines on GI dysfunction in PD are available in published reports, and its mechanism of action is not completely understood. Levels of cytokines (TNF- α , IL-1 β , IFN- γ , IL-6 and VEGF) were assessed employing a cytokine microarray in 6-OHDA, 6-OHDA+DSP-4 and 6-OHDA+pCA rats using ileum tissue.

This type of protocol is highly relevant to the clinical PD condition since it's a way of allowing the lesion to develop before the rats were sacrificed and before the therapeutic effect of EX-4 was evaluated *in vitro*.

3.2. Dose-Response Curve Sham vs. 6-OHDA treated

Figure 3.1 indicates the contractile effect of different concentrations of Ach (0.2 μ M – 3.2 μ M) on isolated rat ileum from Sham and 6-OHDA treated rats. As shown, an increase in AchC concentration caused increasing illial smooth muscle contraction and a maximum response was observed at a concentration of 3.2 μ M. Ileum contraction to AchC was computed based on the percentage and maximal effect induced by concentration of 3.2 μ M which was considered to be 100% response and other responses were observed as percentages of this maximal response.

6-OHDA treatment produced a parallel rightward shift of the AchC dose response curve.

Data presented in Figure 3.1 showed that rats treated with 6-OHDA ($EC_{50} = 1.342 \mu$ M, P-value < 0.0001) alone showed a significant decrease in ileum contractility compared to the Sham treatment ($EC_{50} = 0.3840 \mu$ M).

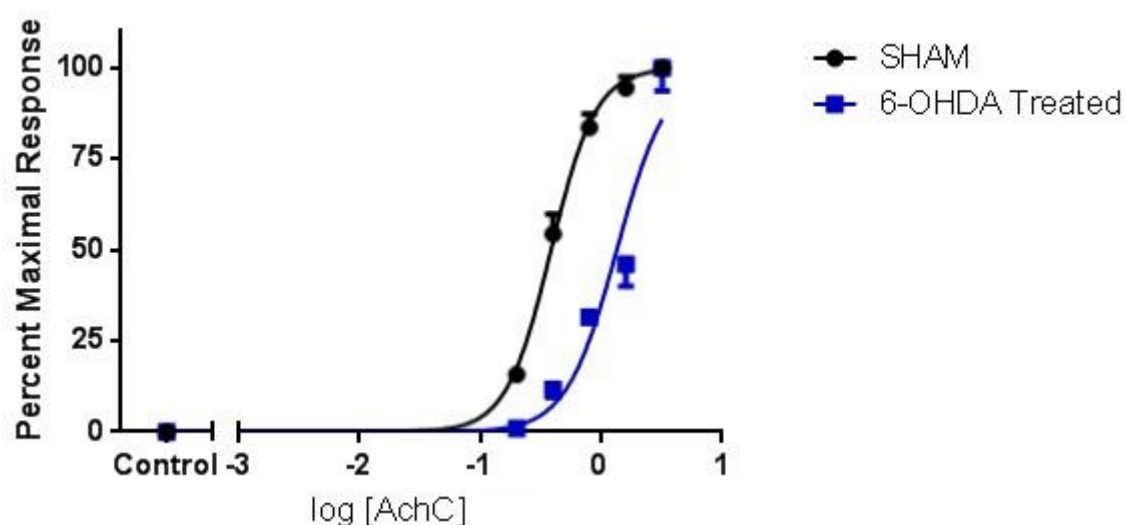


Figure 3.1 - Effect of acetylcholine chloride (AchC, 10 μ M) in the isolated ileum of Sham and 6-OHDA treated rats with a selective dopaminergic lesion. Ordinate scale: Ileum contractile response expressed as % of maximal response. Abscissa scale: log₁₀ concentrations of AchC. Each point represents a two fold increment in concentration. The points are mean and the vertical bars show the SEM (n=3). There was a statistically significant difference in AchC responses between vehicle and 6-OHDA treated tissues. P-value < 0.0001

3.3. Dose-Response Curve Sham vs. 6-OHDA + DSP-4

Figure 3.2 indicates the contractile effect of different concentrations of AchC (0.2 μ M – 3.2 μ M) on isolated rat ileum from Sham and 6-OHDA + DSP-4 treated rats.

6-OHDA + DSP-4 produced a parallel leftward shift of the AchC dose response curve. As shown, an increase in AchC concentration caused increasing ileal smooth muscle contraction and a maximum response was observed at a concentration of 3.2 μ M in Sham animals and at a concentration of 0.2 μ M in DSP-4 + 6-OHDA animals. Ileum contraction to AchC was computed based on the percentage and maximal effect induced by concentration of 3.2 μ M which was considered to be 100% response and other responses were observed as percentages of this maximal response.

The results below showed that rats treated with both DSP-4 and 6-OHDA ($EC_{50} = 0,1623 \mu$ M, P-value < 0.0001) showed a significant increase in ileum contractility compared to the Sham treatment ($EC_{50} = 0.3840 \mu$ M).

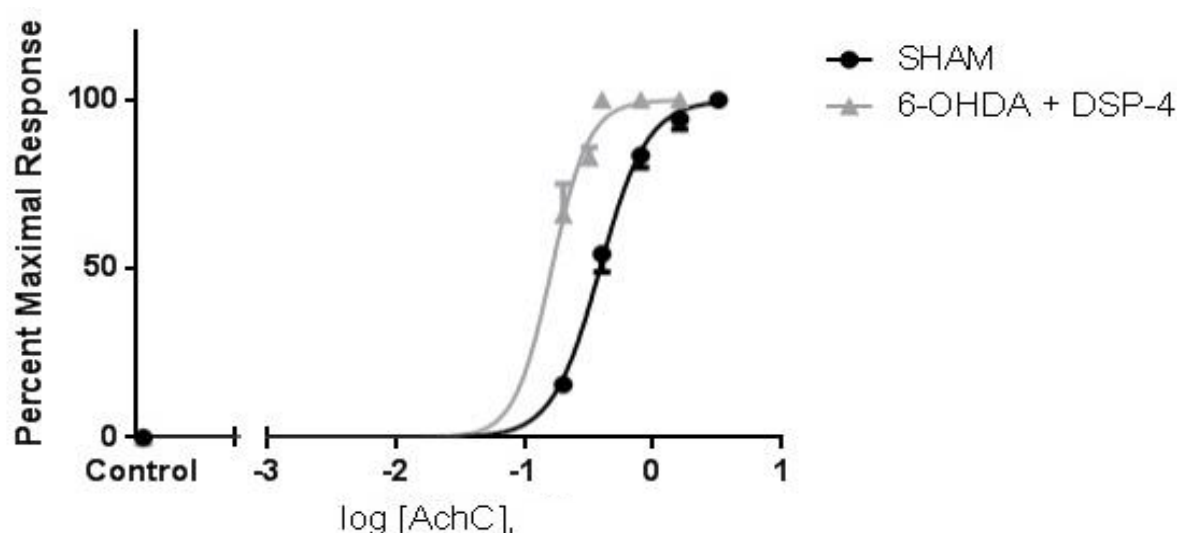


Figure 3.2 - Effect of acetylcholine chloride (AchC, 10 μ M) in the isolated ileum of Sham and 6-OHDA + DSP-4 treated rats with a combined noradrenergic and dopaminergic lesion. Ordinate scale: Ileum contractile response expressed as % of maximal response. Abscissa scale: log₁₀ concentrations of AchC. Each point represents a two fold increment in concentration. The points are mean and the vertical bars show the SEM (n=3). There was a statistically significant difference in AchC responses between vehicle and 6-OHDA + DSP-4 treated tissues. P-value < 0.0001.

3.4. Dose-Response Curve Sham vs. 6-OHDA + pCA

Figure 3.3 indicates the contractile effect of different concentrations of AchC (0.2 μ M – 3.2 μ M) on isolated rat ileum from Sham and 6-OHDA + pCA treated rats.

6-OHDA + pCA produced a rightward shift of the AchC dose response curve. As shown, an increase in AchC concentration caused increasing ileal smooth muscle contraction and a maximum response was observed at a concentration of 3.2 μ M. Ileum contraction to AchC was computed based on the percentage and maximal effect induced by concentration of 3.2 μ M which was considered to be 100% response and other responses were observed as percentages of this maximal response.

The results presented below showed that rats treated with 6-OHDA + pCA ($EC_{50} = 1.360 \mu$ M, P-value < 0.0001) showed a significant decrease in ileum contractility compared to the Sham treatment ($EC_{50} = 0.3840 \mu$ M).

