Exploring the aetiology of pre-motor Parkinson's disease and the efficacy of potential neuroprotective therapies

Marco Sancandi
Research Degree: School of Pharmacy
Department of Pharmacology, UCL school of Pharmacy, London

I, Marco Sancandi, hereby confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my Ph.D. supervisor Dr. Audrey Mercer from “University College London – School of Pharmacy” for believing in me and my abilities, and for her dedicated support and guidance. Audrey continuously provided encouragement, and she was always willing and enthusiastic to assist in any way she could throughout the project. I would also like to thank my second supervisor Dr. Andrew Constanti from “University College London – School of Pharmacy” for his invaluable contributions regarding both the analyses and the writing.

I would like to express my sincere gratitude to my fiancé Valentina, who has always been on my side, supporting me in the most difficult periods with her love and understanding. Furthermore, I must express my profound gratitude to my parents, Stefano and Florence, for providing me with unfailing support and continuous encouragement throughout my years of studying and through the process of researching.

I also wish to thank my colleagues and friends from UCL, starting from Georgia Economides and Adam Phillips who have given me practical suggestions and helpful advice throughout the project. Similarly, thanks should also go to all my friends, from and outside the UK, who gave me the necessary distractions from my research when needed.

Lastly, a special acknowledgment goes to Dr. Claudio Latagliata from “Fondazione Santa Lucia, IRCCS, Rome”. Although he is not directly involved in this project, Claudio was the person that followed, aided, supported, and trained me seven years ago when I first set foot in a lab.

This accomplishment would have not been possible without all of them.

Thank you.

M.S.

Table of Contents

ABSTRACT **12**

IMPACT STATEMENT **13**

CHAPTER 1 - INTRODUCTION **15**

1.1 PD diagnosis *17*

1.1.1 PD differential diagnosis 17

1.2 How does PD develop? *19*

1.2.1 Different theories on how PD pathophysiology spreads 19

1.2.2 LBs are key players in PD neurodegeneration 21

1.2.3 Gut-brain axis in PD 22

1.2.4 Neuroinflammation may be a PD pathogenesis trigger 22

1.3 Motor symptoms: the cornerstones of PD *24*

1.4 The neglected side of PD: non-motor symptoms *26*

1.5 Counteracting PD progression: established and emerging strategies *29*

1.5.1 Dopamine-based medications 29

1.5.2 Beyond dopaminergic treatments 31

1.6 Hyposmia and olfactory circuitry in PD *33*

1.7 Animal models of PD *36*

1.7.1 Different species models of PD 36

1.7.2 Genetic animal models of PD 37

1.7.3 Toxin-based animal models of PD 39

1.8 Exendin-4: glucagon-like peptide-1 (GLP-1) receptors as new therapeutic targets *42*

1.9 Background and aims of the present project *44*

CHAPTER 2 - MATERIALS AND METHODS **45**

2.1 Animals *46*

2.2 Generation of the early-stage PD animal model *46*

2.3 Surgeries and drug administration *47*

2.4 Immunohistochemistry *49*

2.4.1 Immunofluorescence 52

2.4.2 Immunoperoxidase 52

2.4.2.1 Neuroinflammation 53

2.4.2.2 Neuronal distributions 53

2.7 Statistics *54*

CHAPTER 3 - INSIDE THE CORE OF THE OLFACTORY CIRCUITRY **55**

3.1 INTRODUCTION 56

3.1.1 First step: The olfactory bulbs *57*
3.1.2 Second step: the primary olfactory cortex *58*
3.1.3 Monoaminergic modulation of the OB and PC *61*
3.1.4 Exploring the aetiology of hyposmia in the PC and OB *62*
3.1.5 Aims of this chapter *63*
3.2 MATERIALS AND METHODS 65
3.2.1 Experimental Groups *65*
3.2.2 Drugs *66*
3.2.3 Behavioural tests *67*
3.2.3.1 Hidden food test 67
3.2.3.2 Habituation/dishabituation test 68
3.2.3.3 Rotarod test 68
3.2.4 Immunohistochemistry *69*
3.2.4.1 Neuronal distributions in the PC* 70
3.2.4.2 Neuronal distributions in the OB 70
3.2.4.3 Tyrosine Hydroxylase (TH) staining 71
*3.2.5 High Performance Liquid Chromatography (HPLC)** *71*
3.2.6 Statistics *72*
3.3 RESULTS 73
3.3.1 6-OHDA- and DSP-4-induced dopaminergic and noradrenergic loss in the SNpc, striatum, and piriform cortex. *73*
3.3.2 The early-stage PD model displayed hyposmia in the absence of motor symptoms *75*
3.3.3 Neuroinflammation is present in the PC of the early-stage PD model. *77*
3.3.4 Down regulation of interneuronal calcium binding proteins is observed in the PC of the early-stage PD model. *79*
3.3.5 Cellular densities of GAD- and NeuN-immunopositive cells in the PC of the early-stage PD model. *81*
3.3.6 Degradation of the perineuronal nets (PNNs) in the PC of the early-stage PD model *83*
3.3.7 Increased number of dopaminergic interneurones in the OB GL of the early-stage PD model *85*
3.3.8 Increased CBP interneurones in the OB of the early-stage PD model *86*
3.3.9 Cellular densities of GAD- and NeuN-immunopositive cells in the OB of the early-stage PD model. *88*
3.4 DISCUSSION 90
3.4.1 Monoaminergic loss in the midbrain results in dopaminergic and noradrenergic denervation in the PC *91*
3.4.2 The model of early-stage PD displayed olfactory deficits in the absence of motor dysfunctions *93*
3.4.3 Neuroinflammation was observed in the PC of the early-stage PD model. *95*

3.4.4 Reduced expression of CBPs in the PC following dual-toxin treatment 96
3.4.5 Dual-toxin treatment upregulates dopaminergic and CBPs expression in the OB..... 100
3.4.6 The changes observed in the olfactory system of the early-stage PD model are partially prevented by EX-4..... 104
3.4.7 Summary and future directions 106

CHAPTER 4 – ALONG THE OLFACTORY PATHWAY: PFC AND HIPPOCAMPUS 108

4.1 Introduction 109
4.1.1 Cognitive decline in PD 110
4.1.2 Executive functions, PFC, and PD..... 111
4.1.3 Explicit memory, hippocampus, and PD 112
4.1.4 Aims of the present chapter 114
4.2 Materials and Methods 115
4.2.1 Experimental Groups 115
4.2.2 Drugs 116
4.2.3 Immunohistochemistry 116
4.2.4 Determination of the CA1-2-3 borders 117
4.2.5 Statistical analysis 119
4.3 Results 120
4.3.1 Decreased CBP interneurones in the PFC of the early-stage PD model. 120
4.3.2 Cellular densities of GAD- and NeuN-immunopositive cells in the PFC of the early-stage PD model are unaltered. 122
4.3.3 Dual-toxin treatment led to an increase in CB-positive interneurones in CA1 of the early-stage PD model..... 124
4.3.4 Reduced density of GABAergic interneurones in CA2 of the early-stage model. 128
4.4 Discussion 132
4.4.1 Expression of CBPs is decreased in the PFC of the early-stage PD model. 132
4.4.2 CA2 of the early-stage PD model is the first hippocampal region to display structural deficits. 134
4.4.3 Treatment with EX-4 partially prevents the observed deficits in the PFC and hippocampus 136
4.4.4 Summary and future directions 137

CHAPTER 5 - GENE THERAPY: A NEW POTENTIAL TREATMENT FOR PD 139

5.1 Introduction 140
5.1.1 Gene therapy for PD 141

5.1.2 Background and aims of this chapter *145*
5.2 Materials and Methods 147
5.2.1 Animals *147*
5.2.2 Compounds *148*
5.2.3 Surgeries and drug administration *149*
5.2.4 GFP-tagged empty viral vector *150*
5.2.5 TH-immunohistochemistry and unbiased stereology *151*
5.2.6 Statistical analysis *152*
5.3 Results 153
5.3.1 Tolerability of the viral vector *153*
5.3.2 Virus spread following specific pathways *154*
5.3.3 EX-4 viral vector delivered stereotaxically does not prevent dopaminergic loss in the SNpc *156*
5.3.4 EX-4 viral vector delivered intravenously prevents dopaminergic loss in the SNpc *158*
5.4 Discussion 160
5.4.1 The virus travelled from the striatum to several other brain regions while being well-tolerated by the animals *160*
5.4.2 Intravenous injection of EX-4 viral vector prevented cell loss in the SNpc. *162*
5.4.3 Gene therapy limitations *164*
5.4.4 Limitations of the study *165*
5.4.5 Summary and future directions *166*

CHAPTER 6 - FROM THE GUT TO THE BRAIN: EFFECTS OF PROBIOTICS ON EARLY STAGE PD 168

6.1 Introduction 169
6.1.1 The gut-brain axis *169*
6.1.2 The gut-brain axis and probiotics *171*
6.1.2.1 Probiotics and PD 173
6.1.2.2 Symprove 174
6.1.3 Aims of this chapter *175*
6.2 Materials and Methods 177
6.2.1 Experimental Groups *177*
6.2.2 Drugs *178*
6.2.3 Immunohistochemistry *178*
6.2.3.1 TH immunostaining quantification 179
6.2.3.2 Neuroinflammation 180
6.2.4 Statistical analysis *180*
6.3 Results 181

6.3.1 Tolerability of the probiotics *181*
6.3.2 Symprove® partially prevented dopaminergic loss in the SNpc *182*
6.3.3 Symprove did not prevent astrocyte activation in the striatum *184*
6.3.4 Effect of Symprove® on striatal microglial activation *186*
6.4 DISCUSSION 188
6.4.1 Symprove partially prevented dopaminergic loss in the SNpc of an early-stage PD model *190*
6.4.2 Symprove prevented the activation of microglial cells *191*
6.4.3 Limitations and future directions *193*
6.4.4 Summary and conclusions *194*
CHAPTER 7 - DISCUSSION 195
7.1 A novel early-stage PD animal model *197*
7.2 Hyposmia is sustained by cellular and structural changes in the olfactory pathway of the early-stage PD model *199*
7.3 Potential new treatments for early-stage PD *203*
7.3.1 Exendin-4: from type 2 diabetes mellitus (T2DM) to PD 203
7.3.2 Probiotics: another new hope for PD? 205
7.4 Methodological considerations *206*
7.5 Future directions *207*
7.6 Conclusions *211*
BIBLIOGRAPHY 212

List of Tables and Figures

Table 2.1 - Primary antibodies used for Immunofluorescence and Immunoperoxidase staining……48
Table 3.1 - Cellular densities of interneuronal CBPs in the PC of the sham animals, pre-motor PD model and model treated with EX-4. ……76

Figure 2.1 - Timeline for the generation of the early-stage PD animal model.……...43
Figure 2.2 - Coordinates used for stereotaxic striatal injections.……..……...45
Figure 2.3 - Immunohistochemical protocol.……...……..……...47

Figure 3.1 - A schematic view of the olfactory circuitry.……..……..……..……...53
Figure 3.2 - Layers and cell types of the olfactory bulb. ……...……..……..……..55
Figure 3.3 - Schematic cytoarchitecture and basic neuronal types in the Piriform Cortex….56
Figure 3.4 - Experimental timeline..……...……..……...63

Figure 3.5 - Reduction of dopamine and noradrenergic levels in a model of pre-motor PD model..……..71
Figure 3.6 - The pre-motor PD model displayed hyposmia in the absence of motor symptoms. ……73
Figure 3.7 - Neuroinflammation in the PC of the pre-motor PD model.……..…75
Figure 3.8 - Distribution of interneuronal CBPs in the pre-motor PD model……..77
Figure 3.9 - Distribution of NeuN- and GAD-immunopositive neurones in the model of the pre-motor PD model…….....……..……79
Figure 3.10 - Effect of EX-4 on the distribution of perineuronal nets (PNNs) in the PC of the pre-motor PD model…….....……..……81
Figure 3.12 - Increase in TH-immunopositive cells in the glomerular layer (GL) of the pre-motor PD model…….....……..……..82
Figure 3.13 - Distribution of interneuronal CBPs in the pre-motor PD model……..84
Figure 3.14 - Distribution of NeuN- and GAD-immunopositive neurones in the OB of all experimental groups.……..……...86

Figure 4.1 – Experimental timeline.……...112
Figure 4.2. Characterisation of the hippocampal CA2 borders.…..……115
Figure 4.3 - Distribution of CBP-containing interneurones in the PFC.……118
Figure 4.4 - Distribution of NeuN- and GAD67-immunopositive neurones in the PFC…….120
Figure 4.5 - Distribution of interneuronal CBPs in hippocampal CA1. 122
Figure 4.6 - Distribution of interneuronal CBPs in hippocampal CA2. 123
Figure 4.7 - Distribution of interneuronal CBPs in hippocampal CA3. 124
Figure 4.8 - Distribution of NeuN- and GAD-immunopositive neurones in hippocampal CA1. ……..……..126
Figure 4.9 - Distribution of GAD-immunopositive neurones in hippocampal CA2…….127
Figure 4.10 - Distribution of NeuN- and GAD-immunopositive neurones in hippocampal CA3. ……..……..128

Figure 5.1 - Experimental timelines.…..……..…145
Figure 5.2 - Animal weight curves…....……150
Figure 5.3 - GFP-tagged empty AAV vector spreading within the brain……..……152
Figure 5.4 - EX-4 viral vector delivered stereotaxically does not prevent dopaminergic loss in the SNpc.……...154
Figure 5.5 - EX-4 viral vector delivered intravenously does prevent dopaminergic loss in the SNpc.……...……156

Figure 6.4 – Experimental timeline.……173
Figure 6.2 - Animal weight curves……..……177

Figure 6.3 - Reduction of dopaminergic levels in a rat model of pre-motor PD was partially prevented by treatment with Symprove®.……179
Figure 6.4 – Activation of astrocytes in the striatum of the pre-motor PD model, sham + Symprove®, and model + Symprove®…….181
Figure 6.5 – Microglial cells activation in the striatum of the sham + Symprove® and model + Symprove® is similar to that in sham animals treated with placebo and model + Symprove®.……..183

Abbreviations

5-HT: 5-hydroxytryptamin
6-OHDA: 6-hydoxydopamine
CA: cornu ammonis; CB: calbindin
COMT: catechol-O-methyl transferase
CR: calretinin DA: dopamine
DAT: dopamine transporter
DBS: deep brain stimulation
DSP-4: N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
EPL: external plexiform layer
ESPD: early stage Parkinson's disease
ET: external tufted
EX-4: Exendin-4
GABA: gamma-aminobutyric acid
GL: glomerulous layer
GCL: granule cell layer
GLP-1: glucagon-like peptide 1
GLP-1Rs: glucagon-like peptide 1 receptors
L-DOPA: L-dihydroxyphenylalanine
LBs: Lewy bodies
LC: Locus Coeruleus
LOT: lateral olfactory tract
LPS: bacterial lipopolysaccharide
MAO-B: monoamine oxidase-B
MCL: mitral cell body layer
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSs: motor symptoms
NA: noradrenaline
NET: norepinephrine transporter
NMSs: non-motor symptoms
OB: olfactory bulb
OSNs: olfactory sensory neurones
PD: Parkinson's disease
PC: piriform cortex
PFC: prefrontal cortex
PG: periglomerular
PNNs: perineuronal nets
PV: parvalbumin
ROS: reactive oxygen species
SNpc: Substantia Nigra pars compacta
SPECT: single-photon emission computed tomography
sSA: superficial short-axon
TH: tyrosine hydroxylase
TNF- α: tumour necrosis factor α
WFA: wisteria floribunda agglutinin.

Abstract

The present thesis aimed at providing insight into the aetiology and treatment of Parkinson's disease (PD), which symptomatology consists of both motor and non-motor symptoms (NMSs). The latter have been linked to a loss of neurotransmitters other than dopamine and they have been shown to be modulated by treatments that do not act directly on the dopaminergic system, such as the glucagon-like peptide-1 receptor agonist exendin-4 (EX-4). Nevertheless, the aetiology of NMs, alongside with their potential treatments, has yet to be fully investigated. In this study, through injections of the neurotoxins N-N-ethyl-2-bromobenzylamine (DSP-4) and 6-hydroxydopamine (6-OHDA), a rat model of early-stage PD was developed and validated. Animals displayed the NMS hyposmia and memory impairments in the absence of motor symptoms, suggesting the PD model is representative of an early stage of the disease. Next, the effect of partial noradrenergic and dopaminergic denervation in several brain regions within the olfactory pathway was investigated using immunohistochemical techniques. Surprisingly, the combined denervation led to a reduction in the expression of interneuronal calcium binding proteins (CBPs) in the primary olfactory cortex and prefrontal cortex, whilst the expression in the olfactory bulbs was found to be increased, alongside with dopaminergic expression. Additionally, GABAergic cells in CA2 of the PD model were found to be decreased compare with controls. Interestingly, the observed structural changes were partially prevented following treatment with EX-4. Additionally, two preliminary studies were conducted using the early-stage PD model to test two potential new treatments, a novel viral vector and probiotics, and their effectiveness in preventing neuronal loss in the Substantia Nigra. Overall, this rat model of early-stage PD offers a useful means for research into early diagnosis as well as early intervention of PD, possibly resulting in a delay of disease progression together with improved patient's quality of life.