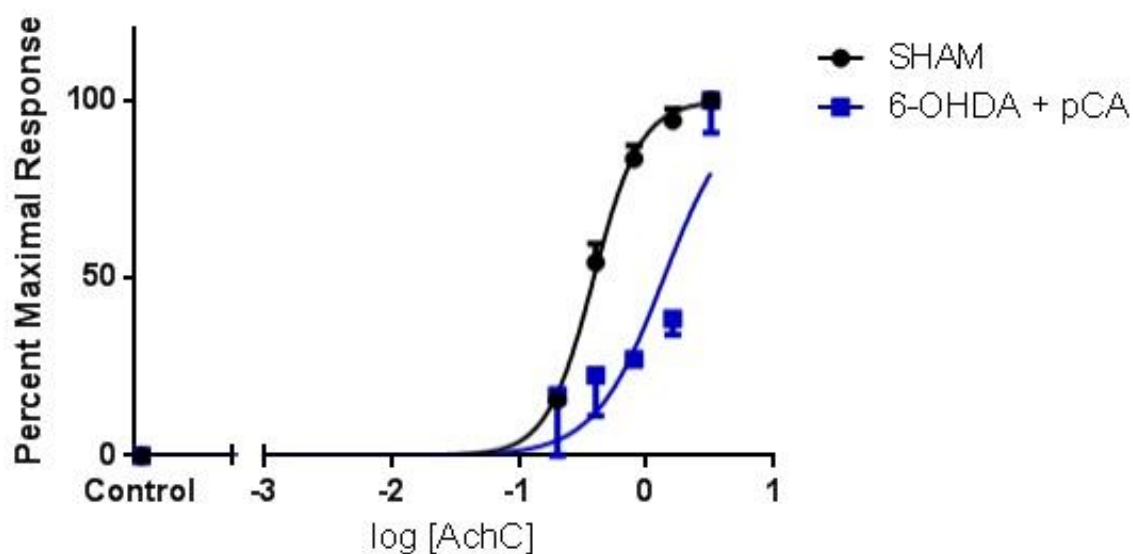


Figure 3.3 - Effect of acetylcholine chloride (AchC, 10 μ M) in the isolated ileum of Sham and 6-OHDA treated rats with a combined serotonergic and dopaminergic lesion. Ordinate scale: Ileum contractile response expressed as % of maximal response. Abscissa scale: log10 concentrations of AchC. Each point represents a two fold increment in concentration. The points are mean and the vertical bars show the SEM (n=3). There was a statistically significant difference in AchC responses between vehicle and 6-OHDA + pCA treated tissues. P-value < 0.0001.

3.5. Effect of EX-4 in ileum contractility

Figure 3.4 indicates the contractile effect of different concentrations of AchC (0.2 μ M – 3.2 μ M) on isolated rat ileum from 6-OHDA treated rats with contractions induced by AchC alone and with contractions induced by AchC and EX-4 together.

As shown, an increase in AchC concentration caused increasing ileal smooth muscle contraction and a maximum response was observed at a concentration of 3.2 μ M. Ileum contraction to AchC was computed based on the percentage and maximal effect induced by concentration of 3.2 μ M which was

considered to be 100% response and other responses were observed as percentages of this maximal response.

The combined use of EX-4 produced a parallel leftward shift of the AchC dose response curve. The decrease in ileum contractility induced by the administration of 6-OHDA was reversed in groups co-treated with EX-4 ($EC_{50} = 0.4379 \mu\text{M}$, $P\text{-value} < 0.005$). 6-OHDA treated animals with contractions induced by AchC alone ($EC_{50} = 1.342 \mu\text{M}$) showed a significant decrease in ileum contractility as presented in the section 3.2.

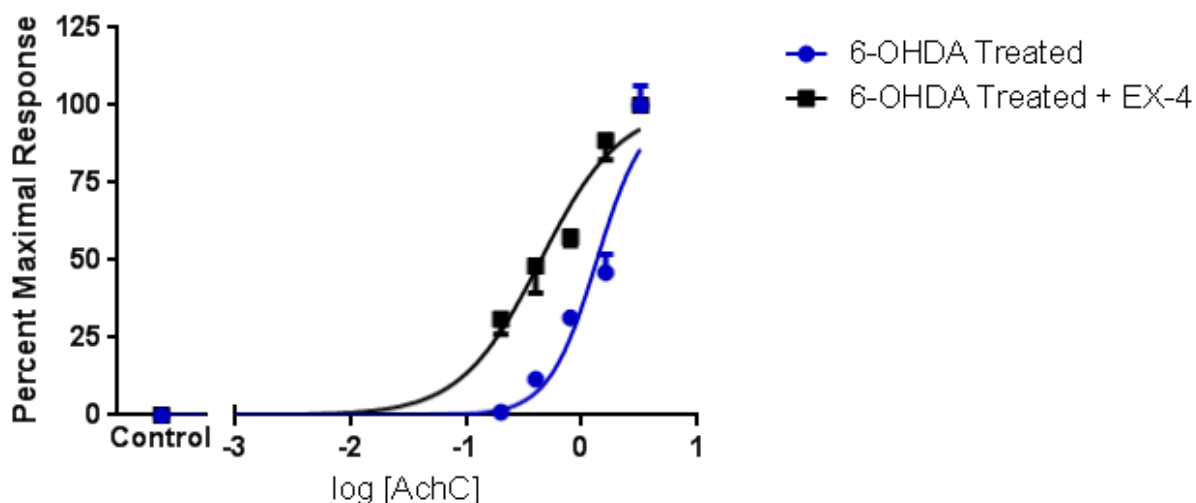


Figure 3.4 - Effect of EX-4 (EX-4, 1 $\mu\text{g/mL}$) in the isolated ileum of 6-OHDA and 6-OHDA+EX-4 treated rats with a selective dopaminergic lesion. Ordinate scale: Ileum contractile response expressed as % of maximal response. Abscissa scale: $\log_{10}$ concentrations of AchC. Each point represents a two fold increment in concentration. The points are mean and the vertical bars show the SEM ($n=3$). There was a statistically significant difference in AchC responses between vehicle and 6-OHDA treated tissues. $P\text{-value} < 0.0001$.

3.6. Effect of EX-(9, 39) in ileum contractility

Figure 3.5 indicates the contractile effect of different concentrations of EX-4 ($2.5 \times 10^{-8} \mu\text{M}$ – 3.2 μM) on isolated rat ileum from vehicle (saline) injected rats with contractions induced by Ex-4 alone and with contractions induced by EX-4 and EX-(9,39) together. As shown, an increase in EX-4 concentration caused increasing illial smooth muscle contraction and a maximum response was observed at a concentration of 3.2 μM . Ileum contraction to EX-4 was computed based on the percentage and maximal effect induced by concentration of 3.2 μM which was considered to be 100% response and other responses were observed as percentages of this maximal response.

The increased in ileum contractility induced by the administration of EX-4 was reversed in groups co-treated with EX-(9,39) ($EC_{50} = 0.2127 \mu\text{g/mL}$, $P\text{-value} < 0.05$).

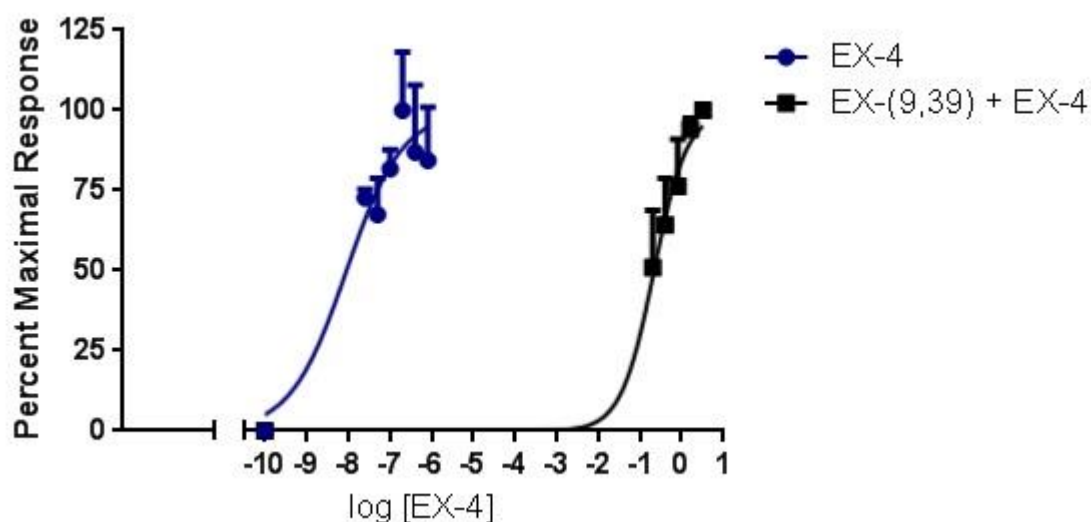


Figure 3.5 - Effect of EX-(9,39) (1 $\mu\text{g/mL}$) in the isolated ileum of vehicle (saline) injected rats. Ordinate scale: Ileum contractile response expressed as % of maximal response. Abscissa scale: $\log_{10}$ concentrations of EX-4 (1 $\mu\text{g/mL}$). Each point represents a two fold increment in concentration. The points are mean and the vertical bars show the SEM ($n=3$). There was a statistically significant difference in responses between EX-4 only and EX-4 + EX-(9,39) treated tissues. $P\text{-value} < 0.005$.

3.7. Gut Cytokine Array

Figure 3.6 shows that $\text{TNF-}\alpha$, $\text{IFN-}\gamma$ and $\text{IL-1}\beta$ protein contents in the ileum were undetectable both in vehicle injected and in treated animals.

The results presented above showed that rats treated with 6-OHDA+DSP-4 (280%, $P\text{-value} < 0.01$) showed a significant increase in the expression of VEGF compared to the SHAM treatment.

Rats treated with 6-OHDA + DSP-4 (178%, $P\text{-value} < 0.05$) showed a significant increase in the expression of IL-13 compared to the SHAM treatment. It is important to note that 6-OHDA + pCA also showed an increase in the expression of IL-13 but these values were not significantly different from Sham treatment group.

It is worth mentioning that all treatment groups injected with a neurotoxin had a decrease in the expression level of IL-6. However, the values obtained were not significantly different from the Sham treatment group. Gut cytokine array data can be viewed in Figure 3.6.

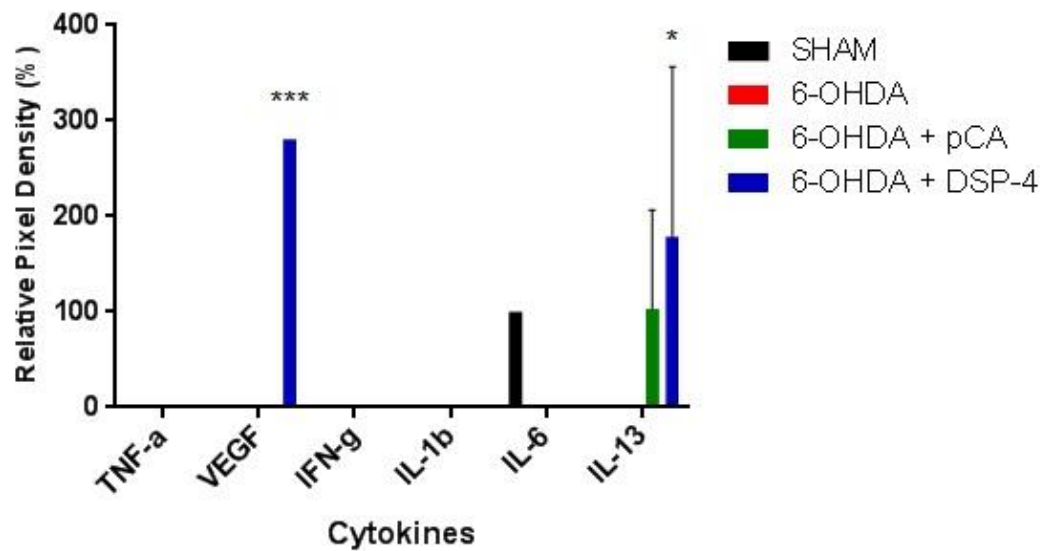


Figure 3.6 – Cytokine array with ileum tissue from Sham, 6-OHDA, 6-OHDA + pCA and 6-OHDA + DSP-4 lesioned rats. Results were analyzed using one-way ANOVA and a post hoc Bonferroni's test to compare differences between groups. (n = 3 per experimental group). Error bars represent SEM.

* Indicates that IL-13 expression of 6-OHDA + DSP-4 treatment group is significantly different from IL-13 expression of Sham treatment group (P-value < 0.05) using Bonferroni's multiple comparison test post hoc.

*** Indicates that VEGF expression of 6-OHDA + DSP-4 treatment group is significantly different from VEGF expression of Sham treatment group (P-value < 0.001) using Bonferroni's multiple comparison test post hoc.

4. Discussion

4.1. Introduction and Project Details

The “traditional” pathology of PD is progressive degeneration of pigmented nigrostriatal dopaminergic neurons. This degeneration produces a loss of DA in the SNpc which results in compromised motor function, which is the principal manifestation of the disease. Current pharmacological therapies are palliative and progressively lose their efficacy over time. In addition to these limitations, existing pharmacological therapies have detrimental side effects to patients.

However, a significant body of literature has shown that noradrenergic and serotonergic transmitter systems are as compromised as dopaminergic neurons in PD. Actually, this extensive damage of the NA and 5-HT systems is now thought to precede degeneration of dopaminergic nigrostriatal neurons and may contribute to non-motor symptoms of PD, mainly deficits in mood and cognition, sleeping disorders and very importantly to this project, gastrointestinal dysfunction.

Since current treatment of PD focuses predominantly on the replacement of DA, the management of the non-motor symptoms, including most of the GI complaints, remains particularly challenging. GI symptoms are a prominent non-motor symptom in PD that occurs in nearly every patient at some point in his or her illness. Symptoms span the entire alimentary tract and include early satiety and nausea from delayed gastric emptying, bloating from poor small bowel coordination, and constipation and defecatory dysfunction from impaired colonic transit. Motility disturbances of the upper GI tract and GI motility as a whole (inclusive of esophagus, stomach and colon) have been identified in the early phase of the disease. Although GI symptoms significantly impact the patients’ quality of life, these symptoms are often not addressed in routine clinical practice. Raising awareness about the GI involvement in PD could contribute to improved patient care and well-being (Greene, 2014; Salat-Foix and Suchowersky, 2012; Voitalla and Goetze, 2011).

The aim of this project was first to use a noradrenergic and/or serotonergic lesions to determine whether these compromise GI function in a “pre-motor” rodent model of PD. The differences in gut contractility were assessed via dose-response curves obtained from data acquired using an organ bath. As the smooth muscle contractile activity is a major regulator of functions of the GI system, malfunction of contractility leads to a host of clinical disorders (Abuirmeileh *et al.*, 2014). This was accomplished by moderately degenerating DA, NA and 5-HT systems through the use of the selective neurotoxins 6-OHDA, DSP-4 and pCA, respectively.

The second goal of the study was to test the efficacy of EX-4 in reversing the alterations in GI contractility exhibited by 6-OHDA insulted animals. EX-4 has demonstrated neuroprotective properties both *in vitro* and *in vivo*, in particular through stimulation of GLP-1 receptors (Bertilsson *et al.*, 2008, Kim *et al.*, 2009, Perry *et al.*, 2002). EX-4 has been shown to be effective in the treatment of neurodegenerative disorders, reversing DA deficiency and stimulating neurogenesis in late-stage rodent models of PD.

Recently, there is increasing recognition of the possible role of neuroinflammation as a major factor in the pathogenesis of PD (McGeer *et al.*, 1988; Whitton, 2007), induced by exposure to either infectious agents or toxicants with proinflammatory characteristics. Elevated levels of proinflammatory cytokines such as TNF- α , IL-1 β , IL-6 and IFN- γ in the striatum in PD brains have also been demonstrated (Whitton, 2007). A cytokine array was performed to assess whether these changes in the cytokine profile in the striatum in PD brains are also present in the gut in groups treated with 6-OHDA, 6-OHDA + pCA or 6-OHDA + DSP-4.