Impact Statement

Parkinson's disease (PD) is a highly debilitating disorder with no present cure and is believed to currently affect more than 10 million people worldwide. The cardinal feature is the loss of dopamine neurones in the midbrain, which plays a vital role in the control of movement. By far, the most common treatment for PD is to temporarily replace dopamine and transiently reactivate dopamine's neurotransmitter functions in the nigral-striatal pathway, by giving L-dihydroxyphenylalanine (L-DOPA) that neurones can convert into dopamine, giving a temporary, though imperfect, restoration of movement. However, the pathology of PD encompasses a much wider neurological base and range of symptoms than can be accounted for by dopamine loss alone. These so-called non-motor symptoms, which are not ameliorated by L-DOPA, include anxiety, depression, cognitive deficits and loss of sense of smell (hyposmia). These are linked to depletion of other key brain chemicals such as noradrenaline, 5-hydroxytryptamine and gamma-aminobutyric acid (GABA). Currently used animal models have focussed heavily on dopamine depletion and motor dysfunctions to replicate the pathology of PD and to develop treatment strategies, mainly because the motor symptoms are the clinically most prominent 'event'. The lack of PD animal models representing the early stage of the disease is therefore currently limiting our current understanding of its aetiology and treatment. This Ph.D. project focussed on developing, validating, and characterising a novel early-stage PD animal model to uncover some of the deficits that underlie the aetiology of hyposmia and test new potential treatments aimed at slowing down PD progression. We

demonstrated that hyposmia in this animal model is sustained by the presence of neuroinflammation in the primary olfactory cortex together with other structural changes, such as a downregulation in the expression of calcium-binding proteins in GABAergic interneurones. Moreover, hyposmia in the PD model was correlated with an increased synthesis of dopamine in the olfactory bulb. These results are the first report of deficits/changes occurring within the olfactory pathway during the early stage of the disease. In addition, our results showed that treatment with the antidiabetic peptide exendin-4 (EX-4), a GLP-1 receptor agonist, twice daily for 7 days, was able to prevent both neuroinflammation and structural changes in both the olfactory cortex and the olfactory bulbs, suggesting that it may be effective in slowing down the progression of the disease. Following these results, we developed a single-dose GLP1 receptor agonist viral vector gene therapy and explored its neuroprotective effect in the Substantia Nigra. Similarly, treatment with the probiotic preparation Symprove® resulted in neuroprotection and anti-inflammatory effects. Overall, we conclude that this rat model of early-stage PD offers a useful means for research into early PD treatments. Future investigation of a GLP-1 therapeutic target system and the effects of probiotic supplements may lead to the possibility of earlier intervention for PD patients and a significant delay of disease progression.

Chapter 1 - Introduction

Parkinson's disease (PD) was first described more than 200 years ago by the English doctor James Parkinson. Today, it is the second most common neurodegenerative disorder and is believed to currently affect more than 10 million people worldwide (Parkinson's Foundation, 2020). PD rarely emerges before 50 years of age, however, the incidence increases 5–10-fold from the sixth to the ninth decade of life (Van Den Eeden *et al.*, 2003; Savica *et al.*, 2013). In addition, the enhancement of general health care has led to longer survival, which is associated with increasing prevalence of neurodegenerative disorders over time, including PD. Early-onset PD, which accounts for 3-5% of all PD diagnoses, is defined as the onset of parkinsonian features before the age of 40 years old, whilst young-onset PD is observed between the second and the forth decade of life (Schrag and Schott, 2006). Lastly, PD is twice as common in men than in women in most populations, suggesting a potential protective effect of oestrogens (Radhakrishnan and Goyal, 2018). The hallmark features of PD are neuronal loss of dopaminergic cells in the substantia nigra pars compacta (SNpc) and the appearance of Lewy bodies (LBs), intracellular aggregates of α-synuclein (Beal, 2010). Clinically, all the cardinal signs of PD relate to motor dysfunctions and include resting tremor, bradykinesia, rigidity and postural reflex impairment, together with other psychiatric and dysautonomia symptoms, such as anxiety and depression, constipation, and olfactory dysfunctions (Wirdefeldt *et al.*, 2011). This set of symptoms is also referred to as "parkinsonism", a term usually used for syndromes with known aetiology that share some or most of signs of PD plus other signs or symptoms that are not characteristic of PD (Srivanitchapoom, Pitakpatapee and Suengtaworn, 2018).

1.1 PD diagnosis

Currently, the diagnosis of PD is entirely based on clinical criteria and no signs of other neurological disease or history of provoking drugs, toxins or infections (Wirdefeldt *et al.* 2012). Therefore, it is very important to find reliable biomarkers that can allow to differentiate PD from other conditions, monitor its progression, and evaluate the effectiveness of a specific therapeutic intervention (Söderbom, 2020). To date, PD biomarkers can be divided into four main categories: clinical, imaging, biochemical, and genetic (Emamzadeh and Surguchov, 2018). Initially, protein biomarkers, such as dopamine metabolites and amino acids, found in blood, serum, and cerebrospinal liquid were considered the most promising (Emamzadeh and Surguchov, 2018), whilst various forms of α-synuclein were considered the most efficient (Liddle, 2018). Lately, however, microRNA-based analysis has been shown to hold promising results, especially when in combination with α-synuclein data (Arshad *et al.*, 2017). Indeed, a miRNA-based biomarker for the early diagnosis of PD was recently discovered in the CSF of early stage PD patients, with the inclusion of α-synuclein in the analysis further strengthening the results (Dos Santos *et al.*, 2018). Similarly, a significant increase in inflammatory- and hypoxia-related microRNAs were observed in plasma-extracellular vesicles of an early-stage PD model, identifying the latter as a potential novel biomarker in early stages of PD (Sancandi *et al.*, 2020).

1.1.1 PD differential diagnosis

The diagnostic criteria for PD have evolved several times during the years with the last update, by the Movement Disorders Society, as recent as 2015. Although PD symptoms will be described in depth in a following paragraph,

briefly, PD is defined as bradykinesia in combination with at least rest tremor or rigidity, and three key points have to be met: 1) absence of absolute exclusion criteria; 2) at least two supportive criteria, and 3) no red flags. If any of the above key points is not met, then the diagnosis will be considered as a differential parkinsonism. Specifically, based on the underlying causes and clinical features, a diagnosis of secondary parkinsonism, atypical parkinsonism, neurodegenerative diseases, or other disease is made (Postuma *et al.*, 2015). Secondary parkinsonism can be caused by several factors, such as lesions of the basal ganglia following ischaemia, neoplasia or infections, and it is usually characterised by an abrupt onset and the coexistence of other symptoms (Srivanitchapoom, Pitakpatapee and Suengtaworn, 2018). Similarly, exposure to toxins or dopamine-blocking agents, such as anti-psychotics, can lead to parkinsonism with clinical features resembling PD (Balestrino and Schapira, 2020). Atypical parkinsonism is a set of disorders, such as dementia with Lewy bodies, progressive supranuclear palsy, multiple system atrophy, and corticobasal syndrome, that share some signs and symptoms with PD but do not respond well to classic PD treatments (Moretti, 2019). Lastly, imaging techniques can help in differentiating PD from other conditions. For example, dopamine transporter single-photon emission computed tomography (SPECT) scans allow the discrimination between PD and essential tremor, whilst MRI may help differentiate PD from other parkinsonism syndromes, such as vascular parkinsonism and parkinsonism that includes multiple system atrophy (Armstrong and Okun, 2020).

1.2 How does PD develop?

Although neither the loss of dopaminergic neurones in the SN nor the deposition of α-synuclein is exclusively linked to PD, their co-existence allows for a definitive diagnosis of PD (Poewe *et al.*, 2017). There are two forms of PD: familial and sporadic. Whilst the former is caused by genetic aberrations, such as the gene coding for α-synuclein (Stefanis, 2012), the cause for sporadic PD remains unknown; however, both genetic and environmental factors are believed to be involved (Rietdijk *et al.*, 2017). Not knowing precisely what triggers neuronal death makes the study of sporadic PD aetiology a challenge. Nevertheless, some important elements contributing to the development of the disease have been identified, such as neuroinflammation, mitochondrial dysfunction, and misfolding and aggregation of α-synuclein (Lee *et al.*, 2010; Drouin-Ouellet and Cicchetti, 2012; Lema Tomé *et al.*, 2013; Niranjan, 2014; Tong *et al.*, 2015; Zaltieri *et al.*, 2015).

1.2.1 Different theories on how PD pathophysiology spreads

In 2003, it was proposed for the first time, that neuronal damage and death in PD may not occur randomly but follow a predetermined pattern (Braak *et al.*, 2003). The Braak's hypothesis suggested that PD progression can be divided into 6 stages. Briefly, the first two stages are characterised by the presence of LBs in the gut, olfactory bulb and anterior olfactory nucleus as well as in medullary nuclei, such as the lower raphe nuclei and the locus coeruleus. In addition, patients start experiencing some non-motor symptoms including hyposmia, autonomic dysfunctions, and disturbed sleep. In stage 3, LBs reach the SNpc, the upper raphe nuclei, and the central subnucleus of the amygdala

with some PD patients suffering from early phase motor dysfunctions. However, the latter are characteristic of stage 4, together with LBs spreading to the anteromedial temporal cortex. The spread of LBs to cortical areas regulating autonomic functions, in high-order sensory association areas and prefrontal cortex, and in primary sensory and motor areas in the last two stages is associated with severe motor dysfunctions, cognitive impairment, and dementia (Del Tredici and Braak, 2016). The Braak's hypothesis, however, is not based on the distribution of neuronal death, but on the distribution of LBs, which does not explain how it relates to the progression of neurochemical changes, and should be therefore cautiously interpreted (Yulug, Ozansoy and Cankaya, 2019). For example, it was argued that Braak's hypothesis suggests for the presence of patterns of synucleinopathy that are often not observed in clinical practice, and no actual relationship between the different stages and the clinical severity of PD has been observed (Burke, Dauer and Vonsattel, 2008). For instance, post-mortem studies in humans have shown the presence of LBs in asymptomatic individuals, while many individuals who had clinically advanced PD would fall into initial Braak's stages (Rietdijk *et al.*, 2017).

Recently, Braak's hypothesis has been questioned by a new theory aimed at explaining PD progression, the threshold theory (Engelender and Isacson, 2017). This theory suggests that PD pathogenesis does not occur due to a neurone to neurone propagation of α-synuclein but, rather, a parallel degeneration of both central nervous and peripheral nervous systems. Neuronal connections between the CNS, brainstem, and the autonomic nervous system, lack of redundant connections; therefore, if some

connections are affected by LBs, the whole system becomes dysfunctional leading to the appearance of non-motor symptoms. In contrast, the circuitry in the midbrain that regulates motor functions has several connections that can compensate for deficits of inputs; thus, delaying the characteristic motor symptoms appear to only when the majority of these connections are affected by LBs (Engelender and Isacson, 2017).

1.2.2 LBs are key players in PD neurodegeneration

Although these two theories differ on the mechanism by which LBs spread, they both agree upon the key role that LBs play in the peripheral nervous system. Indeed, the parasympathetic nerves and the enteric nervous systems were repeatedly found to be among the first structures to be affected by LB pathology, possibly leading to the manifestation of non-motor symptoms before the classical motor symptoms, supporting the hypothesis that PD pathophysiology propagates from the gut to the brain (Yang *et al.*, 2019). Additionally, animal studies have shown that alterations in the gut microbiota were required for the appearance of motor deficits, inflammation, and LB formation, suggesting that gut microbiomes may play a key role also in the onset of motor dysfunctions (Sampson *et al.*, 2016). Similarly, it was reported that alterations in the microbiota composition correlate positively with the severity of postural instability and gait difficulty in PD patients (Scheperjans *et al.*, 2015).

1.2.3 Gut-brain axis in PD

The gut-brain axis links the GI tract to the CNS through biochemical signalling (Gómez-Pinilla, 2008) and disruption of the gut epithelium was shown to lead to intestinal inflammation as shown in both preclinical and clinical studies (Tan, Hor, *et al.*, 2020) (Devos *et al.*, 2013; Houser *et al.*, 2018; Lubomski *et al.*, 2020). GI symptoms in PD patients, such as constipation and bloating, usually arise years before the onset of motor symptoms (Perez-Pardo *et al.*, 2017; Radhakrishnan and Goyal, 2018; Seppi *et al.*, 2019) and an increased intestinal permeability in PD correlates with the presence of intestinal α-synuclein (Forsyth *et al.*, 2011). The combination of gut inflammation and deposition of α-synuclein fibrils in the ENS has therefore been suggested to initiate the neuroinflammatory process that leads to the pathogenesis of PD (Rietdijk *et al.*, 2017; Gazerani, 2019; Lubomski *et al.*, 2020; Tan, Hor, *et al.*, 2020; Castelli *et al.*, 2021). Lastly, it was recently shown that IBS diagnosis is associated with a 44% increase risk of developing PD (Liu *et al.*, 2021), and that treatment with L-DOPA affects the normal microbiota composition of the gut (Weis *et al.*, 2019). However, several factors may be involved and the changes in microbial composition induced by L-DOPA in PD patients require further investigations (Weis *et al.*, 2019).

1.2.4 Neuroinflammation may be a PD pathogenesis trigger

Whilst LBs are often considered as the initiators of the disease, several other factors, such as mitochondrial dysfunction, oxidative stress, and neuroinflammation are thought to be also involved in PD pathogenesis (Liu *et al.*, 2002; Beitz, 2014a; Celardo, Martins and Gandhi, 2014; Klingelhoefer and

Reichmann, 2015; Zaltieri *et al.*, 2015; Ransohoff, 2016; Puspita, Chung and Shim, 2017; Radhakrishnan and Goyal, 2018). Dysfunction of mitochondria can be caused by bioenergetic defects, mutations in mitochondrial DNA, or presence of mutated proteins resulting in mitochondrial changes in size and morphology, alterations in trafficking or transport, and impairment of transcription (Whitton, 2007; Bose and Beal, 2016; Gelders, Baekelandt and Van der Perren, 2018a). Particularly, α-synuclein aggregation occurring inside mitochondria has been shown to result in mitochondrial complex I deficits and oxidative stress (Devi *et al.*, 2008), leading to a vicious cycle in which α-synuclein aggregation and mitochondrial dysfunction exacerbate each other (Poewe *et al.*, 2017). Mitochondrial dysfunction is thought to upregulate the production of reactive oxygen species (ROS), which play crucial roles in the regulation of cellular metabolism, antioxidant defence, and posttranslational modification of proteins (Wang *et al.*, 2013). Additionally, dopaminergic neurones in the SNpc are thought to be particularly vulnerable to metabolic and oxidative stress (Puspita *et al.*, 2017). Although the exact reason behind this vulnerability has yet to be found, it is thought that it may be due either to their pacemaking activity involving cytosolic calcium oscillations and calcium extrusion at the expense of energy (Surmeier *et al.*, 2017) or to the depletion of lysosomes and functional impairment of lysosomal autophagy (Poewe *et al.*, 2017). Neuroinflammation is known to be a key element in the pathogenesis of PD in both PD patients and models (Wang, Liu and Zhou, 2015a; Mehta and Tanner, 2016; McKenzie *et al.*, 2017; Gelders, Baekelandt and Van der Perren, 2018a), although it is not universally regarded as a potential initial trigger (Ransohoff, 2016). Nevertheless, experimental

evidence from both animal models and neuronal cultures has shown that neuroinflammation can also promote α-synuclein misfolding, suggesting that the two processes influence each other, resulting in a cycle similar to that occurring in dysfunctional mitochondria (Gao *et al.*, 2008). A key question is whether the inflammatory processes are the cause or the consequence of PD (Gelders, Baekelandt and Van der Perren, 2018a). Currently, experimental evidence shows that microglia and T lymphocytes actively contribute to neurodegeneration by amplifying and exacerbating ongoing inflammatory processes, inducing extensive cell death in PD models (Appel, 2012).

1.3 Motor symptoms: the cornerstones of PD

Motor symptoms (MSs) represent the most well-known face of PD and, currently, clinical diagnosis only occurs after their appearance (Jankovic, 2008). MSs are mostly caused by a loss of dopamine (DA) in the nigrostriatal pathway (Beitz, 2014a). This decrease is in turn caused by a loss of pigmented neuromelanin-positive neurones in the SNpc, a mescencephalic brain nucleus which synthesises DA (Brissaud, 1925) and projects to the dorsal striatum through the median forebrain bundle (Björklund and Dunnett, 2007). The striatum integrates cortical and thalamic inputs reaching the basal ganglia to then guide motor behaviour. This integration is influenced by DA, which highly innervates the striatum rising from the midbrain nigrostriatal pathway. Indeed, it has been suggested that a single postsynaptic striatal medium spiny neurone could be modulated by up to almost 200 dopaminergic inputs (Matsuda *et al.*, 2009). As a result of these numerous dopaminergic inputs, MSs only develop when approximately 70 % of SNpc neurones are lost,

allowing the diagnosis of PD to be established (Rothwell, 2011). Primary MSs are bradykinesia and akinesia, tremor, rigidity, and gait disturbances, while impairment of handwriting, speech and precision grip are considered to be secondary MSs (Moustafa *et al.*, 2016). Bradykinesia and akinesia refer to functional disturbances of voluntary movements prominently characterized by slowness. Specifically, bradykinesia relates to slowness of a movement that is ongoing, while akinesia refers to failure of starting voluntary, spontaneous, or associated movements (Berardelli *et al.*, 2001). The pathophysiology underlying bradykinesia and akinesia is not completely understood, however it is thought to be linked to failure of the basal ganglia output to reinforce the cortical mechanisms responsible for the preparation of the movement and its execution (Obeso *et al.*, 2000). Tremor is not just a single sign but rather a class of symptoms and it can be further divided into rest tremor, re-emergent tremor, essential tremor, dystonic tremor, and exaggerated physiological tremor (Helmich *et al.*, 2012; Magrinelli *et al.*, 2016). Although very little is known about the pathophysiology of tremor, it is thought to differ from that of bradykinesia and rigidity since the magnitude of tremors does not correlate with DA deficiency and it is not improved by dopaminergic treatments (Hallett, 2012). Additionally, experimental evidence correlated the severity of tremor with a decreased serotonin receptor binding in the median raphe (Doder *et al.*, 2003). Rigidity in PD patients is characterised by increased resting muscle tone to palpation, reduced distension to passive movement, increased resistance to stretching, and facilitation of the shortening reaction (Rodriguez-Oroz *et al.*, 2009). Contrary to bradykinesia and tremor, PD rigidity is associated with changes in the passive mechanical properties of joints,

tendons, and muscles, and abnormalities in peripheral sensory inputs that may influence the response to muscle stretch (Andrews, Burke and Lance, 1972; Valls-Solé, 2000). Lastly, gait disturbances are common in PD patients and a major contributor to motor deficits (Magrinelli *et al.*, 2016). Similarly to the other motor dysfunctions, their pathophysiology has yet to be being fully uncovered and several central and peripheral triggers have been suggested, such as asymmetry of the basal ganglia output, abnormal processing of vestibular and proprioceptive inputs, abnormal spatial cognition, spinal and soft tissue abnormalities, and medication side effects (Castrioto *et al.*, 2014).

Thus, studies often classify PD patients into tremor-dominant, akinesia-dominant, or mixed phenotype category (Mure *et al.*, 2011; Lee *et al.*, 2012), with the hope of linking different clinical profiles, neural substrates, and outcomes to these different motor subtypes (Moustafa *et al.*, 2016). However, it has been recently found that patients can shift from one subtype to an another over time, suggesting that these phenotypes might have common clinical and neural substrates (Rainer Von Coelln *et al.*, 2015; Eisinger *et al.*, 2017).

1.4 The neglected side of PD: non-motor symptoms

Non-motor symptoms (NMSs) linked to PD include, among many, anxiety, depression, cognitive deficits, gastrointestinal symptoms such as constipation and dysphagia, pain, sleep disorders, and hyposmia, which is the loss of sense of smell (Hou and Lai, 2007; Beitz, 2014b; Del Rey *et al.*, 2018; Radhakrishnan and Goyal, 2018; De Rui *et al.*, 2020; Lubomski *et al.*, 2020). These symptoms can arise several years before the MSs used for the diagnosis of PD,

suggesting that DA loss may not be the only trigger (Schapira, Ray Chaudhuri and Jenner, 2017). While MSs are known to be sustained by dopaminergic loss, the aetiology of NMSs has yet to be elucidated (Poewe *et al.*, 2017). Some studies suggested that NMSs may be induced by a degeneration/depletion of other key brain neurotransmitters such as noradrenaline (NA), 5-hydroxytryptamine (5-HT), γ-aminobutyric acid (GABA), and acetylcholine (Hou and Lai, 2007; Delaville, Deurwaerdère and Benazzouz, 2011b; Janusz W Błaszczyk, 2016).

Sensory symptoms are NMSs commonly experienced by PD patients, with almost all patients suffering from at least one as a prodromal symptom, which will then increase in prevalence and severity as the disease progresses (Schapira, Ray Chaudhuri and Jenner, 2017). The first sensory impairments observed in PD patients relate to olfaction, which will be discussed in depth in section 1.7. Briefly, hyposmia or anosmia develop in more than 90% of patients with PD, preceding the onset of the dopamine deficiency-related motor dysfunctions (Bohnen *et al.*, 2008). PD-associated changes in olfaction were shown to be related to modifications in the central olfactory processing areas, with decreased olfactory bulb and piriform cortex volumes observed in PD patients (Wang *et al.*, 2011; Lee *et al.*, 2014; Ille *et al.*, 2015). Other sensory impairments include visual disturbances, such as hallucinations and diplopia (double vision), and their severity has been shown to correlate with PD progression (Nebe and Ebersbach, 2007) and to actually worsen following dopaminergic therapy (Baker *et al.*, 2009). Initially, these disturbances were thought to be drug-induced side effects; however, they have also been observed in untreated PD patients during the prodromal phase

(Pagonabarraga *et al.*, 2016). Visual disturbances are associated with cognitive impairments and dementia in PD progression, and hallucinations have been shown to correlate with cognitive decline in the advanced stage of the disease (Archibald *et al.*, 2013). During the late stage of PD, cognitive deficits and dementia usually arise as a consequence of senescence (Hely *et al.*, 2008). Cognitive impairments in PD patients can affect one of two main areas. The first one relates to planning, working memory, and executive dysfunction, processes associated with decreased DA levels in the frontal lobe-striatum loop, whilst the second one is responsible for attention, semantic verbal fluency, and visual spatial ability, involving dysfunctions of both the temporal lobe and the posterior cortical areas (Fang *et al.*, 2020). In addition to sensory symptoms and cognitive deficits, also neuropsychiatric disorders, such as anxiety and depression, occur in PD from the pre-motor phase to the late stages of the disease, with the two symptoms often overlapping (Poewe, 2008). While anxiety affects up to 60% of PD patients, depression is observed in 35% of PD patients and it generally appears in milder forms than in people without PD (Balestrino and Schapira, 2020). Lastly, sleep disorders and autonomic dysfunctions are also often experienced by PD subjects (Swick, 2012; De Pablo-Fernandez *et al.*, 2017). Most patients with PD experience sleep disturbances, such as insomnia, sleep fragmentation, prolonged awakening, and restless leg syndrome, with their prevalence increasing with the duration of the disease (Louter *et al.*, 2013). Autonomic dysfunctions are also common among PD patients and they were shown to both precede the appearance of motor deficits and worsen as the disease progresses (Beitz, 2014b). Generally, bladder, bowel and sexual dysfunctions, as well as

cardiovascular complications are the most common autonomic issues (Schapira, Chaudhuri and Jenner, 2017).

As mentioned above, NMSs in PD are not only sustained by a dopaminergic loss but, rather, involve degeneration of other neurotransmitters (Poewe *et al.*, 2017). Indeed, experimental evidence has linked neuropsychiatric, autonomic, and sleep disorders to GABAergic dysfunctions (Stefanis, 2012; Sanjari Moghaddam *et al.*, 2017). Furthermore, in the second stage of Braak's hypothesis, when MSs have yet to arise, LBs start to appear and accumulate in serotoninergic neurones (Braak *et al.*, 2003), heralding the subsequent dopaminergic loss in the SNpc (Hornung, 2003). Similar to GABAergic and serotoninergic systems, the noradrenergic system is also severely affected in PD, especially synapse sizes, polymorphism of the synaptic vesicles and marked morphological alterations of the mitochondria (Delaville, Deurwaerdère and Benazzouz, 2011a). Despite the increasing amount of evidence about the involvement of other neurotransmitters, currently, the first line treatment for PD remains aimed at replacing the dopaminergic loss, with NMSs treated with symptom-specific medications (Maiti, Manna and Dunbar, 2017).

1.5 Counteracting PD progression: established and emerging strategies

1.5.1 Dopamine-based medications

Presently, no definitive cure for PD has been found; however, several therapies have been developed that aim at slowing down the disease progression, providing transient relief of the severe symptoms (Maiti, Manna

and Dunbar, 2017). As the most characteristic symptoms of PD are associated with DA loss, the main PD treatments focus on both replacing and transiently reactivating DA signalling through the administration of L-dihydroxyphenylalanine (L-DOPA), a compound that can be converted into DA by specific nerve cells, leading to a temporary, though imperfect, improvement of motor functions (Whitton, 2007). The most utilised drug in this context is levodopa (L-DOPA), that has been shown to be both very effective in reducing the tremors and other MSs but unable to preserve or replace degenerated dopaminergic neurones (Foster and Hoffer, 2004). L-DOPA is associated with several mild side effects, such as nausea, vomiting, or low blood pressure as well as more severe side effects, such as behavioural impediments and generation of toxic metabolites (Paul and Borah, 2016). PD patients require more frequent and higher doses of L-DOPA over time since the brain loses the ability to store extra dopamine for later use as the disease progresses (Chou *et al.*, 2018). Additionally, after long duration of treatment, L-DOPA-induced dyskinesia, which is characterised by choreiform movements, is often observed in PD patients (Pandey and Srivanitchapoom, 2017). Two other main dopaminergic approaches are monoamine oxidase-B (MAO-B) and catechol-O-methyl transferase (COMT) inhibitors, both catalytic enzymes involved in DA breakdown (Sampaio *et al.*, 2018), that have been shown to sustain the response of L-DOPA for up to a year when administered together (Maiti, Manna and Dunbar, 2017). Lastly, DA agonists, such as pramipexole and ropinirole, are also therapeutic agents commonly used in the treatment of PD, which, however, often lead to severe side effects (Borovac, 2016). Specifically, it was shown that more than 40% of individuals treated with DA

agonists suffered from impulse control disorders, such as gambling, abnormal sexual and eating behaviours, and compulsive medication use (Garcia-Ruiz *et al.*, 2014). Furthermore, patients who discontinued the use of such medications were subjected to withdrawal symptoms (Rabinak and Nirenberg, 2010; Pondal *et al.*, 2013). Additionally, young individuals with tremor are often prescribed anticholinergic agents (*e.g.* benztropine, trihexyphenidyl, orphenadrine, procyclidine, and biperiden), though prescription of these drugs requires a close monitoring due to potential side effects, usually related to cognitive impairments (Armstrong and Okun, 2020).

1.5.2 Beyond dopaminergic treatments

Although treatments aimed at replacing DA can restore extracellular DA concentrations, they are unable to reverse functional and anatomical changes in the non-dopaminergic systems; thus, in recent years several new non-DA based strategies have been suggested (Oertel and Schulz, 2016). For example, deep brain stimulation (DBS) has been shown to be a safe and highly effective treatment option for some PD patients regardless of the stage of the disease, mostly due to its ability to modulate specific brain regions depending on the stimulation site (Muthuraman *et al.*, 2018). The treatment involves surgical placement of unilateral or bilateral leads in the subthalamic nucleus or the globus pallidus interna, which are connected to a battery in the chest (Armstrong and Okun, 2020). Furthermore, neurotrophic factors, and glial cell line-derived neurotrophic factor (GDNF) in particular, are considered to be promising molecules for PD treatment and several strategies have been designed to increase GDNF stability and retention in the brain (Del Rey *et al.*,

2018). Microencapsulated GDNF was shown to improve motor functions and restore DA function, through trophic effects on the nigrostriatal pathway, in different PD animal models (Garbayo *et al.*, 2009, 2011, 2016). Similarly, administration of basic fibroblast growth factor was found to stimulate dopaminergic function in surviving synapses and play a neuroprotective role in 6-OHDA hemiparkinsonian rats (Zhao *et al.*, 2014). Another approach is neural transplantation of stem cells, which have self-renewal capacity and ability to differentiate into dopaminergic cells (Romito and Cobellis, 2016). Specifically, mesenchymal stem cells can differentiate into a variety of neuronal phenotypes, such as dopaminergic as well as noradrenergic, serotonergic, and cholinergic cells, making them good candidates for counteracting NMSs (Pantcheva *et al.*, 2015). However, given the key role that neuroinflammation plays in PD, targeting the immune system appears as one of the most promising strategies for treating PD, with several nonsteroidal anti-inflammatory drugs shown to have neuroprotective effects on dopaminergic neurones (Hernán *et al.,* 2006; Rees *et al.*, 2011; Flood *et al.,* 2016). Relatedly, probiotics have recently been shown to exert neuroprotective effects in different models of PD due to their anti-inflammatory effects (Castelli *et al.*, 2020; Lubomski *et al.*, 2020). Additionally, probiotic supplementation in PD patients has been shown to exert benefits in the treatment of constipation, bloatedness, sense of incomplete emptying, and abdominal pain (Knudsen et al., 2017).

Lastly, treatments aimed specifically at managing NMSs in PD patients follow the same guidelines as for the general population (Armstrong and Okun, 2020). For example, depression associated with PD is usually treated using

selective serotonin reuptake inhibitors, selective serotonin-noradrenaline reuptake inhibitors, and tricyclic antidepressants, or cognitive-behavioural therapy and repetitive transcranial magnetic stimulation (Seppi *et al.*, 2019). Treatments for insomnia include melatonin and clonazepam (Howell and Schenck, 2015), whilst autonomic dysfunctions are addressed with probiotics and prebiotic fibre (Armstrong and Okun, 2020).

1.6 Hyposmia and olfactory circuitry in PD

Anxiety, depression, and cognitive deficits are symptoms that characterise several psychiatric and neurological disorders. In contrast, the loss of sense of smell is most commonly associated with PD, especially in the early stage of the disease (Doty, 2017). According to Braak's staging of the disease, Lewy pathology begins in the OBs and then it spreads to other brain regions (Del Tredici and Braak, 2016). Recently, it was shown that α-synuclein fibrils injected into the OBs propagate to 40 different brain regions bilaterally over the course of 12 months, indicating that the olfactory pathway can be a vector of PD pathology (Rey *et al.*, 2016). Indeed, α-synuclein pathology was found to be present across the entire olfactory pathway, including the anterior olfactory nucleus, cortical nucleus of the amygdala, piriform cortex (PC), olfactory tubercle, entorhinal cortex, and orbitofrontal cortex (Ubeda-Bañon *et al.*, 2017; Marin *et al.*, 2018). In addition, a recent meta-analysis found that individuals displaying hyposmia have a 2.12−6.95-fold increased risk of developing PD compared with healthy controls (Sui *et al.*, 2019). Hyposmia, which is one of the main sensory symptoms, is defined as the defect in the ability to perceive, recognise, and discriminate or memorise odours (Doty,

2012). Accordingly, PD patients tested for olfactory functions showed impairments regardless of whether the tests were aimed at odour detection, identification, discrimination or memory (Doty *et al.*, 1992; Mesholam *et al.*, 1998). Similarly, It has been suggested that detection of specific odours might be compromised in PD (Hawkes and Shephard, 1993), however, no experimental evidence was found, suggesting the absence of a specific damage to different olfactory receptor types (Marin *et al.*, 2018). Interestingly, the severity of hyposmia correlates directly with the severity of MSs and NMSs but not with the disease duration (Roos *et al.*, 2019). Furthermore, hyposmia appears to be milder in PD patients with LRKK2 mutations compared with idiopathic cases (Vilas *et al.*, 2020). Additionally, the presence of hyposmia has been shown to allow discrimination between dementia with LBs and dementia observed in patients suffering from Alzheimer's disease (Beach *et al.*, 2020). Structural magnetic resonance imaging studies on morphological changes occurring during hyposmia reported a reduction of the volume of the olfactory bulb in PD subjects that was linked to poor olfactory performances compared with those of control subjects (Wang *et al.*, 2011; Brodoehl *et al.*, 2012; Altinayar *et al.*, 2014; Li *et al.*, 2016). Similarly, a reduction in the volume of the PC of PD patients with hyposmia was observed (Wattendorf *et al.*, 2009; Chen *et al.*, 2014; Lee *et al.*, 2014; Ille *et al.*, 2015), as well as asymmetry in size between the left and right thalamus (Hwang *et al.*, 2019). Paradoxically, the total number of dopaminergic interneurones in the olfactory bulb of PD patients was found to be *increased* compared with control subjects (Huismanet *al.,* 2004). In addition, electrophysiological studies in PD patients have provided evidence for a decline of central brain networks as a causal

factor for olfactory loss in PD (Iannilli *et al.*, 2017). Indeed, hyposmia may not only be linked to the "primary" olfactory regions. Bohnen and colleagues (2008) observed that hyposmia in PD patients is linked to dopaminergic denervation in the hippocampus, and, more recently, a decreased functional connectivity between amygdala and inferior parietal lobule, and fusiform gyrus was observed correlating with the symptom's severity (Yoneyama *et al.*, 2018).

Relatedly, hyposmia does not appear to be sustained entirely and solely by a dopaminergic loss, but progressive degeneration of the other two monoamines as well as cholinergic systems has been suggested to correlate with olfactory impairments (Doty, 2012a; Müller and Bohnen, 2013; Marin *et al.*, 2017). Serotoninergic modulation of the OBs was shown to enhance the activity of the excitatory mitral cells (Huang, Thiebaud and Fadool, 2017). Additionally, α-synuclein aggregates were found in the raphe nuclei, along with marked depletion of serotonin in the OBs and other areas of the olfactory system (Vermeiren *et al.*, 2018). Similarly, LBs were found in cholinergic neurones of the basal forebrain parallel with α-synuclein aggregates and neuronal loss in the SNpc (Versace *et al.*, 2017). Indeed, acetylcholine in the OBs was shown to facilitate olfactory learning, memory, and odour discrimination (Chaudhury, Escanilla and Linster, 2009; Devore, Manella and Linster, 2012). Overall, hyposmia may, therefore, be used to aid clinical diagnosis and monitor PD disease progression (Berendse *et al.*, 2011; Doty, 2012, 2017).

1.7 Animal models of PD

PD is in 95% of cases an idiopathic disease, meaning that a combination of genetic and environmental factors contributes to PD pathology (Ortiz *et al.*, 2017). Therefore, to deepen our understanding of the pathophysiology of this multifaceted disease and expand the currently limited treatment options, experimental models that can replicate different aspects of PD in animals are needed (Konnova and Swanberg, 2018). Over the years, several animal models of PD have been developed, each one with its pros and cons depending on what aspects or characteristics of the disease the investigation focuses on (Jagmag *et al.*, 2016).

1.7.1 Different species models of PD

Three main animal groups are commonly used for studying PD pathophysiology and progression: non-human primates, non-mammalian species, and rodents. The former is used mainly due to their anatomic and genetic similarity to humans, which allows for behavioural assessments and neuroimaging studies (Emborg, 2007). However, non-human primates require more demanding care and higher maintenance costs, hence, only 10% of PD studies are performed on this animal group, which are usually reserved for preclinical evaluation of therapies (Grow, McCarrey and Navara, 2016). Non-mammalian models, such as *Drosophila melanogaster* (fruit fly) and *C. elegans* (nematode worm), have the advantages of being easily genetically manipulated, having a rapid reproductive cycle, requiring low costs of maintenance, and presenting well-defined neuropathology and behaviour (Konnova and Swanberg, 2018). Additionally, their small connectomes allow for the study of fundamental principles governing cellular, genetic, and network

changes resulting from dopaminergic loss (Jagmag *et al.*, 2016). Lastly, rodents can be easily taken care of in laboratory conditions, have associated robust experimental protocols, different suitable routes of drug administration, can be genetically modified and behaviourally assessed (Blesa *et al.*, 2012a). It should come with no surprise that 85% of all the animal studies on PD published since 1990 were performed on either mice or rats (Konnova and Swanberg, 2018). One of the main advantages of rodent PD models is that the nigro-striatal dopaminergic degeneration leads to MSs that can be studied and quantified using a wide range of behavioural tests, such as the open field test, the pole test, and the rotarod test (Schober, 2004). Moreover, mouse and rat models of PD also display several NMSs, allowing the study of their aetiology and potential treatments (Taylor, Greene and Miller, 2010). Indeed, the ideal PD model should present progressive and age-dependent development of both NMSs and MSs, dopaminergic loss in the SNpc and reduced DA levels in the striatum as well as neuroinflammation and LBs, characteristics that are rarely fully found all at once (Vingill *et al.,* 2018). Nevertheless, several animal models currently exist, which can be divided into genetic or neurotoxin-based models, with the choice of the most appropriate to use depending on the hypothesis tested (Imbriani *et al.*, 2018).