4.2. SHAM vs. 6-OHDA treated rats

Intestinal muscle is innervated by both parasympathetic and sympathetic fibres of the ANS. Parasympathetic system supplies preganglionic fibres to the ENS which synapse with the myenteric plexus and with the submucosal plexus. Fibres from the cell bodies in these plexuses travel to the smooth muscle of the gut to control motility and to secretory cells in the mucosa. The parasympathetic system is responsible for maintaining normal intestinal motility through releasing ACh and it is the major one (Costa and Furness, 1982; Uchiyama and Chess-Williams, 2004). Ascending reflex contractions of the small intestine involves predominantly cholinergic neurotransmission ACh causes contraction in the GI through muscarinic (M2 and M3) receptors binding (Sanders, 1998). This experiment shows that the peristaltic reflex involves nervous pathways, i.e., that it is a local reflex which does not involve the spinal cord.

6-OHDA only treated rats exhibited a decrease in the GI propulsive motility, given by an increase in the EC50, when compared to control animals (P-value < 0.0001). The results of the 6-OHDA treated rats indicate that a sole dopaminergic lesion is adequate to produce GI dysfunction. Nonetheless, ACh release might decrease, as detected in the gastric ENS (Zheng *et al*, 2011), as a consequence of the increased activity of dopaminergic neurons. This observation has been found previously by groups utilizing a premotor bilateral 6-OHDA model of PD (Tian *et al*, 2008). Actually, ACh release from the intrinsic cholinergic motor neurons is negatively modulated by DA via activation of D2 receptors. In 6-OHDA treated rats, TH and the DA transporter increase in the stomach, duodenum and colon, as well as DA increases in the gastric myenteric plexus.

Recently, Tian and co-workers observed increased expression of TH, a rate-limiting enzyme in DA synthesis, and DAT in the GI tract of rats possessing bilateral lesions of the SNpc obtained by stereotaxic injection of 6-OHDA. Given the inhibitory nature of DA in the ENS, an excess of DA production caused by 6-OHDA would have been expected to reduce the GI propulsive motility. An enhanced expression of dopaminergic markers would suggest high DA content in the gut in 6-OHDA-treated rats, which might be responsible for the delay in gastric emptying observed in PD patients (Tian *et al*, 2008). It could be possible that the number of enteric dopaminergic neurons might have

increased, instead of decrease, as a result of a compensatory mechanism to mitigate the loss of DA in the CNS.

In this context, it is tempting to speculate that the increase in the expression of dopaminergic neurons could represent an adaptative response to the drop in nitric oxide-immunoreactive neurons as both subsets of neurons are present in the ENS and both of them exert an inhibitory effect on GI motility (Blandini *et al.*, 2009, Colucci *et al.*, 2012).

4.3. SHAM vs. 6-OHDA + DSP-4 treated rats

The influence of the sympathetic nervous system on GI function has long been the subject of investigation. Information about the sympathetic innervation of the GI tract has accumulated gradually from diverse techniques such as nerve stimulation, the application of autonomic agonists and antagonists and the preparation of histochemical stains specific for neurotransmitters. The sympathetic nervous system is an important influence in the physiological control of GI motility (Taubin *et al.*, 1972). NA in the ENS has an inhibitory effect through extrinsic sympathetic neurons. NE inhibits Ach release from motor neurons (via α_2 adrenoreceptors), decreasing parasympathetic nerve release of Ach, evokes inhibitory postsynaptic potentials in submucosal neurons and relaxes and decreases smooth muscle contractions (Li *et al.*, 2006).

In general, sympathetic stimulation causes inhibition of gastrointestinal secretion and motor activity, and inhibits contraction of gastrointestinal sphincters and blood vessels.

DSP-4 insult (i.p., 25 mg/kg) was induced 4 days before exposure to 6-OHDA. This methodology was selected to mimic the sequential condition of neurodegeneration present in PD patients. The purpose was to induce a noradrenergic deficit prior to a dopaminergic insult. The lesions were allowed to progress for a period of two weeks before animals were sacrificed.

DSP-4 + 6-OHDA groups exhibited a lower EC₅₀ when compared to vehicle-injected animals (0.1623 μ M and 0.3840 μ M, respectively) and this decrease reached statistical significance (P-value < 0.0001). A lower EC₅₀ will imply a higher response to Ach and consequently an increase in motor activity and gastric tone. A combined dopaminergic and noradrenergic lesion was able to overcome the inhibitory motor response associated with NA in the GI tract as the DSP4 + 6-OHDA-treatment group exhibited a stronger illial contractility.

Noradrenaline release leads to a more hyperpolarised smooth muscle, less excitable and less able to produce contractions - decreased motility, decreased secretion, decreased blood flow and depressed rates of cell renewal of the mucosa (Li *et al.*, 2006). The results presented in this thesis indicate that the selective degeneration of both central and peripheral noradrenergic nerve terminals resulting from DSP-4 injection combined with a dopaminergic insult are able to induce an increase in GI motility. It appears that the depletion of the inhibitory effect caused by NE in the GI tract is enough to overcome

the possible increase in TH and DAT induced by a lesion of the SNpc obtained by stereotaxic bilateral injection of 6-OHDA as described before.

Although the work of several groups have shown that a decrease in NA transporter binding might have negative effects since it is present in PD patients with symptoms of depression (Remy *et al.*, 2005), the results in this thesis indicate that a combined dopaminergic and noradrenergic lesion enhances gut contraction in our model of PD. Nevertheless, the potential of catecholamines to influence gut or ENS development remains to be explored.

4.4. SHAM vs. 6-OHDA + pCA treated rats

pCA insult (6 mg/kg via i.p. injection) was induced 4 days before exposure to 6-OHDA. This dosing regimen was chosen to mimic the Braak staging scheme of PD progression (Braak *et al.*, 2003a). In this scheme, serotonergic deficit appears prior to dopaminergic degeneration. 5-HT is important for brain functions and mood control but it is also crucial to the function of GI tract. Most of the body's 5-HT is synthesized and stored in the intestine and the presence of different 5-HT receptors within the intestinal wall has been identified (Bornstein, 2012).

pCA + 6-OHDA groups showed a decrease in gut contractility, given by an increase in the EC₅₀ when compared to vehicle-treated animals (1.360 μ M and 0.3840 μ M, P-value < 0.0001). It is worth mentioning that a 6-OHDA injection alone also induced a significant decrease in gut contractility. The partial degeneration of the serotonergic nerve terminals and cell bodies through pCA injection combined with a dopaminergic lesion seems to reduce illial contractions.

Initial studies suggested that 5-HT stimulated gastric and colonic phasic contraction but recent data suggests that 5-HT level explains less than 20% of the variance in colonic motility (Camilleri, 2009). GI disorders may be related to an imbalance of 5-HT in the gut or a faulty communication network between 5-HT in the gut and the brain and spinal cord (Prins, 2011).

Serotonin causes secretion; 5-HT induces secretion across human illial mucosa via receptor of the 5-HT₃ subtype. 5-HT₃ receptors are distributed throughout the human, guinea pig, rat and mouse intestine and play an important role in the excitability of the ENS (Kelley *et al.*, 2014). There is strong evidence that 5-HT₃ receptors mediate fast excitatory synaptic potentials in some enteric neurons of the myenteric and submucosal plexus (Zhou and Galligan, 1999). Although 5-HT receptors are located on enteric nerves within the myenteric plexus as well as on vagal and spinal afferents, it can also be found in the brain and spinal afferents (Gaman and Kuo, 2008). Mucosal application of 5-HT activates local reflex pathways via 5-HT receptors and enhances peristalsis via the same receptors (Camilleri, 2009). However, the actual roles of 5-HT in the GI tract have been maddeningly difficult to identify. It appears that there is no consistent message or clear mechanistic interpretation that can be gleaned from these findings. Further research is necessary in large numbers of patients with clearly defined

phenotype and genotype, given the potential genetic variations in rates of synthesis and re-uptake of 5-HT. These differences may be due to different disease phases. Our observations were made 2 weeks after 6-OHDA injection (15 mg/kg, i.c) , and thus, represent the early stages of PD.