1.7.2 Genetic animal models of PD

Genetic models have mainly focussed on the role of synuclein alpha (SNCA), Parkin, PINK1, PARK7, LRRK2, and VPS35, genes that have been confirmed as monogenic PD variants (Marras *et al.*, 2016). SNCA was the first gene to be associated with familial PD together with the observation that the encoded protein α-synuclein was aggregating into LBs (Spillantini *et al.*, 1997), *de facto*

starting the line of research on α-synuclein models and pathology (Konnova and Swanberg, 2018). Nevertheless, it has yet to be determined whether the spreading of α-synuclein is indeed a driving factor for neuronal degeneration and progression in PD or if it is the result of other modifications (Killinger and Kordower, 2019). Parkin, PINK1, and PARK7 genes are involved in the regulation of mitochondrial functions in the cell, and animals lacking these genes have shown progressive MSs together with dopaminergic loss in the SNpc (Dave *et al.*, 2014). Additionally, overexpression of these autosomal recessive genes, has been shown to exert some beneficial effects in animal models (Konnova and Swanberg, 2018). Similarly, progressive reduction in DA release and dysfunctional dopaminergic neurotransmission have been found in knock-in models expressing mutated versions of LRRK2 (Tong *et al.*, 2009; Yue *et al.*, 2015). These models, however, do not replicate all PD hallmarks; thus, LRRK2 models are mainly used to study the interplay between different genetic mutations and environmental factors (Vingill, Connor-Robson and Wade-Martins, 2018). Furthermore, LRRK2 kinase inhibitors have been proposed as a potential therapeutic option in PD, and their effect has been explored in macaques following exposure to the nigral neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) with promising results (Henderson *et al.*, 2015). Lastly, the MitoPark mouse model was specifically created to study the involvement of mitochondrial dysfunction in the aetiology of PD. Specifically, this is a knock-out mouse with a mutation in the mitochondrial transcription factor A, a gene involved in mitochondrial DNA maintenance, selectively in the dopaminergic neurones in the SNpc (Ekstrand and Galter, 2009). The key point of this model is the adult-onset of

parkinsonian symptoms, both NMs and MSs, in a progressive manner that mimics the time-dependent decline observed in human PD (Branch *et al.*, 2016).

1.7.3 Toxin-based animal models of PD

Neurotoxin-based models of PD work by affecting either mitochondrial complex I or complex III (Schober, 2004). The main toxin models selectively disrupt or destroy catecholaminergic systems using agents such as MPTP, rotenone (insecticide), or 6-hydroxydopamine (6-OHDA) (Dauer and Przedborski, 2003). The former is first converted into MPP^+ by astrocytes and then uptaken into dopaminergic neurones by the selective dopamine transporter (DAT) inducing neuronal death by targeting the mitochondrial complex I (Schildknecht *et al.*, 2017). However, it should be noted that MPTP effectiveness varies depending on the strain of the animals, with some strains exhibiting an acute, but not progressive, dopaminergic loss and others being more resistant overall (Vingill *et al.,* 2018). Additionally, MPTP-induced dopaminergic degeneration in mice correlates with motor deficits which, however, are reverted within a few days post-acute injection, creating limitations on the duration of behavioural studies (Sedelis, Schwarting and Huston, 2001). Similarly to MPTP, rotenone acts by inhibiting mitochondrial complex I, however, it also induces LBs in dopaminergic neurones in the SNpc (Betarbet *et al.*, 2000), mimicking more accurately the pathophysiological characteristics of PD, and it is suitable for systemic administration (Imbriani *et al.*, 2018). Rotenone has therefore been suggested to replicate more precisely the environmental risk factors than toxins which require to be injected directly into the brain such as 6-OHDA. This model is nevertheless still widely used

since it induces MSs such as bradykinesia, postural instability and rigidity as well as NMSs including sleep disturbances, autonomic dysfunctions, neuropsychiatric conditions and hyposmia (Vingill *et al.,* 2018). 6-OHDA shares a similar structure with DA with the addition of an hydroxyl group, making it toxic for DA cells through a combined effect of ROS and quinones (Blesa *et al.*, 2012b). 6-OHDA induces neuronal degeneration involving the processing of hydrogen peroxidase and hydroxyl radicals in the presence of iron (Sachs and Jonsson, 1975). Separate injections of iron have been shown to produce neurotoxic effects comparable to those induced by 6-OHDA supporting the involvement of iron in 6-OHDA-induced neuronal degeneration (Youdim, Ben-Shachar and Riederer, 1991). Since 6-OHDA cannot cross the blood-brain barrier, it requires to be injected directly into the brain region of interest, such as the SNpc or the striatum, where it is taken up by the catecholaminergic nerve terminals and results in inhibition of mitochondria complex I (Imbriani *et al.*, 2018). Indeed, this compound shows a high affinity not only for the DAT but also for the noradrenaline transporter (NET), necessitating a co-injection of a selective noradrenaline reuptake inhibitor in order to protect the noradrenergic neurones from been damaged (Rodriguez-Pallares *et al.*, 2007). 6-OHDA is often injected unilaterally due to its high toxicity and the otherwise appearance of severe adipsia and aphagia, which can lead to the animal's death due to an inability to look after itself (Hernandez-Baltazar *et al.,* 2017). Indeed, unilateral injections of 6-OHDA into the SNpc trigger dopaminergic loss within 12 hours, reaching the striatum within 2-3 days (Thiele *et al.,* 2012). Although 6-OHDA injected into the SNpc induces DA depletion, nigrostriatal DA cell loss, and some of the neurobehavioural

deficits observed in PD patients, it does not appear to affect other brain regions, such as lower brain stem areas, and the locus coeruleus (LC) (Blesa *et al.*, 2012b). Nonetheless, administration of 6-OHDA into the striatum has been shown to replicate more faithfully the comprehensive symptomatology of PD, including NMSs such as cognitive impairment, depression and anxiety, as well as motor symptoms, suggesting that it affects other regions and pathways than the dopaminergic ones only (Tadaiesky *et al.*, 2008a; Santiago *et al.*, 2010; Bonito-Oliva, Masini and Fisone, 2014; L. Chen *et al.*, 2014). Whilst 6-OHDA is used to induce DA loss, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) is employed to trigger NA loss from inputs arising selectively from the locus coeruleus (LC) (Delaville, Deurwaerdère and Benazzouz, 2011b). This toxin crosses the blood-brain barrier, allowing its systemic administration, and is taken up into neuronal terminals by the NET, where it induces cell death by alkylation of unknown vital neuronal structures (Ross and Stenfors, 2014). The addition of NA depletion through DSP-4 in 6-OHDA-lesioned rats has been shown to induce several NMSs such as hyposmia and cognitive deficits (Khakpour-Taleghani *et al.*, 2009; Delaville *et al.,* 2010). Moreover, the combination of dopaminergic and noradrenergic degeneration in the prefrontal cortex was observed to trigger anxiety in a rodent model of early stage PD (Tadaiesky *et al.*, 2008), suggesting that both dopaminergic and noradrenergic innervation are involved in the aetiology of MNSs.

1.8 Exendin-4: glucagon-like peptide-1 (GLP-1) receptors as new therapeutic targets

Studying the aetiology of NMSs in PD is of particular importance since it may allow to identify and address the underlying deficits from a very early stage, slowing down the disease progression long before the appearance of the characteristic and debilitating MSs (Doty, 2017). As previously stated, NMSs are linked to several factors, hence, there is a need for new drugs that are multitarget and do not simply aim at restoring DA loss. A drug that has shown such properties is exendin-4 (EX-4), a glucagon-like peptide 1 (GLP-1) analogue, currently licenced for treatment of type II diabetes (Athauda and Foltynie, 2016). GLP-1 has been shown to regulate glucose homeostasis and facilitate insulin signalling via activation of GLP-1 receptors (GLP-1Rs) (Nadkarni *et al.,* 2014), a G-protein coupled receptor associated with cAMP-dependent activation of protein kinase A (PKA) and of cAMP-regulated guanine nucleotide exchange factor (Grieco *et al.*, 2019). This peptide is produced both peripherally in the ileum and centrally in the nucleus of the solitary tract and the OBs, although to what extent peripherally versus centrally derived GLP-1 activates GLP-1Rs has yet to be determined (Daniels and Mietlicki-Baase, 2019). These receptors are expressed in several brain regions, such as frontal cortex, PC, hypothalamus, thalamus, hippocampus, cerebellum, and substantia nigra, suggesting that GLP-1 exerts some important neuroregulatory roles within the CNS (Cork *et al.*, 2015). Indeed, GLP-1R activation in the brain is thought to trigger neurotrophic effects, neuroprotection, synaptic plasticity and to reduce neuroinflammation (Kim *et al.*, 2017, Grieco *et al.*, 2019).

Endogenous GLP-1 has a short half-life due to its rapid inactivation by the circulating enzyme dipeptidyl peptidase IV into a metabolite that is no longer able to activate GLP-1Rs (Holst *et al.,* 2011). In contrast, the GLP-1 agonist EX-4, found naturally in the saliva of the Gila monster (a venomous lizard native to the south-western USA and Mexico), has been shown to be resistant to this enzyme (Athauda and Foltynie, 2016). In addition, EX-4, despite being a peptide, can cross the blood brain barrier, allowing systemic administration (Athauda and Foltynie, 2018). EX-4 has proven to be neuroprotective in various animal disease models, such as hypoxic-ischaemic encephalopathy (Rocha-Ferreira *et al.*, 2018), cerebral ischemia (Zhang *et al.*, 2016; Rocha-Ferreira *et al.*, 2018), spinal cord injury (Li *et al.*, 2015) and several PD models (Bertilsson et al., 2008; Harkavyi et al., 2008; Li et al., 2009; Kim et al., 2009; Cao et al., 2016; Yun et al., 2018, Chen et al., 2018). Furthermore, clinical trials on PD patients reported that the activation of GLP1Rs through EX-4 improved motor functions, sleep quality, and cognition, while reducing depression compared with patients treated with conventional PD medication (Aviles Olmos et al., 2013; Aviles Olmos et al., 2014). Similarly, subcutaneous injections of EX-4 were found to improve, up to 12 weeks after exposure, motor functions of PD patients in the first randomized, double blind, placebo-controlled trial (Athauda et al., 2017). Notably, the positive effects were not only limited to motor dysfunctions, but also to mood and emotional well-being (Athauda *et al.*, 2018). Lastly, participants of the trial treated with EX-4 had significantly higher tyrosine phosphorylation of insulin receptor substrate 1 than placebo-matched patients, suggesting that the results observed in clinical trial may be elicited by an insulin-based molecular mechanism (Athauda *et al.*,

2019). However, the effects of EX-4 on the underlying pathophysiology of PD are yet to be fully elucidated.

1.9 Background and aims of the present project

Unfortunately, early stages of PD have not received as much attention as the late stages, where motor dysfunctions are already present impairing patient's quality of life. Additionally, PD research has focussed the attention on the characterisation of the deficits in the dopaminergic pathways, disregarding both other brain regions and other neurotransmitters that may be involved in the aetiology of NMSs. The project described in the present thesis was developed to help provide further insights into the deficits and treatment of the early-stage stage of the disease, specifically focussing on the NMS hyposmia. To this end, through depletion of the monoaminergic system, a dual neurotoxin animal model of early stage PD has been developed, in which NMSs, such as hyposmia, are observed in the absence of MSs (Sancandi *et al.*, 2018). The aims of the present project were therefore to investigate the deficits displayed in the above dual neurotoxin model that may underlie the loss of sense of smell and test new potential treatments, such as EX-4 and also probiotics, to see whether they can prevent these deficits.

Chapter 2 - Materials and Methods[1]

[1] This chapter contains the explanation of the techniques and procedures shared across all the experiments and it is intended as a scaffolding chapter. Please refer to individual chapters for more relevant and specific materials and methods.

2.1 Animals

Male albino Wistar rats (200–250 g) were purchased from either Harlan Laboratories, Inc., UK or Charles River Laboratories, UK. Animals were housed in groups of 4/5 in the Biological Service Unit (BSU) of the UCL School of Pharmacy. Animals had *ad libitum* access to food (standard rodent diet) and water. Conditions of humidity (40-60%), temperature (18-22°C) and a 12 hr light-dark cycle (light phase from 7 am to 7 pm daily) were kept constant in the BSU in line with the Home Office regulations. All experiments were approved by the Bloomsbury AWERB and carried out in accordance with UK Home Office [and European Communities Council Directive of 24 November 1986 (86/609/EEC)] guidelines (PPL No. 70/8199 and PP3144142).

2.2 Generation of the early-stage PD animal model

N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4), a selective noradrenaline neurotoxin, and 6-hydroxydopamine (6-OHDA), a neurotoxin used to deplete both noradrenergic and dopaminergic neurones, were purchased from Sigma (Sigma Aldrich, Gillingham, UK). DSP-4 was delivered at a concentration of 25 mg/kg in saline solution and injected intraperitoneally (i.p.) 4 days prior to the 6-OHDA injection, while 6-OHDA was dissolved at a concentration of 5 mg/ml in saline solution containing 0.9% ascorbic acid and delivered bilaterally in the striatum (Figure 2.1). The 3-day gap between injections was intended to replicate the pathophysiological progression of the disease observed in humans, in which the noradrenergic system is the first one to be affected followed by the dopaminergic one (Espay, LeWitt and Kaufmann, 2014). The optimum doses for DSP-4 and 6-OHDA

were chosen to induce *partial* reduction of NA and DA levels based on previous studies (Jonsson et al., 1981; Prezedborski et al., 1995), mimicking the early stage of the disease in which dopaminergic loss in the Substantia Nigra does not exceed 70%.

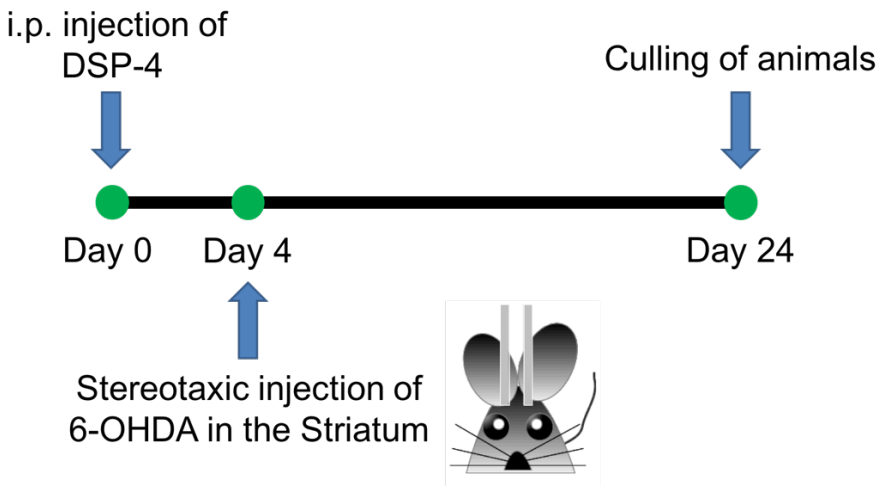

Figure 2.5 – Timeline for the generation of the early-stage PD animal model. *On day 0 animals were given an i.p. injection of either saline (sham) or DSP-4 (model). 3 days later, animals underwent stereotaxic surgery to receive bilaterally either saline (sham) or 6-OHDA (model) into the striatum.*

2.3 Surgeries and drug administration

The rats were anesthetised with Isoflurane (5% v/v in O_2 for induction and 2% v/v in O_2 for maintenance delivered through a fitted anaesthetic nose mask) and were then placed in a stereotaxic frame to restrain the head and kept warm using a heating blanket. Animals received a subcutaneous injection of 0.2 ml of both non-steroidal anti-inflammatory drug Rimadyl (Zoetis) as a general analgesic and the local anaesthetic bupivacaine hydrochloride (Marcain). The head was then shaved and the skull exposed to reveal the bregma, which is defined as the point of intersection of the sagittal suture with the curve of best fit along the coronal suture (Paxinos et al., 1985). Two holes

were then pierced using an electric drill on top of the striatum (coordinates: anterior-posterior (AP) +1.0 and medio-lateral (ML) ± 3.0 from bregma; dorso-ventral (DV) -6.5), according to the atlas of Paxinos and Watson (Figure 2.2, Paxinos and Watson, 1982). To confirm the site of the injections, one hemisphere per animal was cut perpendicularly to the hole left by the needle. Stereotaxic injections were given using a 10 µl Hamilton syringe (Hamilton Company, USA). 6-OHDA was dissolved at a concentration of 5 mg/ml in saline solution containing 0.9% ascorbic acid. Each animal received 15 µg of 6-OHDA or saline with ascorbic acid per striatum at a flow rate of approximately 1 µl/min. The needle was then left *in situ* for 5 minutes to improve diffusion and prevent efflux of the toxin before slowly withdrawing from the brain. The skin over the wound was then closed with surgical sutures (Coated VICRYL 4-0 75 cm, ETHICON®) and 1 ml of saline solution was injected subcutaneously to compensate for potential fluid loss. Animals' weights were then monitored daily. Sutures were removed 7-10 days after surgery. Animals were culled 19 days after surgery and brains were harvested for further immunohistological analysis.

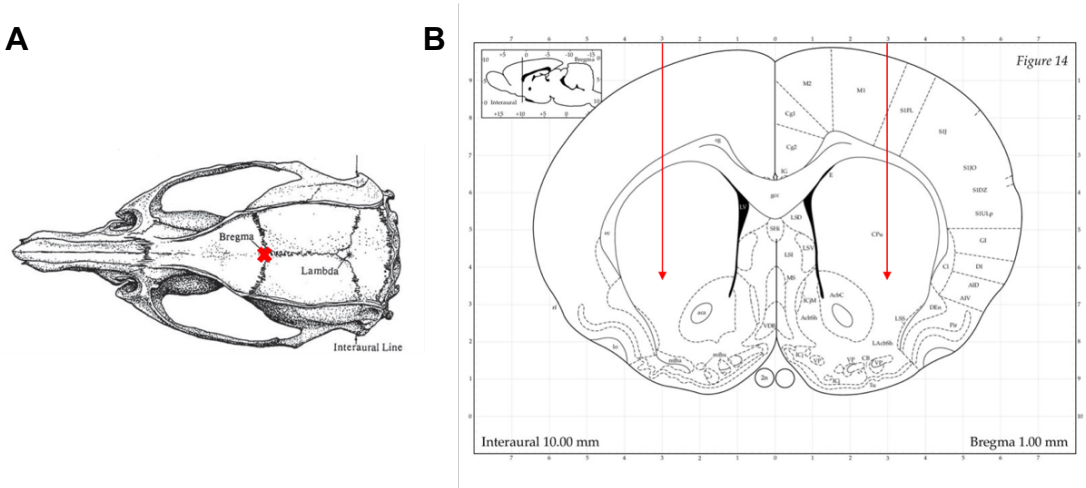

Figure 6.2 - Coordinates used for stereotaxic striatal injections. *A) Top down view of the rat skull delineating the location of bregma indicated by the red 'X'. B) Stereotaxic coordinates*

used for injection in the right and left striatum indicated by the red arrows (anterior-posterior +1.00mm, mediolateral +3.00mm, ventral-dorsal -6.5mm).