4.5. Effect of EX-4 on gut contractility

In order to evaluate the effect of EX-4 on gut contractility, EX-4 was added to the organ bath compartment. EX-4 co-treatment was able to reverse the lack of sensitivity to AchC induced by 6-OHDA injection. According to the data obtained with the organ bath technique, EX-4 addition to the organ bath was able to increase ileum sensitivity and gut motility with a decrease in EC50 value when compared to tissue injected with AchC only – EC50 = 0.4379 μ M and EC50 = 1.342 μ M, respectively (P-value < 0.005). This finding is important since it highlights the ability of EX-4 to reduce gastric immobility in rodents. With additional studies, this hypothesis might be attributed to an increase in enteric neurogenesis due to EX-4 administration. Although the mechanism of action whereby EX-4 is able to exert its neuroprotective effects is not yet known, EX-4 seems to be able to recover neuronal phenotype. It is hypothesized that EX-4 is exhibiting anti-inflammatory, anti-apoptotic and neurotrophic properties similar to GLP1-R activation in pancreatic β cells (Perry *et al.*, 2002; Perry *et al.*, 2004). GLP-1R has been identified and is expressed in a wide range of tissues including stomach, intestine, nodose ganglion neurons of the vagus nerve and several regions of the CNS including the hypothalamus and brainstem (Calvo *et al.*, 1995; Goke *et al.*, 1995). It is possible that EX-4 might inhibit apoptosis by activating GLP-1Rs in the gut which in turn would cause a diminution of pro-apoptotic and pro-inflammatory proteins including active caspase 3 and nuclear factor kappa B (NF κ B) and an up-regulation of pro-survival factors including Bcl-2 and Bcl-xL. The cytoprotective effect of EX-4 might be coupled to the following: (1) activation of cAMP/PKA with subsequent phosphorylation and activation of cAMP response element binding protein (CREB) and induction of the Akt-PKB growth and survival pathway and (Jhala *et al.*, 2003) (2) activation of Akt-PKB and subsequent prevention of caspase activation and inhibition of NF κ B (Buteau *et al.*, 2004).

Possible mechanisms of EX-4 are summarized in Figure 4.1.

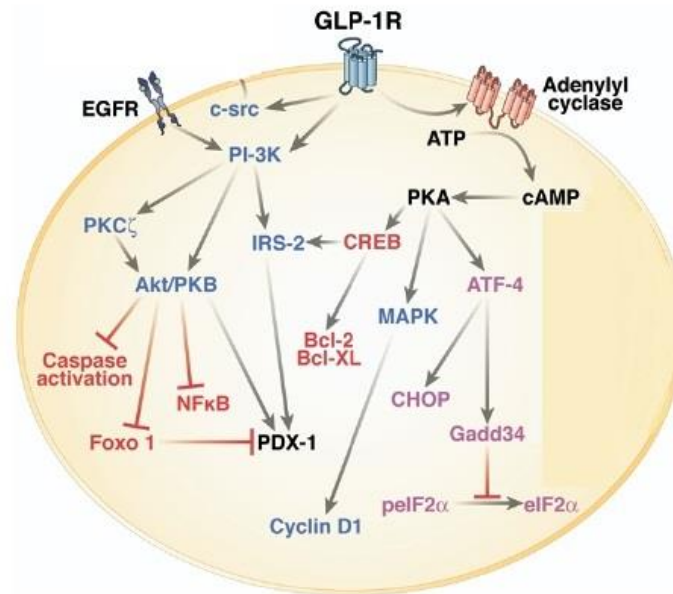


Figure 4.1 - Proposed mechanism of action of GLP-1R dependent signal transduction pathways in the intestinal cell. The major pathways presented are inhibition of apoptosis (red), ER stress reduction (purple) and proliferation and neogenesis (blue). Adapted from Baggio and Drucker, 2007.

4.6. Effect of EX-(9, 39) in ileum contractility

GLP-1Rs are metabotropic G-protein coupled receptors, they are positively coupled to AC through $G_s\alpha$ subunit with subsequent production of cAMP, although they are also capable of signaling through other transduction pathways (Drucker *et al.*, 1987). GLP-1R has been identified and is expressed in a wide range of tissues including α -, β -, and δ -cells of the pancreatic islets, lung, heart, kidney, stomach, intestine and several regions of the CNS (Baggio and Drucker, 2007).

To test whether EX-4 is able to increase ileum contractility and induce contractions through GLP-1R stimulation, a GLP-1R selective antagonist was co-added to the organ bath. Administration of EX-(9, 39) into the organ bath of groups co-treated with EX-4 would demonstrate whether or not the therapeutics effects of EX-4 are, indeed, governed by GLP-1R stimulation. Exendin-(9, 39), an N-terminally truncated peptide derivative of EX-4, binds the GLP-1R and functions as a specific GLP-1R antagonist (Goke *et al.*, 1993). EX-(9, 39) or mice with a targeted disruption of the GLP-1R gene (GLP-1R $-/-$) often are used to examine the physiologic consequences of transient and sustained loss of GLP-1R signaling, respectively (Baggio and Drucker, 2007).

Contractility in the ileum removed from rats pre-treated with vehicle (saline) only and with EX-(9,39) ($EC_{50} = 0.2127 \mu\text{g/mL}$) injected into the organ bath together with EX-4 was significantly lower than the vehicle group without EX-(9,39) addition ($EC_{50} = 8.894 \times 10^{-9} \mu\text{g/mL}$).

Inhibition of GLP-1R signaling with EX-(9, 39) appears to exhibit a potent inhibitory effect on intestinal contractility. Beneficial effects of GLP-1 on the cardiac muscle contractility function became increasingly evident in previous works (Bose *et al.*, 2005; Zhao *et al.*, 2006). GLP-1R agonists increase heart rate and in the lung activation of GLP-1R signalling enhances secretion (Baggio and Drucker, 2007). It is appealing to think that this has the same effect in the GI tract. The cellular mechanisms that mediate an increase in gut motility are less clear but might include the following: (1) inhibition of K_{ATP} channels, which leads to cell depolarization. It has been suggested that GLP-1 reduces K_{ATP} channel activity by elevating the sensitivity of K_{ATP} channels to ATP (Suga *et al.*, 2000; Gromada *et al.*, 1998); (2) increases in intracellular Ca^{2+} levels resulting from GLP-1-dependent influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels; (3) increases in mitochondrial ATP synthesis, which leads to further membrane depolarization and (4) closure of voltage-dependent K^+ (K_v) channels and consequent reductions in K_v currents, thereby preventing repolarization. The primary effector of GLP-1 agonists-induced activation of GLP-1Rs might be cAMP, and cAMP mediates its stimulatory effect via two distinct mechanisms: (1) PKA-dependent phosphorylation of downstream targets and (2) PKA-independent activation of exchange proteins directly activated by cAMP 2 (Epac2).

A substantial body of literature indicates that the effect of EX-4 and GLP-1 on gastric emptying is mediated by the vagus nerve and involves GLP-1Rs located in the CNS and/or vagal afferent fibers that relay sensory information to the brainstem (Baggio and Drucker, 2007).

4.7. Cytokines expression in the GI tract

It is believed and a concept that it is gaining attention is that idiopathic PD results from combinations of multiple risk factors that include age, environmental toxins, genetic predisposition, and possibly inflammation (Sulzer, 2007). Previous studies suggested that preexisting neuroinflammation is a risk factor for the development of PD (Koprach *et al.*, 2008). The central tenet being that each successive risk factor in turn engages compensatory mechanisms and eventually compromises neuronal health beyond recovery (Isacson, 1993). Infiltration of activated immune cells and increased cytokine production define the immunophenotype of GI inflammation (Shajib *et al.*, 2013). It is plausible that the GI tract is a major site and source of oxidative stress and neuroinflammation since the GI tract is the largest interface between neural tissue and the environment and the epithelial lining of the stomach is thin and is susceptible to lesions and chronic infection (Braak *et al.*, 2003b). More importantly, the ENS neuronal network is in close proximity to the potentially injurious factors such as bacterial products capable of inducing inflammatory pathways. Several chronic autoimmune intestinal diseases including inflammatory bowel disease and celiac disease are associated with increased intestinal permeability also known as “leaky” gut. Thus, gut

leakiness in patients with genetic susceptibility to PD may be a pivotal early step promoting a pro-inflammatory environment contributing to the initiation and/or progression of the PD process. One particularly detrimental consequence of increased intestinal permeability is the translocation of bacteria and bacterial products, like LPS, which creates a proinflammatory environment and increases the oxidative stress burden in the ENS (Forsyth *et al.*, 2012).

Various abnormalities have been documented in terms of increased activation of and infiltration by immune mediators and cells, providing evidence for an intense local immune response (Bandlapalli *et al.*, 2011; Kellow *et al.*, 1999). The involvement of inflammation in PD pathogenesis is supported by the occurrence of infiltration of activated microglia and T lymphocytes in *post-mortem* PD brains (Mc Geer *et al.*, 1988). Activated lymphocytes and macrophages release various mediators, including NO, interleukins and proteases which can stimulate the ENS and eventually result in abnormal secretion and motor response in the gut. (Bandlapalli *et al.*, 2011). Various mediators are released during the inflammatory response that have the ability to induce changes in visceral perception, secretion and motility. Cytokines regulate and coordinate the immune response, but also play an important role in the development of anxiety or depression in PD as well as motor dysfunction, mediated by the CNS and it also amplifies and perpetuates the local immune response (Prins, 2011).

It is also well established that inflammation and pro-inflammatory cytokines like IL-1 β , IFN- γ and TNF- α are one major source of neuroinflammation. Indeed, neuroinflammation is present within the brain of early PD patients and considerable evidence supports a role for neuroinflammation in PD pathogenesis.

In a set of experiments, the protein levels of TNF- α , IFN- γ , IL-6, IL-1 β , IL-13 and VEGF were assessed by a cytokine profile array in samples from rat ileum removed from Sham, 6-OHDA, 6-OHDA + DSP-4 and 6-OHDA + pCA treated rats. No expression levels of TNF- α , IFN- γ , IL-6, IL-1 β were observed in the ileum of Sham, 6-OHDA, 6-OHDA + pCA or 6-OHDA+DSP-4 rats. It has been shown that PD patients of a Japanese cohort with a higher expression of IL-1 β had a later age of disease onset compared to lower expressing patients. IL-1 β can modulate the release of Ach or NA, neuromediators located in the rat myenteric plexus. These data might suggest that IL-1 β may be important in PD onset and that more research needs to be done to determine its exact role (Nishimura *et al.*, 2000). The fact that the “pre-motor” model used in this project aims to represent the early stage of the disease might influence the lack of IL-1 β expression.

It would be of interest to repeat the experiment with a high number of samples since previous studies showed that for each pro-inflammatory cytokines, levels of expression were strikingly variable between patients, some showed levels similar to control subjects while others displayed a 4- to 6- fold increase (Devos *et al.*, 2013). IL-1 β might release other cytokines, including IL-6 and TNF- α , which could in turn affect GI intestinal motility (Aubé *et al.*, 1996). In fact, it has been shown that IL-1 β induces IL-6 expression in rat intestinal smooth muscle cells (Khan *et al.*, 1995). The low protein

levels might be the consequence of a negative feedback regulation mechanism. A real-time quantitative PCR could be used to measure mRNA expression of inflammatory cytokines. Real-time quantitative PCR is considered to be the method of choice for quantification of cytokines profiles in cells but also tissues (Giulietti *et al.* 2001). Nevertheless, because cytokines are secreted proteins, the more biologically relevant experiment is to measure their extracellular levels by enzyme-linked immunosorbent assay.

IL-13 is a cytokine of increasing interest to gastroenterologists because of its developing role in gastrointestinal inflammation. Recent data show that IL-13 may play an important role in a novel innate immune response since it can be released by signals from an injured or inflamed epithelium, of particular relevance to the gut. The excessive production of IL-13 is thought to lead to its deleterious effects, particularly as IL-13 has demonstrated toxic effects on colonic epithelial tissues (Mannon and Reinisch, 2012). IL-13 induces apoptosis of cells. In the mouse brain, it has been found that IL-13R1 was expressed in the DA neurons of the SNpc, which are preferentially lost in PD. Mice deficient for IL-13R1 exhibited resistance to loss of DA neurons in a model of chronic peripheral inflammation using bacterial LPS (Morrison *et al.*, 2012). 6-OHDA + DSP-4 (P-value < 0.05) treated group demonstrated a significantly greater increase in IL-13 expression compared to the vehicle-injected group. Although 6-OHDA + pCA group revealed an increase in IL-13 expression as well, this increase did not reach statistic significance. These results indicate that a sole dopaminergic lesion is inadequate to produce a significant increase in IL-13 expression in gut tissue. These results also show that an additional noradrenergic or serotonergic lesion further potentiates neuroinflammation in the ENS.

Bruno Conti's team is studying a gene encoding a protein known as IL-13 receptor alpha 1 chain (IL-13R α 1), located in the PARK2 *locus*, which has been linked to PD. IL-13R α 1 is a receptor chain mediating the action of IL-13. With further studies, the researchers identified that in the mouse brain, IL-13R α 1 is expressed only on the surface of dopaminergic neurons. Conti and co-workers found that mice lacking IL-13R α 1 had decreased inflammation levels and neuronal loss got better (Morrison *et al.*, 2012). This could be a basic mechanism of the increased susceptibility and preferential loss of dopaminergic neurons to neuroinflammation.

If further research confirms the IL-13R α 1 acts in similar way in human dopaminergic neurons as in mice and in the ENS as in the CNS, the discovery could cover the way to addressing the underlying cause of PD. Researchers might, for instance, find that drugs that block IL-13R α 1 are useful in preventing loss of dopaminergic cells during neuroinflammation.

Some chronic inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, are characterized by severe gut inflammation and are accompanied by the formation of new, excessive and abnormal blood vessels from pre-existing ones. This intestinal pathological angiogenesis enhances gut

inflammation by allowing a higher influx of immune cells into the affected tissue. These newly formed vessels become inflamed and can directly secrete pro-inflammatory cytokines and chemokines that attract more immune cells, further amplifying the gut inflammation. VEGF, a key pro-angiogenic protein, has a major role in physiological and pathological angiogenesis (Ardelean *et al.*, 2014). In patients diagnosed with inflammatory bowel diseases, VEGF is increased in serum and GI tissues, suggesting that it might contribute to the pathogenesis of PD disease as well. VEGF induces angiogenesis of human intestinal microvascular endothelial cells *in vitro* as well as an inflammatory phenotype in the intestinal epithelium (Scaldaferri *et al.*, 2009). Agents that block VEGF signaling might reduce intestinal inflammation in patients with PD. A recent study using an anti-angiogenic inhibitory form of VEGF-A for therapy in a model of experimental ulcerative colitis, showed reduced angiogenesis and a decrease in inflammatory scores by expression of this endogenous inhibitory VEGF-A isoform (Cromer *et al.*, 2013). Inhibitory VEGF molecules might play an important role in maintenance of gut homeostasis.

6-OHDA + DSP-4 group demonstrated a significant increase (P-value = 0.0003) in VEGF expression. These data indicate that a sole dopaminergic lesion or a dopaminergic lesion combined with a serotonergic impair are not able to increase angiogenesis; however when a dopaminergic lesion is combined with a NA lesion, pathological angiogenesis emerges. These results indicate that possible angiogenesis in PD is not strictly governed by DA reduction but requires additional NA deficit to produce impairments. In addition, it has been shown that NA exhibits antioxidant and anti-inflammatory properties (Rommelfanger and Weinshenker, 2007).

In contrast, Herrán *et al.* showed that the treatment of 6-OHDA-lesioned rats with both glial cell line-derived neurotrophic factor (GDNF) and VEGF microspheres resulted in a pronounced TH-positive neuron recovery, demonstrating regenerative effects (Hérran *et al.*, 2013).

There was a decrease in the expression of IL-6 in 6-OHDA, 6-OHDA + pCA and 6-OHDA + DSP-4 treatment groups relative to vehicle injected controls; however, this decrease did not reach statistical significance.

The *in vivo* effects of IL-6 on enterocytes and the intestinal tract have not been elucidated. IL-6 administration has been associated with induction of anti-apoptotic proteins along with decreased caspase activity. Some studies suggest that IL-6 treatment promotes less intestinal injury while IL-6 null mice exhibited increased apoptosis relative to *wild-type* controls.

For instance, IL-6 signaling is an important regulator of Intestinal Bowel Disease pathogenesis, mainly through induced resistance of cells against apoptosis through upregulation of anti-apoptotic factors such as Bcl-2 and Bcl-xl (Waldner *et al.*, 2012).

One can assume that loss of IL-6 results in increased activation of pro-apoptotic and/or necrotic pathways in enterocytes after injury. Therapies that increase IL-6 or its signaling pathways may help manage GI symptoms associated with increased apoptosis, necrosis and gut injury (Jin *et al.*, 2010).

Because IL-6 is pleiotropic, it can be either neuroprotective or neurotoxic depending upon the surrounding environment (Sawada *et al.*, 2006). Chen and co-workers reported that men with elevated plasma levels of IL-6 have an increased risk of developing PD (Chen *et al.*, 2008). One can speculate that in rats injected with vehicle only, IL-6 may be non-toxic acting for neuroprotection.