2.4 Immunohistochemistry

Transcardial perfusion generates optimal results by reducing the background staining (Hughes et al., 2000). Therefore, animals were anaesthetized with inhaled isoflurane (4% v/v in O_2) followed by an i.p. injection of euthatal (Merial, Harlow, UK) at a lethal dose of 60 mg/kg. Absence of the righting reflex indicated that the level of general anaesthesia sufficed. The heart was surgically exposed and a butterfly cannula (Butterfly-21, Hospira Venisystems, Ireland) was inserted into the left ventricle. Transcardial perfusion was then started by pumping ice-cold artificial cerebrospinal fluid (aCSF) containing in mM: 124 NaCl, 25.5 $NaHCO_3$, 3.3 KCl, 1.2 KH_2PO_4, 1 $MgSO_4$, 2.5 $CaCl_2$, 15 mM D-Glucose equilibrated with 95% O_2/5% CO_2. Brains were then removed and fixed overnight (4% paraformaldehyde, 0.2% saturated picric acid solution, 0.025% glutaraldehyde solution in 0.1 M phosphate buffer). Figure 2.3 shows the key steps of the immunohistochemical protocol. Briefly, 50 µm parasagittal or coronal sections containing the region of interest were cut using a vibratome. One in every 8 to 12 sections, depending on the size of the area of interest, was collected and 4-5 slices per animal were used per staining (please refer to individual chapters for exact numbers). Sections were incubated first in 1% H_2O_2 for 30 minutes and then in 1% sodium borohydride ($NaBH_4$) for 30 minutes to decrease background staining and then in either 1% bovine serum albumin for the Biotinylated Wisteria floribunda agglutinin (WFA) staining used to label perineuronal nets (PNNs) or in 10% normal goat serum (NGS) for all other antibodies for another 60 min to block nonspecific

antibody binding. Sections were incubated overnight at 4°C in a mixture of primary antibodies and triton X-100 (Sigma Aldrich) [1% Triton for GAD-67 - 0.1% for all other antibodies] made up in phosphate buffer solution. Primary antibodies used in this study are listed in Table 1. All antibodies were tested for specificity using negative controls.

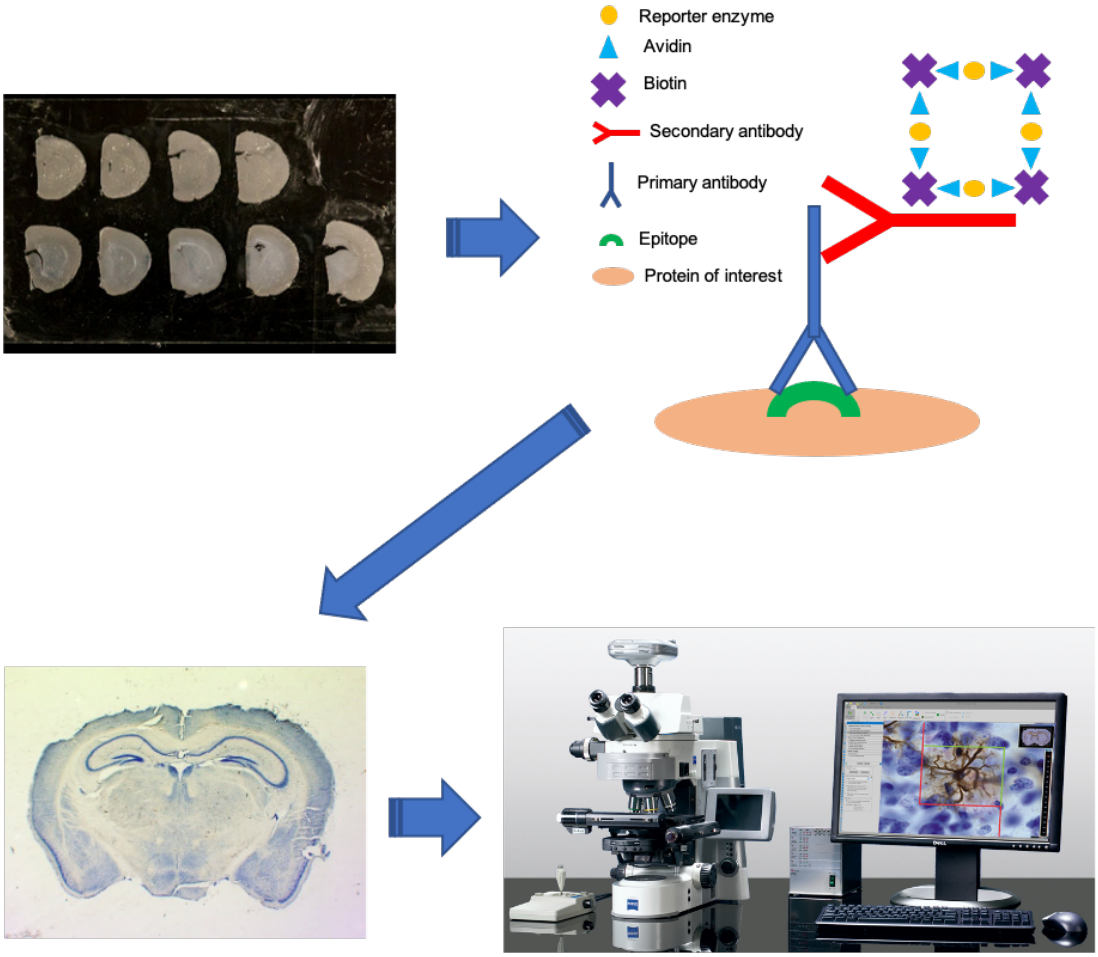

Figure 2.7 – Immunohistochemical protocol. *Fixed brains were sliced using a vibratome and 1 in every 8-12 slices was collected. Next, through a combination of primary and secondary antibodies, proteins of interest were stained and subsequently imaged with a microscope. In the immunofluorescence protocol the secondary antibody is tagged with a fluorophore rather than the avidin-biotin complex.*

Antibody	Immunogen	Manufacturer/ Investigator	Species	Catalogue/ Lot number	Dilutions
Parvalbumin	Purified frog muscle parvalbumin	Sigma	Mouse	P3088 Clone PARV-9 Lot #048K4752	1:1000
Calretinin	Recombinant rat calretinin	Millipore	Rabbit	AB5054/LV1532272	1:1000
Calbindin	Recombinant chick CaBP	Gift from Dr K. Baimbridge (2000)	Rabbit	R9501	1:1000
GAD67	Synthetic peptide from mouse GAD67 (amino acids (87-106))	Sigma	Mouse	MAB 5406 Lot #2549419	1:1500
TH	SDS-denaturated rat tyrosine hydroxylase purified from pheochromocytoma	Sigma	Rabbit	T8700-1VL Lot #SLBL8773V	1:7500
GFAP	Purified glial filament	Millipore	Mouse	MAB 3402 #2549419	1:7500
Iba1	C-terminus of Iba1	Wako	Rabbit	019- 19741	1:1000
α-actinin-2	Rabbit skeletal alpha-actinin	Sigma	Mouse	L1516	1:5000

Table 2.1 – Primary antibodies used for Immunofluorescence and Immunoperoxidase staining.

2.4.1 Immunofluorescence[*]

Sections for fluorescence microscopy were then incubated for 2 h in a mixture of fluorescently-labelled secondary antibodies, anti-mouse fluorescein isothiocyanate (FITC) (1:160, Sigma) and goat anti-rabbit Texas Red (1:500, Vector Laboratories) made up in PBS. All fluorescently-labelled sections were then mounted on slides in Vectashield (Vector Laboratories) and studied using a confocal microscope (Zeiss LSM 710). 6 to 7 Z-stacks from the PC of each slice in each animal were obtained using a x10 objective and immunopositive cell bodies were counted using a cell counting plug-in in ImageJ software. Cellular densities were expressed as the number of cells per mm^3 ± SEM.

2.4.2 Immunoperoxidase

Following incubation in primary antibodies, sections for immunoperoxidase staining were incubated overnight in secondary antibodies, biotinylated goat anti-mouse or anti-rabbit antibody (1:500, Vector Laboratories) made up in PBS. To visualise the stained neurones, sections were washed in 0.1M PB and PBS, incubated in ABC (Vector Laboratories) for at least 2 hours, washed in TRIS buffer, and then in 3, 3' diaminobenzidine (DAB, Sigma Aldrich). The stained neurones were then revealed using H_2O_2. Sections from the different experimental groups were processed together using the same immunoreagents and the DAB reaction was stopped adding TRIS buffer at the same time to allow comparison between groups. Sections were then placed onto Superfrost slides, dehydrated, cleared with Histoclear and mounted using

[*] This part of the project was performed by Emma Schul (PhD student, UCL SOP)

DPX (Sigma Aldrich). Finally, slides were stored in sealed boxes to prevent potential oxidation of the slices until needed for stereology.

2.4.2.1 Neuroinflammation

Levels of GFAP and Iba1 immunohistochemical staining were measured by quantitative thresholding image analysis as previously described (Rahim et al., 2012). Four non-overlapping images of the PC in each section were captured using a DMR microscope and Leica Application Suite V4 (Leica Microsystems) at 20x magnification with constant light intensity, microscope calibration and video camera settings. Image-Pro Premier (Media Cybernetics, Cambridge, UK) was used to analyse the images and measure immunoreactivity using a constant threshold that was applied to all images for each respective antigen. Data are presented as the mean percentage area of immunoreactivity ± SEM. 3D reconstructions of Iba1-immunopositive cells for each experimental group (*n* = 8 per animal) were obtained with a Neurolucida software (MBF Bioscience) and morphological characteristics of these cells were analysed using Sholl and branched structure analyses.

2.4.2.2 Neuronal distributions

The optical fractionator probe was used to determine the number of immunopositive neurones in all regions of interest (StereoInvestigator, MicroBrightField) using a Nikon microscope coupled to a computer-controlled x-y-z motorized stage and an MBF video camera system. Unbiased stereology was carried out on 5 slices per animal (Please refer to individual chapters for specific *n* numbers). The parameters for the stereological quantification were

adapted depending on the brain region analysed (please refer to individual chapters for specifics). Only neurones with visible nuclei and dendrites were counted.

2.7 Statistics

All data in this study were analysed using IBM SPSS Statistics Version 22.0. The Shapiro-Wilk test and the Kolmogorov-Smirnov test were carried out prior to statistical analysis to determine whether the data followed a normal distribution. The parametric one-way ANOVA or unpaired t-test were then used when the data followed a normal distribution and the non-parametric Kruskal-Wallis test was used in the case of a non-normal distribution.

Chapter 3 - Inside the core of the olfactory circuitry

3.1 Introduction

The olfactory system represents one of the oldest sensory modalities in the phylogenetic history of mammals. Initially, an odour is detected by the olfactory sensory neurones (OSNs) in the olfactory epithelium, where odorant receptors are located (Nagayama, Homma and Imamura, 2014). Over 1000 different functional odorant receptors have been identified in the mouse genome (Adipietro *et al.*, 2012). Interestingly, each OSN expresses only a single odorant receptor, hence, different odours trigger distinct subgroups of OSNs (Lodovichi and Belluscio, 2012). Activated OSNs then transmit the information firstly to the olfactory bulb (OB) and then to the olfactory cortex via the lateral olfactory tract, and to other cortical areas (Figure 3.1).

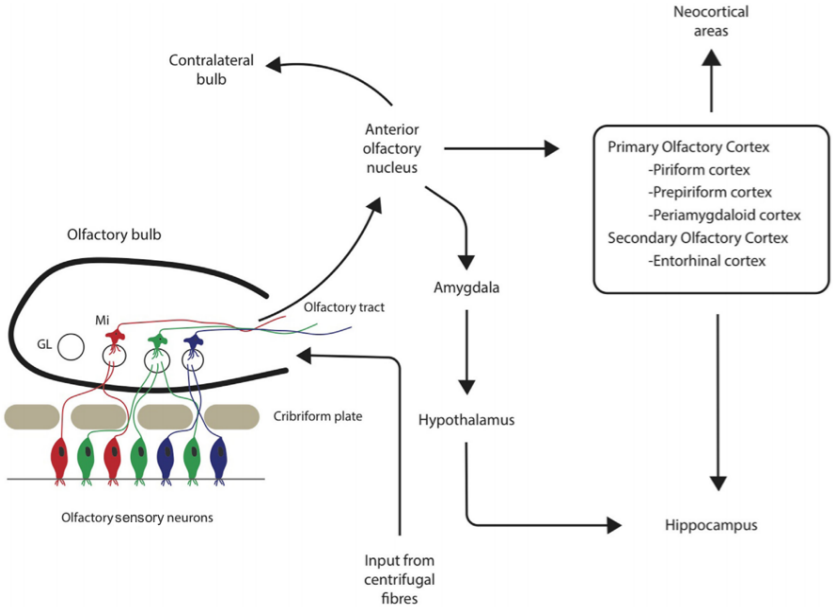

Figure 3.1 *– (Adapted from Franks et al., 2015).* ***A schematic view of the olfactory circuitry.*** *First, the odour is detected by the olfactory sensory neurones, and transmitted to the dendrites of mitral cells (Mi) in the glomerulous layer (GL) of the olfactory bulb. Next, the information reaches the anterior olfactory nucleus, via the lateral olfactory tract. From there, the information is transmitted to other cortical and subcortical structures, such as the amygdala and the primary olfactory cortex before being processed in the hippocampus and in the neocortex.*

3.1.1 First step: The olfactory bulbs

Within the OB, information is processed by several types of neurones located in different layers (Figure 3.2). Specifically, four layers have been distinguished: glomerular layer (GL), external plexiform layer (EPL), mitral cell body layer (MCL) and granule cell layer (GCL) (Kosaka and Kosaka, 2016). The GL comprises three morphological distinct cell types, that are mostly interneurones and do not innervate brain regions outside the OB: periglomerular (PG) cells, external tufted (ET) cells, and superficial short-axon (SSA) cells (Nagayama, Homma and Imamura, 2014). Particularly, PG cells have been shown to be GABAergic interneurones that express either the calcium-binding proteins (CBPs) calretinin (CR) or calbindin (CB) and co-release DA, forming a local inhibitory microcircuit (Kosaka and Kosaka, 2005). After being processed by GL cells, odour signals are transmitted to the tufted cells in the EPL and mitral cells in the MCL. Tufted and mitral cells share many morphological properties, such as the primary dendrites that extend to only one of the several thousand glomeruli in the GL, meaning that these cells receive odour information from only one type of odorant receptor, preserving the peculiar “single cell- single odorant receptor” property (Masurkar and Chen, 2010). Similarly to GL, the EPL includes interneurones that mostly express CR, with few expressing PV (Kosaka and Kosaka, 2010). Moreover, PV-positive interneurones have been shown to form reciprocal connections with mitral/tufted cells (Huang *et al.*, 2013; Kato *et al.*, 2013; Miyamichi *et al.*, 2013). The bottom layer of the OB, the GCL, contains mostly granule cells, which are inhibitory neurones (Mori, 2010). These cells are expressing GABA,

and only a subset localised in the superficial GCL also express CR (Batista-Brito *et al.*, 2008).

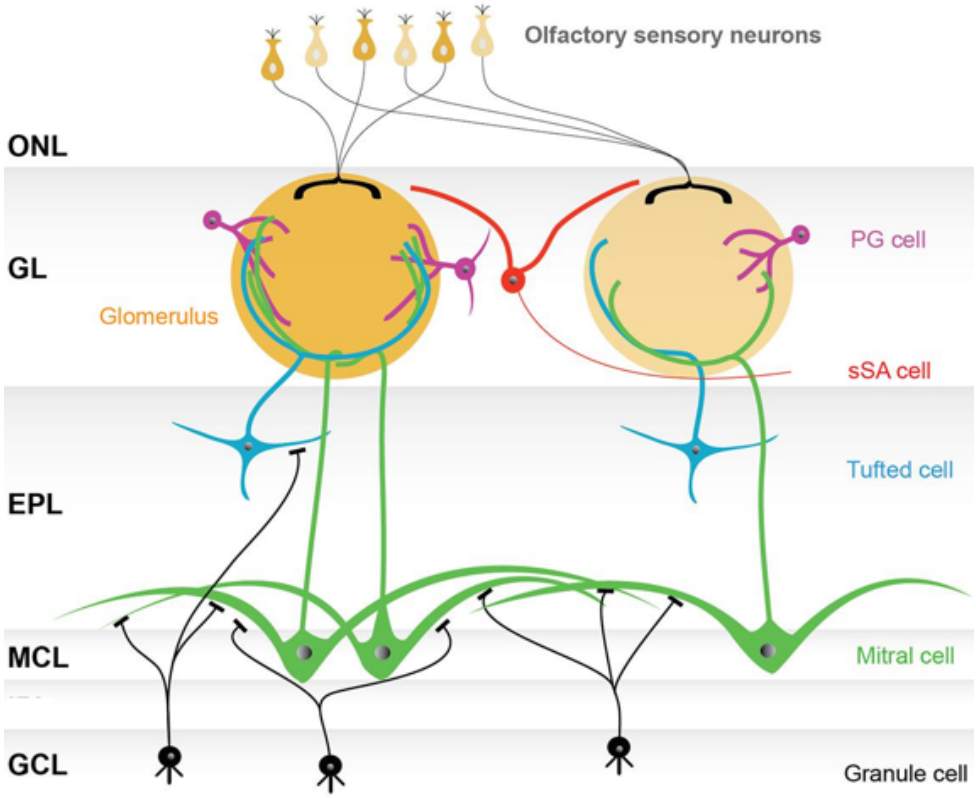

Figure 3.2 – (*Adapted from* Nagayama et al., 2014*)**. **Layers and cell types of the olfactory bulb.** Axons of olfactory sensory neurones make synapses in the glomerular layer (GL), where each glomerulus represents a single odorant receptor. Neurones surrounding glomeruli in the GL are called juxtaglomerular cells (JG cells), consisting of three morphologically distinct cell types: periglomerular (PG) cells, external tufted (ET) cells (not shown), and superficial short-axon (sSA) cells. In the GL, the information is transmitted to tufted cells, whose somata are located in the external plexiform layer (EPL), and mitral cells, located in the mitral cell layer (MCL). The most internal layer of the olfactory bulb is the granule cell layer (GCL), that contains mostly inhibitory neurones.*

3.1.2 Second step: the primary olfactory cortex

After being processed in the OB, the sensory information is then transmitted to cortical structures such as the olfactory cortex, the olfactory tubercle, cortical amygdala, and entorhinal cortex by a myelinated fibre tract, the so-called lateral olfactory tract (LOT) (Murthy, 2011) (see Figure 3.1). Specifically,

the main cortical area designated for the olfactory perception, particularly for discrimination and memory, is the olfactory or piriform cortex (PC) (Strauch and Manahan-Vaughan, 2018). The PC occupies the ventrolateral surface of the brain and being archaecortex, it is composed of three main layers: a first meagrely populated superficial layer (layer 1), a second main input layer, which contains the densely packed somata of glutamate-releasing superficial pyramidal neurones and semilunar cells (layer 2), and the deepest layer, which also comprises pyramidal neurones but at a lower density (layer 3) (Bekkers and Suzuki, 2013) (Figure 3.3).