There is an inflammatory cytokine profile observed in this “pre-motor” rodent model of PD, namely a significant increase in the expression on IL-13 and VEGF.

Previous studies proposed that levels of pro-inflammatory cytokines were not related with the presence of enteric Lewy pathology, which reflects disease severity (Lebouvier *et al.*, 2010). It has been found that GI inflammation in PD does not increase over time and is independent of clinical severity (Devos *et al.*, 2013). Such a scenario is not impossible as enteric inflammation may be high at disease onset when the activated cells release their cytokines, then decreasing while maintaining ongoing disease activity.

Nevertheless, results from recent studies suggested that cytokines are differently regulated among PD patients and one can therefore speculate that PD has its own specific cytokine signature.

Recent observations by Braak *et al.* suggest that early lesions characteristic of PD are developed in two specific locations: the DMV and the anterior olfactory nucleus.

A current theory, the so-called Braak’s theory postulates that PD originates in the GI tract. Braak and co-workers determined that the appearance of AS-positive Lewy pathology initially occurs, in the earliest stage of PD, in both the ENS and in the ENS (Braak *et al.*, 2003b). This led Braak and co-workers to propose that the GI tract might be a portal of entry for a putative PD pathogen, triggering pathological changes in the submucosal/myenteric neurons. Indeed, many neuroactive substances are taken up in this way, often through receptor-mediated endocytosis at the presynaptic button.

Conceivably, such a neurotropic pathogen could induce conformational changes in normal AS molecules, thereby provoking their aggregation, without tending to self-aggregation itself or without its becoming a component of the pathological inclusion bodies (DeArmond and Prusiner, 1997). Thus, provided it were capable of passing the brush border and mucosal barrier of the GI tract, neuronal and fiber pathways exist, anatomically speaking, by which a pathogen could enter enteric neurons and then be conveyed to unmyelinated fibers of the DMV, thereby overcoming the distance from the mucous membrane of the digestive tract to the CNS via retrograde axonal transport (Braak *et al.*, 2003b).

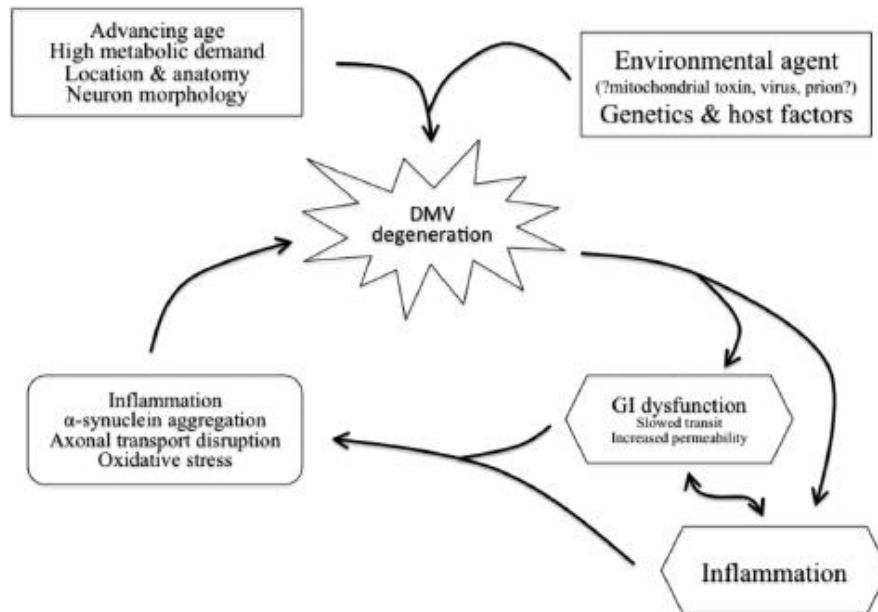


Figure 4.2 – DMV degeneration may induce a vicious cycle of neuronal damage in PD. There is a complex relationship between α -synuclein, axon transport abnormalities, mitochondrial dysfunction, inflammation and oxidative stress. Adapted from Greene, 2014.

In PD, Lewy pathology is distributed from the esophagus to the rectum and affects both myenteric and submucosal plexus (Beach *et al.*, 2010). Later, it was suggested that the putative PD pathogen could enter through the nose (by inhalation) or through the gut (through the intake nasal secretions) and progress to the CNS via enteric neurons (Hawkes *et al.*, 2009).

Thus, the involvement of the GI tract in PD is of great interest as a contributing factor to the development and progression of PD (Figure 4.2).

4.8. Future Work

There are still several questions to be answered from the findings of this study. First and foremost, it would be very important to study gastric contractility by other type of methods, namely *in vivo* gastric myoelectric activity techniques. Electrogastrography, which is a non-invasive method for the measurement of gastric activity, and gastric emptying with dynamic scintigraphy, in which a small amount of radioactivity is used to obtain pictures of the GI tract, could be used to measure GI contractility in the “pre-motor” rodent model used in this project. Tanaka *et al.* measured gastric emptying rates in 20 treatment-naïve early PD patients (median age 70.5 years, median disease duration 0.9 years), 40 patients with advanced treated PD (median age 67.0 years, median disease duration 6 years), and 20 healthy controls (median age 69 years). Gastric emptying, measured using

the ^{13}C -acetate breath test, was significantly slower in both PD groups compared with controls ($P < 0.001$) but there was no significant difference between early and advanced disease (Tanaka *et al.*, 2011). The gold standard for gastric emptying and small intestinal transit is scintigraphy (Goetze *et al.*, 2006).

Physiological parameters and gastrointestinal function tests as fecal daily output, food and water consumption and weight gain should be measured daily in injured rats (not only 6-OHDA but 6-OHDA+DSP-4 and 6-OHDA+pCA groups as well) and in groups co-treated with EX-4. Blandini *et al.* used a unilateral nigrostriatal lesion by 6-OHDA injection to achieve complete cell loss in the SNc and the striatum in rats. Fecal output in the experimental group was noted to be significantly reduced compared with sham controls at 4 weeks (Blandini *et al.*, 2009).

It would be of extreme importance for therapeutic options to assess with other type of methods whether the possible neuroprotective effects of EX-4 in the GI tract are ruled by the GLP-1R. Specific deletion of GLP-1R using anti-sense mRNA for the GLP-1R would demonstrate whether or not the therapeutic effects of EX-4 are governed by GLP-1R stimulation. Variation of the dosage of neurotoxins could also be employed to interpret changes in GI function due to neurochemical manipulation.

It would also be of interest to address possible cell proliferation markers as proliferating cell nuclear antigen (PCNA) or *ex vivo* BrdU labelling.

Real-time PCR could be used to quantify mRNA and investigate the changes of mRNA encoding TH and DAT, DA- β -hydroxylase and NET, TPH and SERT in the GI tract of PD rats and in the GI tract of rats co-treated with EX-4.

In vitro work in gastrointestinal cell lines to understand which cell signaling pathways are activated specifically upon GLP-1R activation should be carried out.

Immunohistochemical analysis with apoptotic markers as cleaved caspase-3 (c-cas-3) or translocation of apoptosis-inducing factor (AIF) could be employed to verify and ensure that enteric neurons are indeed damaged. Tissue level assays should be carried out in the gut to determine the extent of damage that was produced due to toxin administration performed in this study. Tissue content analysis could also be performed in other GI tract regions such as the colon, which also has been implicated in GI dysfunction (Marrinan *et al.*, 2013).

Lesions in the ENS were previously found both in PD cases and in asymptomatic patients who only had Lewy bodies in the lower brain stem (Braak *et al.*, 2003b). Thus, pathology in the ENS could serve as a marker of early disease or disease progression - it could be possible to use colonic biopsies in order to predict who will develop motor symptoms of PD. Identify PD before patients present significant cell loss could slow down, stop or even reverse the disease.

Robert Nussbaum developed a transgenic mouse expressing a mutated version of the human α -synuclein gene. This strain developed AS aggregation in the gut at three months of age. The mice had signs of constipation and reduced defecation similar to what is seen in PD patients. Because this

mutation causes some familial forms of PD, the results provide a link between PD and a disease process in the gut. This mouse model mimics what has been found in the early stages of PD (Kuo *et al.*, 2010). In the same year Pascal Derkinderen's group examined colonic biopsies from 29 PD patients and 10 controls. They found Lewy pathology in one of the two layers of the gut (the submucosal plexus) in 21 of the patients and none of the controls (Lebouvier *et al.*, 2010).