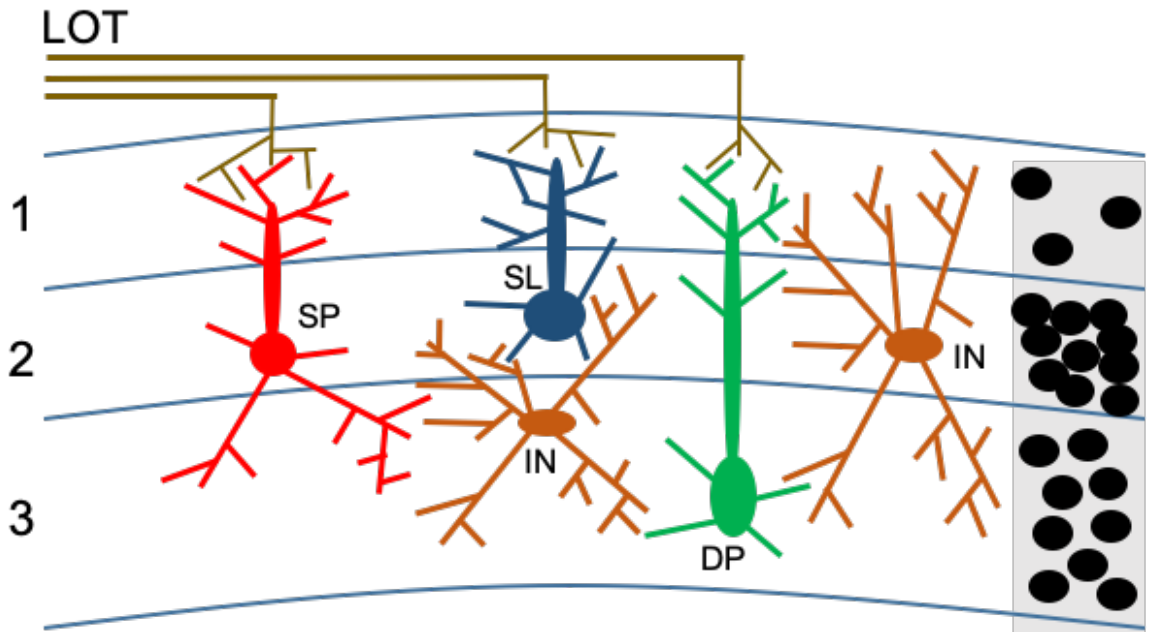

***Figure 3.3 - Schematic cytoarchitecture and basic neuronal types in the Piriform Cortex**. Fibres from the lateral olfactory tract (LOT) terminate in layer 1 of the PC. Semilunar (SL) and superficial pyramidal (SP) cells have their somata concentrated in layers 2. In contrast, deep pyramidal (DP) cells are located in layer 3. Lastly, GABA-releasing interneurones (IN) can be found in layers 2 and 3. Black circles on the right represent the relative density of neuronal somata in different layers.*

The portion of the PC that receives input fibres from the LOT is the most lateral part of layer 1, whereas the dense associational and commissural fibres from neurones within the PC and elsewhere are situated throughout the other PC

layers (Hagiwara *et al.*, 2012; Maier, Wachowiak and Katz, 2012). However, it has been observed that the PC displays a uniform scattering of GABA-releasing interneurones, that provide feedforward or feedback synaptic inhibition of principal cells across all layers (Suzuki and Bekkers, 2007). Indeed, the PC contains five distinctive interneuronal populations that are spread across all layers, with the large majority being located in layers 2 and 3 (Suzuki and Bekkers, 2010a,b). Bitufted cells, that are mostly found in layer 2, express the neuropeptide vasoactive intestinal peptide (VIP) and/or CR (Ekstrand *et al.*, 2001), while fast-spiking multipolar cells express mainly CB and PV (Suzuki and Bekkers, 2010a). In addition, it has been observed that the PC contains also perineuronal nets (PNNs), which are highly condensed extracellular matrix aggregates that surround the cell bodies and proximal dendrites of most PV-positive GABAergic interneurones (Alpár *et al.*, 2006). Specifically, they are formed of four families of extracellular matrix molecules: chondroitin sulfate proteoglycans (CSPGs), tenascin glycoproteins, hyaluronan, and link proteins (Carulli *et al.*, 2006). PNNs are mainly involved in the control of CNS plasticity and they have been observed to be coupled to highly active neurones suggesting a supporting role to neural function (Wintergerst, Faissner and Celio, 1996; Morris and Henderson, 2000). Moreover, experimental evidence suggested that PNNs are linked to synapse formation (Pyka *et al.*, 2011) and that they may exert a neuroprotective role from oxidative stress by forming a protective shield (Cabungcal *et al.*, 2013; Suttkus *et al.*, 2014).

3.1.3 Monoaminergic modulation of the OB and PC

Both the OB and the PC are modulated by several inputs arising from different brain areas through the release of neurotransmitters such as dopamine and noradrenaline (Stettler and Axel, 2009; Wilson, 2009; Bekkers and Suzuki, 2013). Indeed, noradrenergic and dopaminergic inputs have been shown to modulate the olfactory circuitry (Fallon, Riley and Moore, 1978). For example, noradrenergic projections from the LC have been shown to target mitral and tufted cells in EPL (Kosaka and Kosaka, 2016), and the OB hosts the most abundant group of dopaminergic cells in the CNS (Pignatelli and Belluzzi, 2017). Furthermore, layers 2 and 3 of the PC receive inputs from the DA nigrostriatal pathway and noradrenergic projections from the LC (Fallon, Riley and Moore, 1978; Bouret and Sara, 2002). Whilst DA fibres terminate on a small cluster of pyramidal neurones in layer 3, some noradrenergic projections branch into axons running parallel to the pial surface within layer I then descend into layer 2 where they end (Datiche and Cattarelli, 1996). Dopamine and noradrenaline have been found to exert similar modulatory effects within the PC. Noradrenaline was shown to contribute to odour learning and perception through both a selective suppression of excitatory synapses (Hasselmo *et al.*, 1997) and an enhancement in the spontaneous activity of inhibitory cells (Kawaguchi and Shindou, 1998). DA transmission within the PC has also been found to suppress the after hyperpolarization of pyramidal cells via cross reactivity with dopaminergic receptors (Malenka and Nicoll, 1986) and to increase the activity of inhibitory interneurones by enhancing their spontaneous inhibitory potentials (Gellman and Aghajanian, 1993).

3.1.4 Exploring the aetiology of hyposmia in the PC and OB

The mechanisms underlying olfactory dysfunctions in PD are still currently unknown (De Rui *et al.*, 2020). Although most of the emphasis in the aetiology of PD is ascribed to DA loss, hyposmia does not show any improvement with classical DA medications, such as L-DOPA (Seppi *et al.*, 2019). Furthermore, degeneration of several neurotransmitter systems, with a probable consequent development of neuroinflammation, has been proposed to play a key role in the aetiology of hyposmia (Doty, 2017; Song *et al.*, 2019; De Rui *et al.*, 2020). Indeed, loss of 5-HT signalling may affect GABA transmission in the olfactory regions, such as OB and PC (Marek and Aghajanian, 1994; Ciranna, 2006), therefore leading to hyposmia. Recently, the interest in the role of the GABAergic system in PD has increased, since the discovery that GABAergic levels were decreased in the cerebral spinal fluid of PD patients, suggesting for a possible role of GABAergic system dysfunctions in the pathogenesis of PD (Błaszczyk, 2016). Indeed, experimental evidence showed that GABAergic interneurones are essential for odour detection and functionally distinct GABAergic circuits within the olfactory bulbs play different roles in olfactory coding, with the tonic inhibition exerted by these cells regulating the sensitivity of odour detection and odour perception (Murueta-Goyena *et al.*, 2019). Furthermore, glutamatergic and CBP-positive cells were found to be severely affected within the olfactory cortex of a transgenic mouse model of PD (Doty, 2012a) and a deficit in GABAergic neurones in post-mortem brains from PD patients was also observed, with the CBP-positive cells being the most vulnerable to neurodegeneration in the olfactory pathway (Ubeda-Bañon, Saiz-Sanchez, de la Rosa-Prieto, Argandoña-Palacios, *et al.*,

2010). Additionally, interneurones that express CBPs in the olfactory regions are thought to be particularly affected by the deposition of α-synuclein compared with dopaminergic cells in both PD patients and mice over-expressing the A53T human α-synuclein (Ubeda-Bañon *et al.*, 2010; Taguchi *et al.*, 2020). Human studies have observed a significant decrease of somatostatin (SOM)-expressing interneurones and an increase in the number of PV-expressing interneurones in the anterior olfactory nucleus of PD patients compared with healthy controls (Saiz-Sanchez *et al.*, 2020). Similarly, it has been observed that the degeneration of PNNs forestalls the loss of calcium binding protein-positive interneurones, especially PV-positive cells, in various neurological disorders such as Alzheimer's disease (Baig, Wilcock and Love, 2005a) and schizophrenia (Berretta *et al.*, 2015a). Nevertheless, a reduction in PNN levels has yet to be detected in PD subjects. Lastly, non-neuronal cells, such as microglia, pericytes, and astrocytes, in the same region, were also recently shown to contain α-synuclein inclusions, suggesting that they may play an important role in the progression of PD (Stevenson *et al.*, 2020).

3.1.5 Aims of this chapter

The study of the deficits underlying the early-stage stages of PD is of vital importance to determine the aetiology of the disease and potential drug targets. Hence, we first hypothesised that through the injection of both noradrenergic and dopaminergic toxins, it is possible to develop a PD animal model that recapitulates the symptomatology, such as the presence of hyposmia in the absence of MSs, and pathophysiology characteristic of early-stage PD. In addition, based on the studies conducted on human tissues, we

hypothesised that neuroinflammation and a defect in GABAergic cells sustain the hyposmia. Lastly, we also hypothesised that treatment with EX-4 would result in neuroprotective effects. Therefore, the experiments presented in the present Chapter aimed at: 1) validating an early-stage PD animal model for the study of NMSs; 2) investigating the effects exerted by a monoaminergic denervation in the PC and OB; and 3) evaluating the effectiveness of EX-4 in preventing the damages caused by the dual-toxin treatment. To this end, after PD was induced in the animals using a combination of two neurotoxins, animals were tested with behavioural protocols to validate the presence of NMSs in the absence of MSs, thus, validating the early-stage. Brain samples were then collected and analysed using immunohistochemistry and unbiased stereology to assess whether the deficits observed in the early-stage in human studies were displayed by this animal model. Additionally, and most interestingly, EX-4's effectiveness on the observed deficits in our early-stage PD model was investigated for the first time.

3.2 Materials and Methods

3.2.1 Experimental Groups

To achieve the aims of this study, a rat model of early-stage PD was developed using the combination of two neurotoxins. Rats were randomly divided into three groups: sham, DSP-4 + 6-OHDA (early-stage model), and DSP-4 + 6-OHDA + EX-4. The protocol used is summarised in Figure 3.4. Briefly, on day 0, behavioural tests were conducted to acquire baseline values for all the animals. Next, on day 1, animals were injected intraperitoneally either with saline (controls) or DSP-4 (early-stage model). Three days later, they underwent stereotaxic surgeries in which either vehicle or 6-OHDA were delivered directly into the striatum (bilaterally). The three-day interval between the two toxins was intended to mimic the human progression of the disease in which the noradrenergic system is affected prior to the dopaminergic one. Two weeks following the surgeries, animals were injected intraperitoneally twice daily with either EX-4 (Sigma Aldrich) or saline for 7 days (Figure 3.4). Finally, three weeks after the surgeries, animals were culled and the brains were collected for analyses. All animals were tested for hyposmia using both the hidden and habituation/dishabituation tests. Each experimental group was then subdivided into smaller groups that were tested with one other test (rotarod test, novel object recognition test or sucrose preference test) to avoid confounding results. Data presented in this thesis relates to the rotarod test. Immunohistochemistry was performed on all animals.

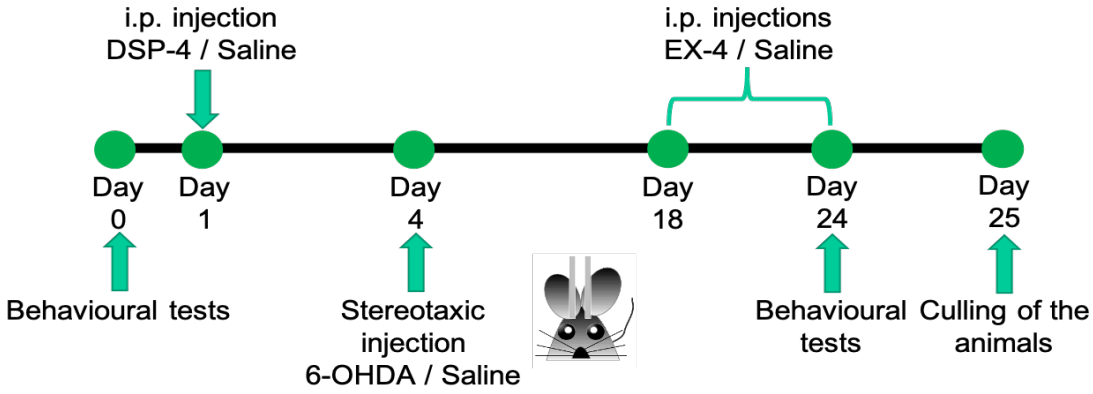

Figure 3.4 – Experimental timeline. *On day 0, animals underwent behavioural tests to acquire the baseline. The following day, they received the injection of either DSP-4 early-stage model, 25 mg/Kg) or saline (sham). 3 days later, stereotaxic surgeries were performed to bilaterally deliver in the striatum either 6-OHDA (early-stage model, 15 μg) or saline (sham). Next, from day 18, animals were treated daily for a week with either EX-4 (early-stage model) or saline (sham and early-stage model). On day 25 animals were culled and the brains collected for further analyses.*

3.2.2 Drugs

The toxins used to induce the noradrenergic and the dopaminergic lesions, N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4) and 6-hydroxydopamine (6-OHDA) respectively, were purchased from Sigma (Sigma Aldrich, Gillingham, UK). DSP-4 was delivered at a concentration of 25 mg/kg in saline solution and injected intraperitoneally (i.p.) 4 days prior to the 6-OHDA injection, while 6-OHDA was dissolved at a concentration of 5 mg/ml in saline solution containing 0.9% ascorbic acid and delivered bilaterally in the striatum. DSP-4 and 6-OHDA doses were chosen to induce only a partial reduction of NA and DA levels to mimic the early stage of the disease (Jonsson et al., 1981; Prezedborski et al., 1995). EX-4 (Sigma Aldrich) was administered twice daily via intraperitoneal injection 14 days after surgery (with saline as a vehicle) at a dose of 0.5 μg/kg for a period of 7 days.

3.2.3 Behavioural tests

Behavioural tests were carried out at 2 different time points: baseline and 18 days after surgery (Figure 3.4). All animals were tested for hyposmia using both the hidden food test, to measure the general olfaction, and the habituation dishabituation test, to investigate odour memory and discrimination. Motor skills were investigated using the rotarod test (ROT). Animals were acclimatised to the testing room for 1 hour prior to each behavioural test. In addition, all animals were handled and exposed to the testing arenas to diminish environmental novelties during the 7-day acclimatising period preceding the baseline tests.

3.2.3.1 Hidden food test

The protocol for the hidden food test was adapted from Yang and Crawley (2009). This test relied on the animals' natural tendency to use olfactory cues. By burying the food in bedding material, visual clues are eliminated and the animal solely relied on olfactory clues. Before the test, animals were fasted overnight. To acclimatise the animals, they were placed in a box (40x20x30 cm) containing 1 cm of sawdust for 3 minutes. They were then transferred to a different box containing 1 cm of sawdust and a banana chip as a treat hidden beneath the sawdust. The latency*, i.e.* the time spent locating the treat, was recorded. Boxes were cleaned with 30% EtOH and sawdust was replaced between animals. Five animals per experimental group were also tested with the treat with the view to assess for lack of motivation.

3.2.3.2 Habituation/dishabituation test

The habituation dishabituation test assessed the general olfactory abilities and more specifically, the ability to discriminate between odours and the ability to learn odours. Dishabituation was a measure for odour discrimination and was characterised by a sudden increase in exploring activity upon the presentation of a new scent. Habituation indicated the ability to learn a new odour and was characterized by a decrease in exploration time over three trials with the same odour. The protocol for this test was adapted from Yang & Crawley (2009). Prior to testing, animals were acclimatised in a transparent closed filtered top cage (40x20x30 cm) for 10-30 minutes, as described by Bonito-Olivo et al. (2014). Subsequently, all animals were tested with 5 different scents (water/ paprika/ vanilla/ social1/ social2) over 3 trials in the closed cage. The vanilla and paprika odours were prepared by diluting vanilla essence (Waitrose, UK) or paprika powder (Waitrose, UK) in distilled water (1:100); each social odour was obtained by swiping the tip of a cotton swab across the bottom of the cage from same sex animals that were not included in this study. Each odour was presented on a cotton swab in 3 consecutive trials for 2 minutes separated by 1 minute intervals between trials. The cotton swabs were freshly prepared for every exposure. All tests were recorded with a video camera. Exploratory time (measured in seconds) was defined as the time during which the animals' nose was in contact or <2mm of the cotton swab and measured for each trial.

3.2.3.3 Rotarod test

Motor skills of the animals were investigated using the rotarod test. Animals were placed on a rotating drum which accelerated over time. Animals were trained on an automated 4-lane rotarod unit (Panlab, Harvard Apparatus, UK)

for 3 consecutive days until performance was stable. An accelerating rotarod protocol was used in which the animal was placed on a rod that accelerated smoothly from 4 to 40 rpm over a period of 5 minutes. The latency to fall, indicated by the time that each animal was able to stay on the rod, and the speed of the rod were registered automatically by a trip switch under the floor of each rotating drum.

3.2.4 Immunohistochemistry

Briefly, 50 μm parasagittal sections containing the PC were cut using a vibratome. One in every 12 sections was collected and 3-4 slices were used per animal and per staining. Similarly, 50 μm coronal sections containing the OB were cut, one slice in every 8 was collected and 4-5 slices per animal were used. Sections were incubated first in 1% H_2O_2 for 30 minutes and then in 1% sodium borohydride ($NaBH_4$) for 30 minutes to decrease background staining and then in either 1% bovine serum albumin for the Biotinylated Wisteria floribunda agglutinin (WFA) staining used to label perineuronal nets (PNNs) or in 10% normal goat serum (NGS) for all other antibodies for another 60 min to block nonspecific antibody binding. Sections were incubated overnight at 4^{o}C in a mixture of primary antibodies and triton X-100 (Sigma Aldrich) [1% Triton for GAD-67 - 0.1% for all other antibodies] made up in phosphate buffer solution (Primary antibodies are listed in Chapter 2).

3.2.4.1 Neuronal distributions in the PC[2*]

6 to 7 Z-stacks from the PC of each slice in each animal [1 in 12 PC sections-3-4 slices per animal] were obtained using a x10 objective and immunopositive cell bodies were counted using a cell counting plug-in in ImageJ software. Cellular densities were expressed as the number of cells per mm^3 ± SEM.

3.2.4.2 Neuronal distributions in the OB

CBPs, GAD-67, and NeuN immunostainings were carried out on coronal sections containing the OB according to the immunoperoxidase protocol described in Chapter 2. 1 in 8 slices per animal were collected and processed. Sections were dehydrated and mounted using DPX. The optical fractionator probe was used to determine the number of immunopositive neurones (StereoInvestigator, MicroBrightField) using a Nikon microscope coupled to a computer-controlled x-y-z motorized stage and an MBF video camera system. Unbiased stereology for CBPs was carried out on 5 slices per animal (n = 6 per experimental group) with the following parameters: counting frame 150 μm x 130 μm; grid size 850 μm x 600 μm; section thickness 50 μm, dissector height 18 μm, and high resolutions lens X10. Similarly, the following parameters were used for the unbiased stereology for GAD-67 and NeuN staining: counting frame 150 μm x 130 μm; grid size 500 μm x 500 μm; section thickness 50 μm, dissector height 15 μm, and high resolutions lens X40. Only neurones with

[2*] This part of the project was performed by Emma Schul (former PhD student UCLSOP)

visible nuclei and dendrites were counted. Data are displayed as cell density per mm^3 ± SEM.

3.2.4.3 Tyrosine Hydroxylase (TH) staining

TH immunostaining was carried out on coronal sections containing the SNpc and on coronal sections containing the OB according to the immunoperoxidase protocol described in Chapter 2. 1 in 4 (SNpc) or 1 in 8 (OB) slices per animal were collected and processed. Sections were dehydrated and mounted using DPX. The optical fractionator probe was used to determine the number of TH-immunopositive neurones in both regions (StereoInvestigator, MicroBrightField) using a Nikon microscope coupled to a computer-controlled x-y-z motorized stage and an MBF video camera system. Unbiased stereology was carried out on 5 slices per animal (*n* = 6 per experimental group) with the following parameters: counting frame 60 μm x 60 μm; grid size 200 μm x 200 μm; section thickness 50 μm, dissector height 12 μm. Only neurones with visible nuclei and dendrites were counted. Data are displayed as cell density per mm^3 ± SEM.

3.2.5 High Performance Liquid Chromatography (HPLC)[3*]

Dissected rat brain tissues (SNpc, striatum and PC) were weighed and homogenised in appropriate volumes of homogenising solution (0.1M perchloric acid containing 400 mM sodium metabisulphite) and then microcentrifuged 10,000g for 20 min at 4 °C. Determination of DA and NA

[3*] This part of the project was performed by RenaSci Limited, UK.

levels was performed with a Jasco PU-980 pump HPLC coupled to electrochemical detection (Coulochem II- ESA model 5011 analytical cell), equipped with a Capital Hypersil column (250 x 4.6mm id 5 μm). The mobile phase consisted of 0.01 M sodium dihydrogen orthophosphate dehydrate, 0.9 mM 1-octanesulfonic acid sodium salt, 0.1% dibutylamine, 12.5% methanol, and pH 3.2 at a flow rate of 1ml min^{-1}. The height of the peaks produced by oxidation of NE and DA was compared with that produced by known standards. Concentrations of the monoamines were expressed as ng/g ± SEM. Data were captured using Antec's Scientific Clarity software.

3.2.6 Statistics

All data in this study were analysed using IBM SPSS Statistics Version 22.0. The Shapiro–Wilk test and the Kolmogorov–Smirnov test were carried out prior to statistical analysis to determine whether the data followed a normal distribution. The parametric one-way ANOVA, repeated measures ANOVA or unpaired t-test were then used when the data followed a normal distribution and the non-parametric Kruskal–Wallis test were used in case of a non-normal distribution. Statistical significance was set at $P < 0.05$.

3.3 Results

3.3.1 6-OHDA- and DSP-4-induced dopaminergic and noradrenergic loss in the SNpc, striatum, and piriform cortex.

Figure 3.5A shows representative photomicrographs of TH-immunopositive cells in the SNpc of each experimental group, sham, 6-OHDA + DSP-4, 6-OHDA + DSP-4 + EX-4. Unbiased stereology revealed that the dopaminergic lesion significantly decreased TH immunoreactivity by 43.5 ± 5.3% in the SNpc of the early-stage model compared with that observed in sham animals ($P < 0.01$) (Figure 3.5B). Injections of EX-4 partially prevented the neuronal loss in this region (Figures 3.5A and 3.5B). Similarly, quantification of DA and NA levels through HPLC revealed a significant reduction in the SNpc, 47.6 ± 2.4% and 49.9 ± 1.6% respectively, as well as in the striatum of the early-stage PD model compared with those measured in the sham animals [unpaired t-test, $P_{DA} < 0.01$, $P_{NA} < 0.001$] (Figures 3.5C and 3.5D). In addition, DA and NA levels were found to be reduced also in the PC by 39.8 ± 1.1% and 40 ± 1.4% respectively, of the early-stage PD model compared with those in the shams [unpaired t-test, $P_{DA} < 0.0001$, $P_{NA} < 0.001$] (Figures 3.5C and 3.5D).

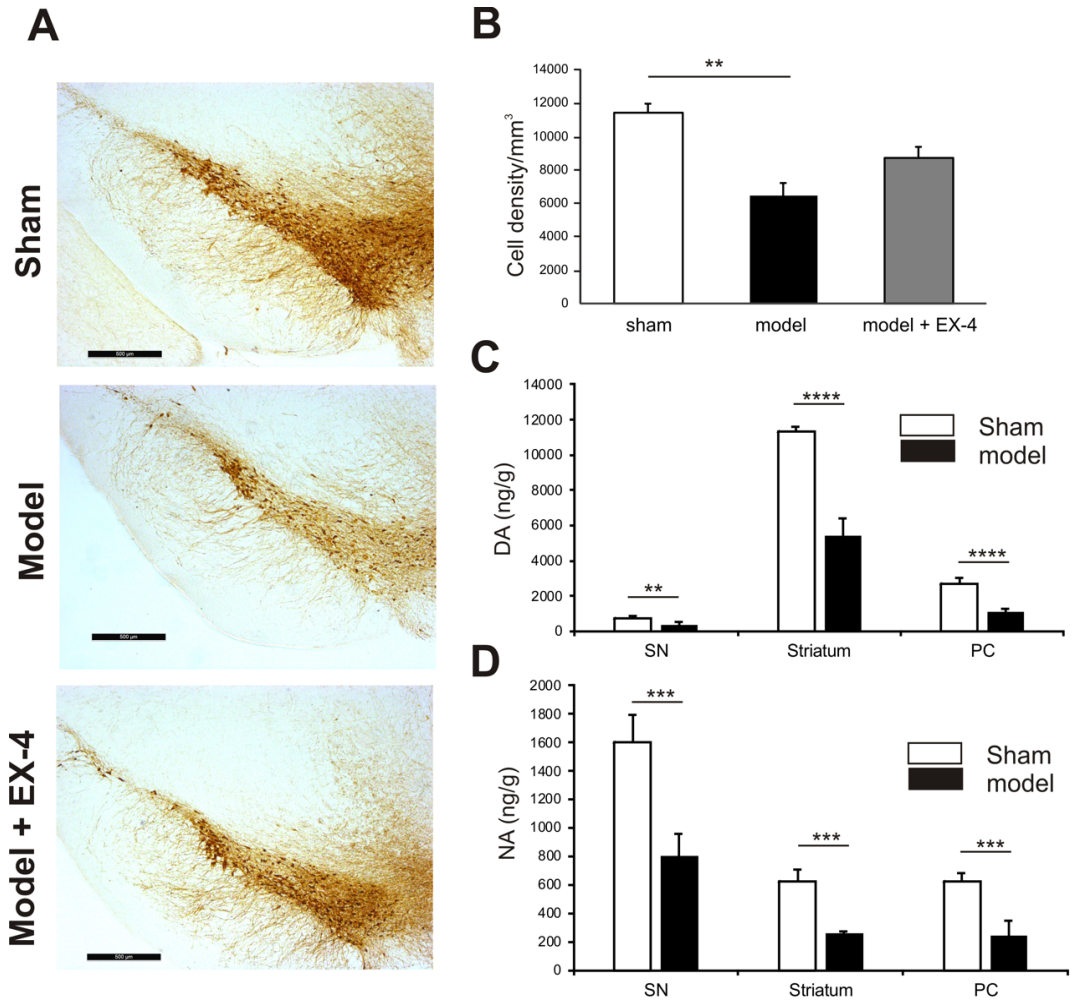

Figure 3.5 - Reduction of dopamine and noradrenergic levels in a model of early-stage PD. A *Representative images of TH -staining in the Substantia Nigra pars compacta (SNpc) in the sham-operated animals (top panel), early-stage PD model (middle panel) and model treated with EX-4 (bottom panel). Scale bars represent 500 µm.* ***B*** *Number of TH-positive cells in the SN expressed as cell density per mm^3 (n= 6 animals per experimental group). A decrease in the number of TH-positive cells was observed in the early-stage PD model (unpaired t-test ** P < 0.01). This decrease was partially prevented by treatment with EX-4 (unpaired t-test $P_{shamvsmodel + EX-4} > 0.05$; $P_{modelvs\ model + EX-4} > 0.05$).* ***C*** *HPLC measurements of tissue levels of dopamine (DA) in SN, striatum and piriform cortex (PC) expressed as ng/g wet weight. Levels of DA were significantly reduced in the three regions (unpaired t-test n = 4-5 animals per group ** P < 0.01, **** P < 0.0001).* ***D*** *HPLC measurements of tissue levels of noradrenaline (NA) in SN, striatum and piriform cortex (PC) expressed as ng/g wet weight. Levels of NA were significantly reduced in the three regions (unpaired t-test n = 4-5 animals per group *** P < 0.001).*

3.3.2 The early-stage PD model displayed hyposmia in the absence of motor symptoms

Motor skills, which were assessed using the rotarod test, were not affected in the model and model + EX-4 groups (One-way ANOVA, $P > 0.05$) (Figure 3.6A) and in sham animals (data not shown). General olfactory function was investigated using the hidden food test. The early-stage model displayed an increased latency to finding the treat compared with the sham animals (One-way ANOVA, $P < 0.05$) (Figure 3.6B). Treatment with EX-4 prevented this increase ($P_{\text{sham vs model +EX-4}} > 0.05$). Open food tests with the treat in view were conducted to rule out a lack of motivation to find the treat and latencies were similar in the three experimental groups (data not shown). The habituation/dishabituation test was used to assess general olfactory function (measure of the exploratory time for each odour during each trial), odour memory (habituation) and odour discrimination (dishabituation). Figure 3.6C shows that experimental groups differed in the progressive decline of the exploration times for social 1 and social 2 ($F_{\text{social1 (18,32)}}=3.115$, $P < 0.01$; $F_{\text{social2 (18,34)}}=3.087$, $P < 0.01$). Treatment with EX-4 increased the exploration time on the first trial for social 1 ($P < 0.05$). The ability to discriminate between odours was evaluated by the increase in exploration time when a new odour was presented. The early-stage model displayed reduced exploration time for the novel presentation of paprika and vanilla compared with Sham animals ($P_{\text{paprika}} < 0.05$; $P_{\text{vanilla}} < 0.05$).

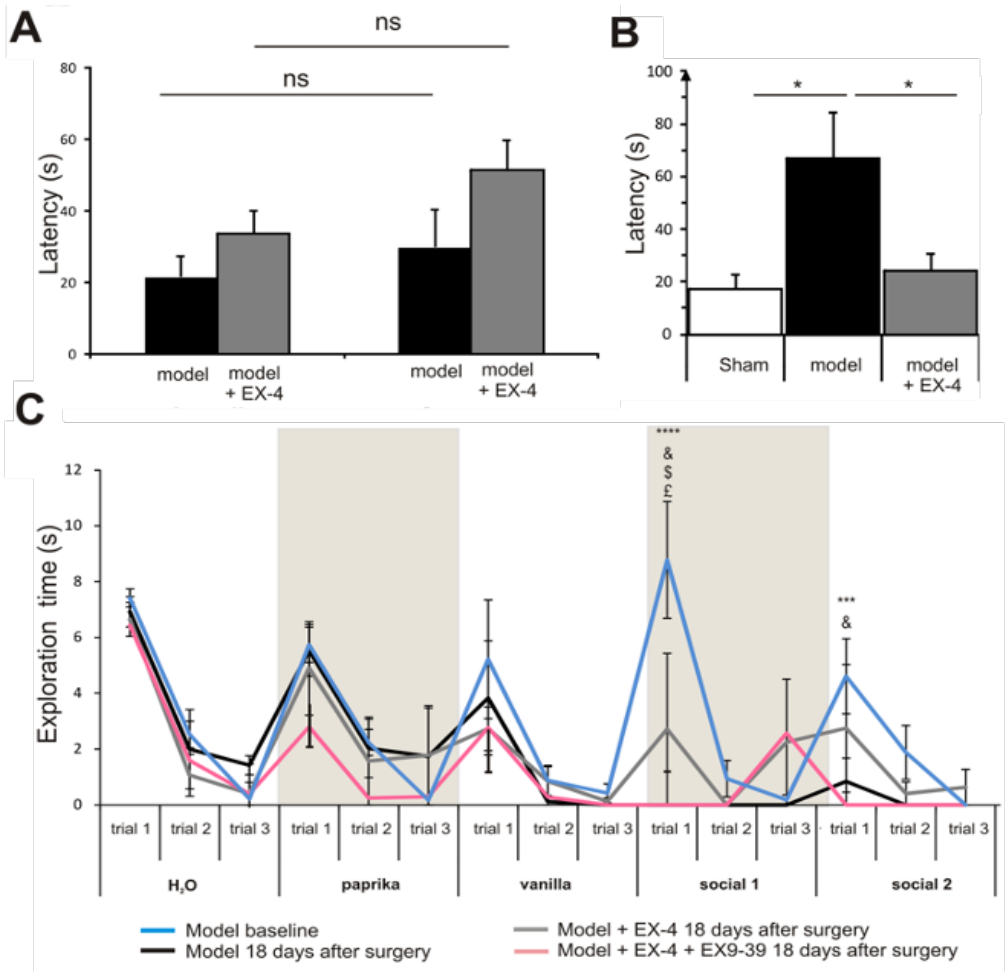

Figure 3.6[4] – The early-stage PD model displayed hyposmia in the absence of motor symptoms. A) *Locomotion in the early-stage model receiving either saline or EX-4 treatments was tested at baseline and after treatment) and no significance difference was found. Data are presented as mean ± SEM. The latency at which the animals fell from the rotating rod was similar to that observed in sham animals (One-way ANOVA; P > 0.05).* ***B)*** *Hyposmia was tested first using the hidden food test. Data are represented as mean ± SEM. The time taken by the early-stage model animals to find the hidden treat was increased compared with that by the sham animals (One-way ANOVA, P < 0.05). Hyposmia was prevented by treatment with EX-4.* ***C)*** *Olfactory deficits were also tested using the habituation/dishabituation test. The early-stage model did not explore the vanilla odour during the trials 2 and 3 or the two social odours presented. EX-4 prevented the non-response following the first presentation of the social 1 and 2 odours (two-way ANOVA with repeated measures- *** P < 0.001, **** P < 0.0001 for early-stage r model at baseline compared with PD model at 18 days after surgery;* ***&*** *for PD model at 18 days after surgery compared with PD model + EX-4;* ***$*** *for PD model +EX-4 compared with PD model + EX-4 + EX9-39;* ***£*** *P < 0.05 for early-stage model at baseline compared with PD model + EX-4).*

[4] The present image was adapted from Sancandi *et al.* (2018), thus, section C includes data on the use of EX-4 antagonist even though the latter was not used in the work presented in this thesis.

3.3.3 Neuroinflammation is present in the PC of the early-stage PD model.

The presence of neuroinflammation in the PC of the early-stage PD model was assessed with staining for the glial fibrillary acidic protein (GFAP) and the ionised calcium binding adaptor molecule 1 (Iba1). The former was used to detect activated astrocytes and the latter activated microglial cells (Figure 3.7A and 3.7B respectively). A stronger staining was observed for GFAP-immunopositive astrocytes in the PC of the early-stage PD model compared with that in the sham animals (Figure 3.7Ab $P < 0.0001$), with astrocytes of the PC of the model showing enlarged somata, extensive branching processes and overlapping domains (insert in Figure 3.7Aa). Treatment with EX-4 prevented the astrocytic activation in the PC of the animals injected with the toxins (Figure 3.7Ab). In contrast, the intensity of Iba1 staining in the PC of the early-stage PD model was significantly decreased compared with that in the sham animals (Figure 3.7Bb $P < 0.05$). Microglial cells in the PC of the model exhibited a decreased number of processes and less complex and shorter branches than in the sham animals, suggesting microglial activation ($P < 0.05$- Figure 3.7Ca,b,c,d,e,f). The size of the somata in the model was, however, similar to that in the sham animals ($P > 0.05$ - Figure 3.7Cg). Interestingly, EX-4 treatment did not significantly prevent microglial activation in the model ($P > 0.05$ Figure 3.7Bb).