It could be possible to increase the recognition of the immune system in order to facilitate the recognition of injured enteric neurons and remove them from the ENS so that they do not reach the brain.

Intestinal inflammation in humans or animals is accompanied by motility changes, which may reflect alteration in the function of smooth muscle or the ENS, or both (Vermillon *et al.*, 1993). So it would be interesting to test whether there is a correlation between GI contractility dysfunction and an inflammatory cytokine profile in the "pre-motor" model used in this project.. For example, previous studies suggested that IL-1 β is a potent inhibitor of rat jejunal, illial and colonic smooth muscle contraction in response to Ach (Aubé *et al.*, 1996). Moreover, it has been shown that exogenous IL-1 β inhibits both Ach and noradrenaline release from rat myenteric nerves. It would also be interesting to test whether targeting intestinal pathological angiogenesis may reduce chronic gut inflammation and therefore decrease GI dysfunction. Hence, VEGF-treated therapies could ameliorate GI symptoms. Anti-VEGF treatment decreased gut inflammation including tissue IL-1 β , suggesting that anti-angiogenic therapy might have anti-inflammatory properties (Ardelean *et al.*, 2014).

Finally, inasmuch as EX-4 is known to have anti-inflammatory, anti-apoptotic and neurotrophic properties (Kim *et al.*, 2009; Perry and Greig, 2004; Perry *et al.*, 2002), it would be interesting to see whether EX-4 is able to abolish the increase observed in some inflammation-related cytokines not only in the gut but in every region of the GI tract.

Further insight on the mechanisms of inflammation in PD will help to further develop alternative therapeutic strategies that will specifically and temporally target inflammatory processes without abrogating the potential benefits derived by neuroinflammation, such as tissue restoration. Altogether, neuroinflammatory processes might represent a target for neuroprotection in PD

In terms of pharmacotherapy, a range of medications is available potentially to restore some of the alterations in GI motor function identified in PD. A class of drugs currently under evaluation includes agents with potentially for stimulation of motor activity and transit such as 5-HT₃ agonists (Kellow *et al.*, 1999).

4.9. Final Remarks

In the last decade, the so-called non-motor symptoms have attracted substantial scientific and clinical attention, partially because of new hypotheses about the onset and course of the disease (Woitalla and Goetze, 2011). These non-motor symptoms are important to recognize, as they can lead to even more serious complications and impair quality of life. Symptoms associated with GI dysfunction that had previously attracted little attention are now the focus of interest. These problems can cause malnutrition, aspiration, pneumonia, and difficulty in swallowing and retaining pills, all of which can lead to problems that are even more serious. In the ENS, the lesions occur throughout the gastric myenteric and submucosal plexuses. There is an uninterrupted series of susceptible neurons that extend from the ENS to the CNS, which implies that environmental toxins might be able to pass the mucosal barrier of the GI tract and may act on the enteric nerve endings of the vagal neurons, resulting in retrograde degeneration of the DMV (Braak *et al.*, 2006).

The work presented in this thesis suggests that GI dysfunction in PD affects not only the DA system but also the NA and 5-HT pathways.

In this thesis it was suggested that EX-4 might be able to recover motility dysfunctions in the rat GI tract produced by intracerebral injection of 6-OHDA alone.

With further studies, these results could help suggesting that EX-4 could be used as an early treatment option for one of the premotor symptoms of the disorder. In addition, due to the ability of EX-4 to preserve the functional integrity of the DA, NA and 5-HT systems (Harkavyi *et al.*, 2008; Rampersaud, 2010), it would be highly beneficial and convenient to start treatment as soon as diagnostic is made. This is important, as recent literature has suggested that PD affects not only the DA system but also the NA and 5-HT pathway. Although the exact mechanism of action of EX-4 is not known, this does not distract from its neuroprotective abilities. EX-4 might offer a mean to treat one of the most prominent non-motor symptoms experienced by PD patients that greatly impairs the patient's quality of life. When other research had proven the usefulness of EX-4 in counteracting GI dysfunction in PD, it remained not completely clear if EX-4 would be of value in such situations.

Consistent findings obtained by various animal models of PD suggest that neuroinflammation is an important contributor to the pathogenesis of the disease and may further propel the progressive neuronal cell death. A cytokine microarray performed with illial tissue removed from animals subjected to different types of treatments showed significant differences between groups. Additional studies are essential since neuroinflammatory processes might represent a target for neuroprotection in PD. Future therapeutic designs must take into account the multifactorial nature of PD, including the varied roles of the adaptive and innate immune responses.

From a clinical perspective, GI symptoms in PD have a number of therapeutic options and can be managed with good clinical care. Clarification of certain aspects, including the role of GI function abnormalities in determining an individual's risk of developing PD, whether the GI tract is involved in

the development and progression of PD as proposed by Braak's hypothesis is essential to concisely explain a complex phenomenon, such as the GI involvement in PD.

The unravelling of the complexity of the brain-gut communication is beginning to afford a better understanding of the GI pathophysiology of PD and further research is likely to led to its more specific treatment.

Finally, results obtained in other studies have already led to the first EX-4 clinical trial with support of The Cure Parkinson's Trust, UK (Harkavyi *et al.*, 2008, Bertilsson *et al.*, 2008, Kim *et al.*, 2009).

5. References

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Appendix

Appendix I – Proteome Profile™ Array Rat Cytokine Array Panel A

Day 1

- 1) Pipette 2.0 mL of Array Buffer 6 into each well of the 4-Well Multi-dish to be used.
- 2) Remove each membrane to be used from between the protective sheets and place in a well of the 4-Well Multi-dish. The numbers on the membrane should be facing upward.
- 3) Incubate for one hour on a rocking platform shaker (each membrane should rock end to end in its well).
- 4) Prepare samples by adding up to 1 mL of each sample to 0.5 ml Array Buffer 4 in separate tubes. Adjust to final volume of 1.5 ml of Array Buffer 6 as necessary. Add 15 µl of reconstituted Detection Antibody Cocktail to each prepared sample. Mix and incubate at room temperature for one hour.

NB for tissue lysate suggested sample amount is 100-400 µg

- 5) Aspirate Array Buffer 6 from the wells of the 4-Well Multi-dish and add sample/antibody mixtures prepared in step 4). Place the lid on the 4-Well Multi-dish.
- 6) Incubate overnight at 2-8 °C on a rocking platform shaker.

Day 2

- 7) Remove each membrane using flat-tip tweezers and place into individual plastic containers with 20 mL 1X Wash Buffer to wash 3x. Rinse the 4-Well Multi-dish with deionized or distilled water and dry thoroughly.
- 8) Dilute the Streptavidin-HRP (1:2000) in Array Buffer 6. Pipette 2 mL of diluted Streptavidin-HRP into each of the 4-Well Multi-dish.
- 9) Remove each membrane from its wash container. Allow excess buffer to drain from the membrane. Return the membrane to the 4-Well Multi-dish containing the diluted Streptavidin-HRP. Cover the wells with the lid.
- 10) Incubate for 30 minutes at room temperature on a rocking platform shaker.

- 11) Wash each array as described in step 7).
- 12) Remove each membrane from its wash container. Allow excess Wash Buffer to drain from the membrane by blotting the lower edge onto paper towels. Place each membrane on the bottom sheet of the plastic sheet protector with identification number facing up.
- 13) Pipette 1 mL of the prepared Chemi Reagent Mix evenly onto each membrane.
- 14) Cover with the top sheet of plastic sheet protector. Gently smooth out any air bubbles and ensure Chemi Reagent Mix is spread evenly to all corners of each membrane. Incubate for 1 min
- 15) Position paper towels on top and sides of plastic sheet protector containing the membranes and carefully squeeze out excess Chemi Reagent Mix.
- 16) Remove the top plastic sheet protector and carefully lay an absorbent lab wipe on top of the membranes to blot off any remaining Chemi Reagent Mix.
- 17) Leaving the membranes on the bottom plastic sheet protector, cover the membranes with plastic wrap taking care to gently smooth out any air bubbles. Wrap the excess plastic wrap around the back of the sheet protector so that the membrane and sheet protector are completely wrapped.
- 18) Expose membranes to X-ray film for 1-10 minutes. Multiple exposure times are recommended.