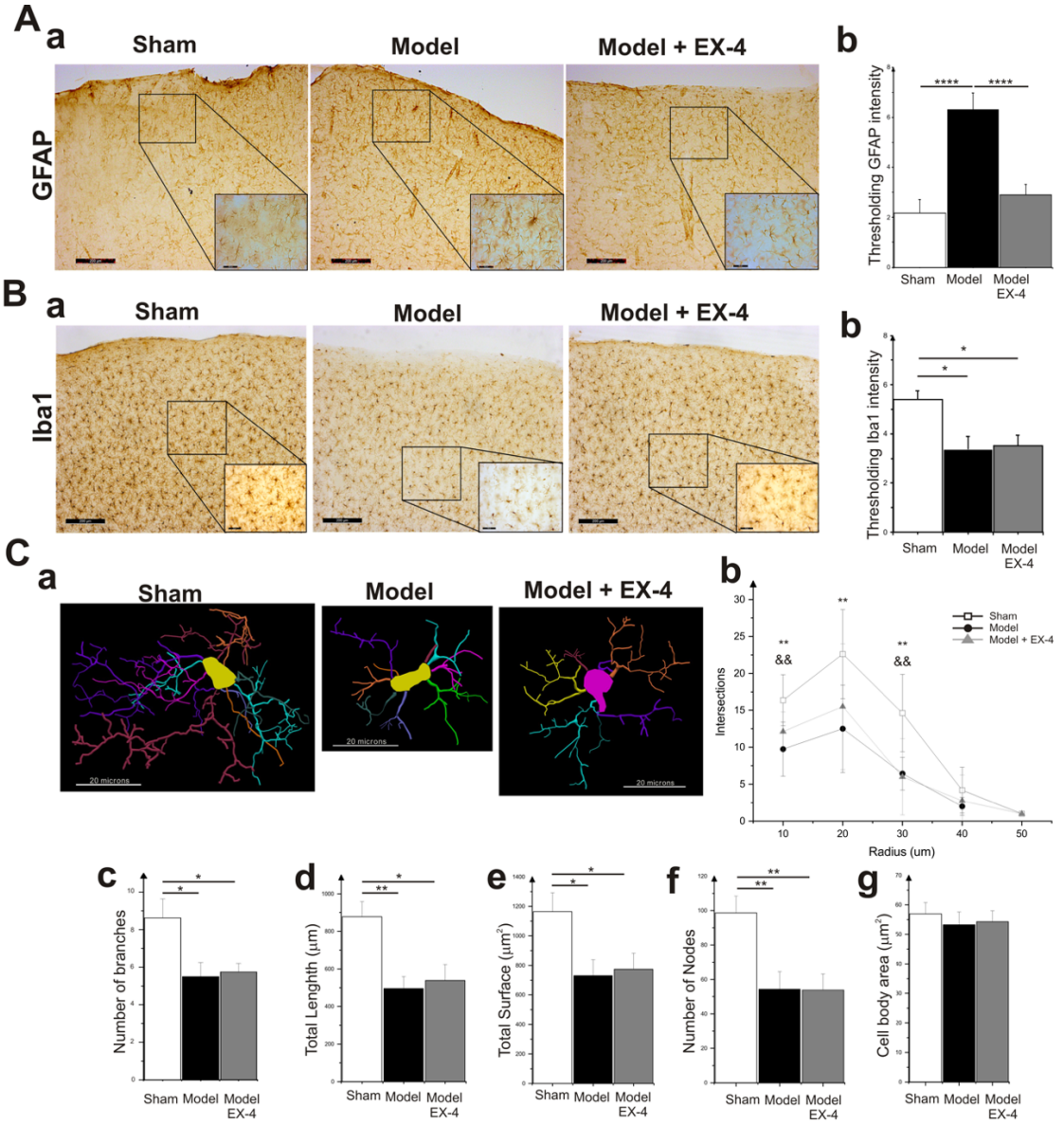

Figure 3.7 - Neuroinflammation in the PC of the early-stage PD model. A *GFAP staining in the early-stage PD model.* ***Aa*** *Representative immunohistochemical staining of GFAP in each experimental group. Scale bars for overview and for inserts represent 200 µm and 50 µm respectively.* ***Ab*** *GFAP staining intensity was stronger in the early-stage model compared with that in sham animals indicating activation of astrocytes. This increase was prevented by treatment with EX-4 (One way ANOVA, **** P < 0.0001). Data are represented as mean ± SEM.* ***B*** *Iba1 staining in the early-stage PD model.* ***Ba*** *Representative immunohistochemical staining of Iba1 in each experimental group. Inserts show high magnification images of Iba1-positive cells. Scale bars for overview and for inserts represent 200 µm and 10 µm respectively.* ***Bb*** *Intensity of Iba1 staining in the PC of early-stage PD model was significantly decreased compared with that in the Sham animals, indicative of an activation of microglia. This decrease was not prevented by treatment with EX-4 One way ANOVA, * $P_{sham\ v\ smodel+\ EX-4} < 0.05$, $P_{model\ vs\ model\ +\ EX-4} > 0.05$). Data are represented as mean ± SEM.* ***C*** *Morphological properties of Iba1-immunopositive cells.* ***Ca*** *Examples of Neurolucida reconstructions of Iba1-positive cells. Scale bars represent 20 µm.* ***Cb*** *Sholl analysis of Iba1-positive cell. Data are represented as mean ± SD (n = 8 per experimental group- One way ANOVA - ** P < 0.01 for early-stage model compared with sham; && P < 0.01 for sham compared with early-stage PD model treated with EX-4.)* ***Cc, Cd, Ce, Cf, Cg*** *The number of branches (**Cc**), total length (**Cd**), total surface (**Ce**) and the number of nodes (**Cf**) of Iba1-positive cells were decreased in early-stage PD model compared with those in the sham animals. Treatment with EX-4 did not prevent this decrease. The size of cell somata was similar in the sham, model and model + EX-4 (**Cg**). Data are represented as mean ± SD [n = 8 per experimental group- One way ANOVA * P < 0.05, ** P < 0.01].*

3.3.4 Down regulation of interneuronal calcium binding proteins is observed in the PC of the early-stage PD model.

Interneurones expressing calcium binding proteins (PV, CB, CR) were analysed in the PC of the early-stage PD model and compared with those in control animals. Cell densities in the PC of all experimental groups are presented in Table 3.1, whilst Figures 3.8A-3.8C show representative examples of the distributions of PV-, CB- and CR-immunopositive interneurones of all experimental groups. All CBP-containing interneurones in the PC of the early-stage model were significantly reduced compared with controls (Table 3.1, Figures 3.8A-3.8C Kruskal-Wallis test $P < 0.05$). Treatment with EX-4 prevented the observed loss of the PV, CB, CR-immunopositive cells (Kruskal-Wallis $P < 0.05$).

	SHAM + saline	Model + saline	Model + EX-4
GAD-67	2587.8 (2247.6 – 2927.9)	2610.7 (1908.9 – 3312.5)	2178.0 (1742.1 – 2613.9)
PV	1294.6 (576.5 - 2012.7)	278.7 **** (136.6 - 420.8)	749.1 #### (581.7 - 916.6)
CB	843.7 (591.3 – 1096.1)	110.6 **** (26.1 – 195.1)	834.2 #### (403.6 – 1264.8)
CR	736.6 (487.1 – 986.0)	171.8 **** (80.2 – 263.3)	772.2 #### (473.8 – 1070.6)

Table 3.1 - Cellular densities of interneuronal CBPs in the PC of the sham animals, early-stage PD model and model treated with EX-4. *Data are represented as the median and interquartile range of the number cells per mm^3 that are immunopositive for the indicated marker (n=4-5 animals/per experimental group). GAD-67, glutamic acid decarboxylase-67; PV, parvalbumin; CB, calbindin; CR, calretinin; [Kruskal-Wallis test was used, post hoc analysis for comparisons with Sham animals. * P <0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001; post hoc analysis model compared with model treated with EX-4 #### P <0.0001].*

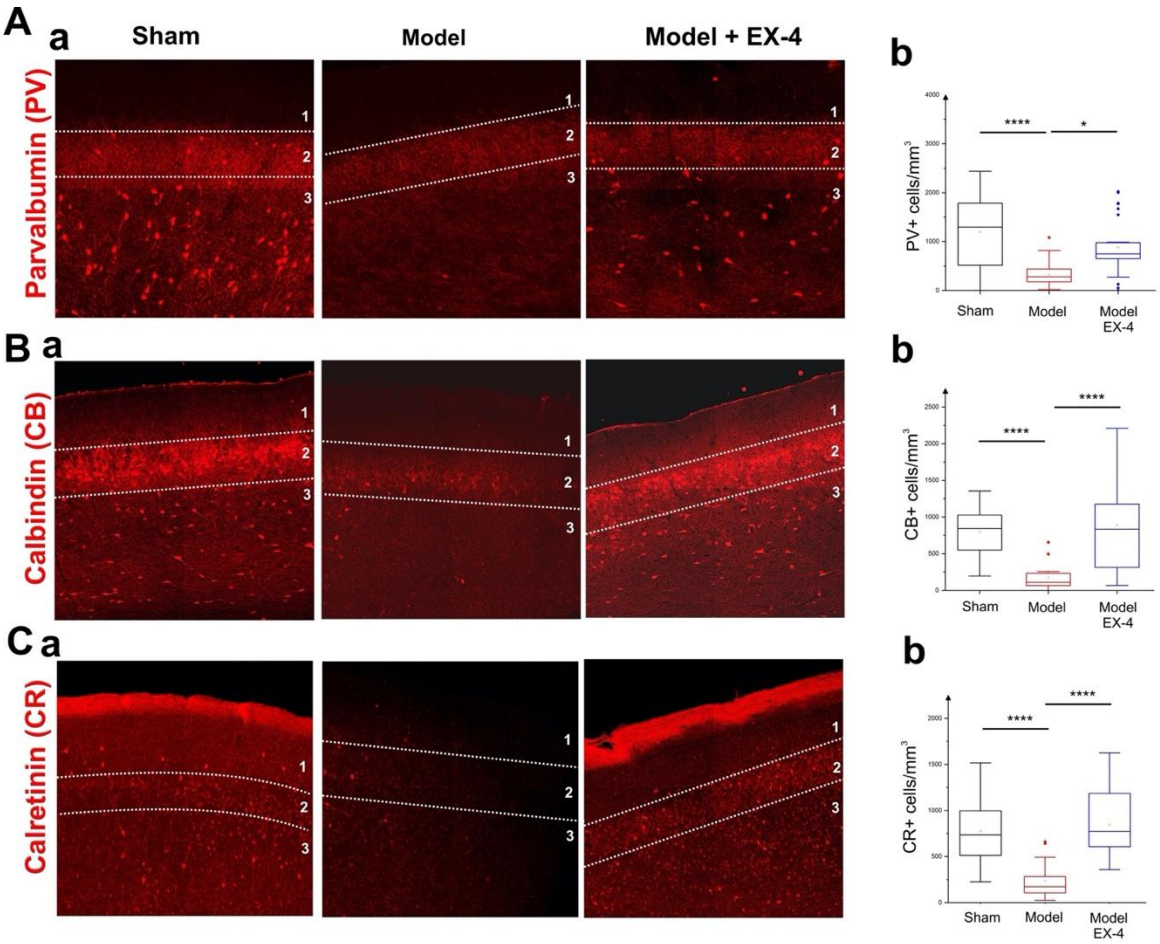

Figure 3.8 - Distribution of interneuronal CBPs in the early-stage PD model. A *EX-4 prevented the loss of parvalbumin (PV)-immunopositive interneurones in the PC of the early-stage model.* ***Aa*** *Examples of PV-immunostaining in the anterior PC in each experimental group.* ***Ab*** *Cell densities in the PC of all experimental groups. The number of PV-positive interneurones was significantly decreased in the early-stage PD model compared with that in the sham animals. Treatment with EX-4 prevented this decrease.* ***B*** *EX-4 prevented the loss of Calbindin (CB)-immunopositive interneurones in the PC of the early-stage model.* ***Ba*** *Examples of CB-immunostaining in the anterior PC for all experimental groups.* ***Bb*** *Cell densities in the PC of all experimental groups. A significant decrease in the number of CB-positive interneurones was observed in the early-stage model compared with the sham animals. Treatment with EX-4 prevented this decrease.* ***C*** *EX-4 prevented the loss of calretinin (CR)-immunopositive interneurones in the PC of the early-stage model.* ***Ca*** *Examples of CR-immunostaining in the anterior PC for all experimental groups.* ***Cb*** *Cell densities in the PC of all experimental groups. A decrease in the number of CR-positive interneurones was observed in the early-stage model compared with the sham animals. Treatment with EX-4 prevented this decrease. [Kruskall Wallis test *P < 0.05, **** P < 0.0001]. All data are presented as mean ± SEM.*

3.3.5 Cellular densities of GAD- and NeuN-immunopositive cells in the PC of the early-stage PD model.

Since all the interneurones investigated express GABA as neurotransmitter, the number of GAD-67-immunopositive neurones was analysed to determine whether the decreased number of the CBP-containing interneurones in the PC of the model was due to a down-regulation of the CBPs or to a reduction in the number of interneurones. The density and distribution of GAD-67-positive cells did not differ among the experimental groups, suggesting a down regulation of the CBPs in the model rather than a cell loss (Table 3.1- Figures 3.9Aa and 3.9Ab). The overall number of neurones in the PC was also studied. Surprisingly, the number of NeuN-immunopositive cells was significantly increased in the model compared with controls (Figures 3.9Ba and 3.9Bb - $P_{\text{sham vs model}} < 0.0001$). This unexpected increase was not apparent after treatment with EX-4 (Figures 3.9Ba and 3.9Bb - $P_{\text{model vs model + EX-4}} < 0.0001$).

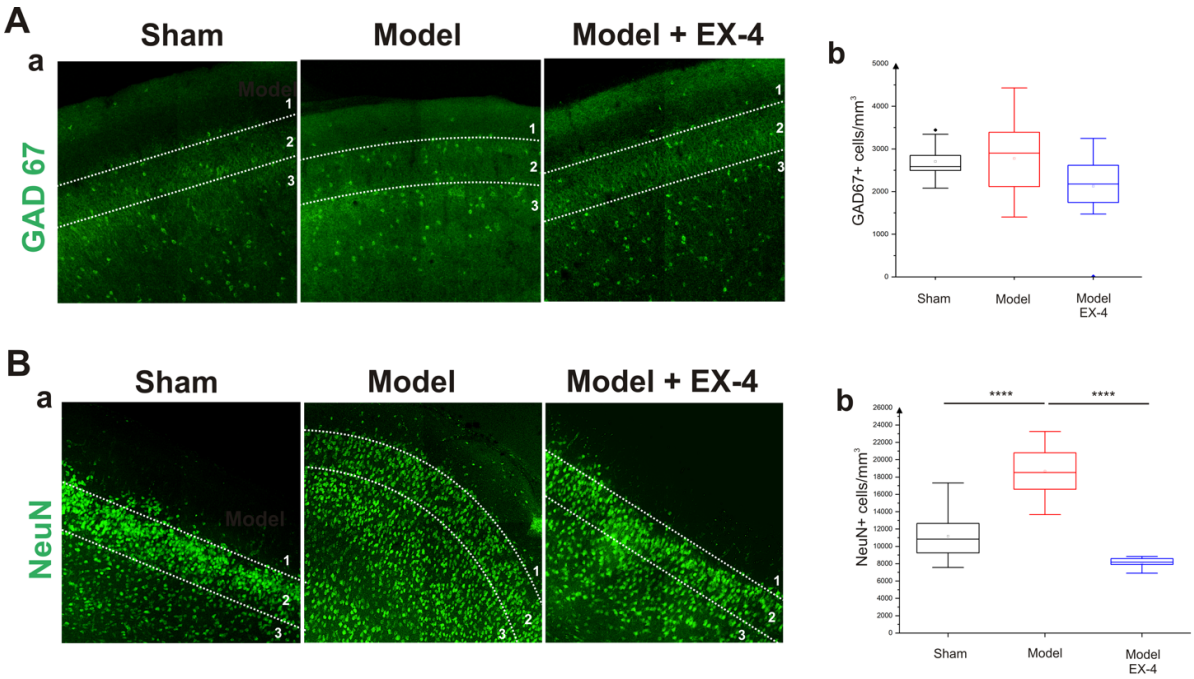

Figure 3.9 - Distribution of NeuN- and GAD-immunopositive neurones in the model of early-stage PD. A *Distribution of GAD-67-immunopositive interneurones in the PC of the early-stage PD model.* ***Aa*** *Examples of GAD-67-immunostaining in the anterior PC of all experimental groups.* ***Ab*** *Cell densities in the PC. The distributions were similar in all experimental groups.* ***B*** *Distribution of NeuN-immunopositive interneurones in the PC of the early-stage model.* ***Ba*** *Examples of NeuN-immunostaining in the anterior PC of all experimental groups.* ***Bb*** *Cell densities in the PC. The density of NeuN-positive neurones in the early-stage PD model increased compared with that in the sham animals. This increase was prevented by treatment with EX-4. [Kruskall Wallis test, **** P < 0.0001]. All data are presented as mean ± SEM.*

3.3.6 Degradation of the perineuronal nets (PNNs) in the PC of the early-stage PD model

Lastly, given the reduction of PV-positive interneurones and the fact that they are commonly surrounded by PNNs (Beretta et al., 2015), the distribution and density of PNNs in the PC of the early-stage PD model were investigated using WFA, a lectin that labels residues of glycoproteins within the extracellular matrix of the neurones. Examples of the WFA+/PV+ staining of neurones in the anterior PC of the sham animals, early-stage PD model and model treated with EX-4 are displayed in the top panels in Figure 3.10A. The number of PV+/WFA+ neurones was observed to be decreased at 19 days after surgery ($P < 0.0001$) (Figure 3.10Ba). Treatment with EX-4 prevented the loss of both PV+ and WFA+ neurones in the early-stage model ($P < 0.05$) (Figure 3.10Ba). The PC also contained a small population of PV-immunonegative (PV-) cells that were also surrounded by PNNs. A decrease in PV-/WFA+ cells in the PC of the early-stage model was observed at 19 days after surgery (Figure 3.10Bb). Treatment with EX-4 prevented the loss of these PV-/WFA+ cells ($P < 0.0001$). The WFA-immunopositive neurones were shown to be either GABAergic (see GAD-67+/WFA+ staining in bottom panels in Figure 3.10A and Figure 3.10Bc) or GAD-67-immunonegative (Figure 3.10Bd). Treatment with EX-4 prevented the loss of GAD-67+/WFA+ cells only ($P < 0.001$) (Figure 3.9Bc).

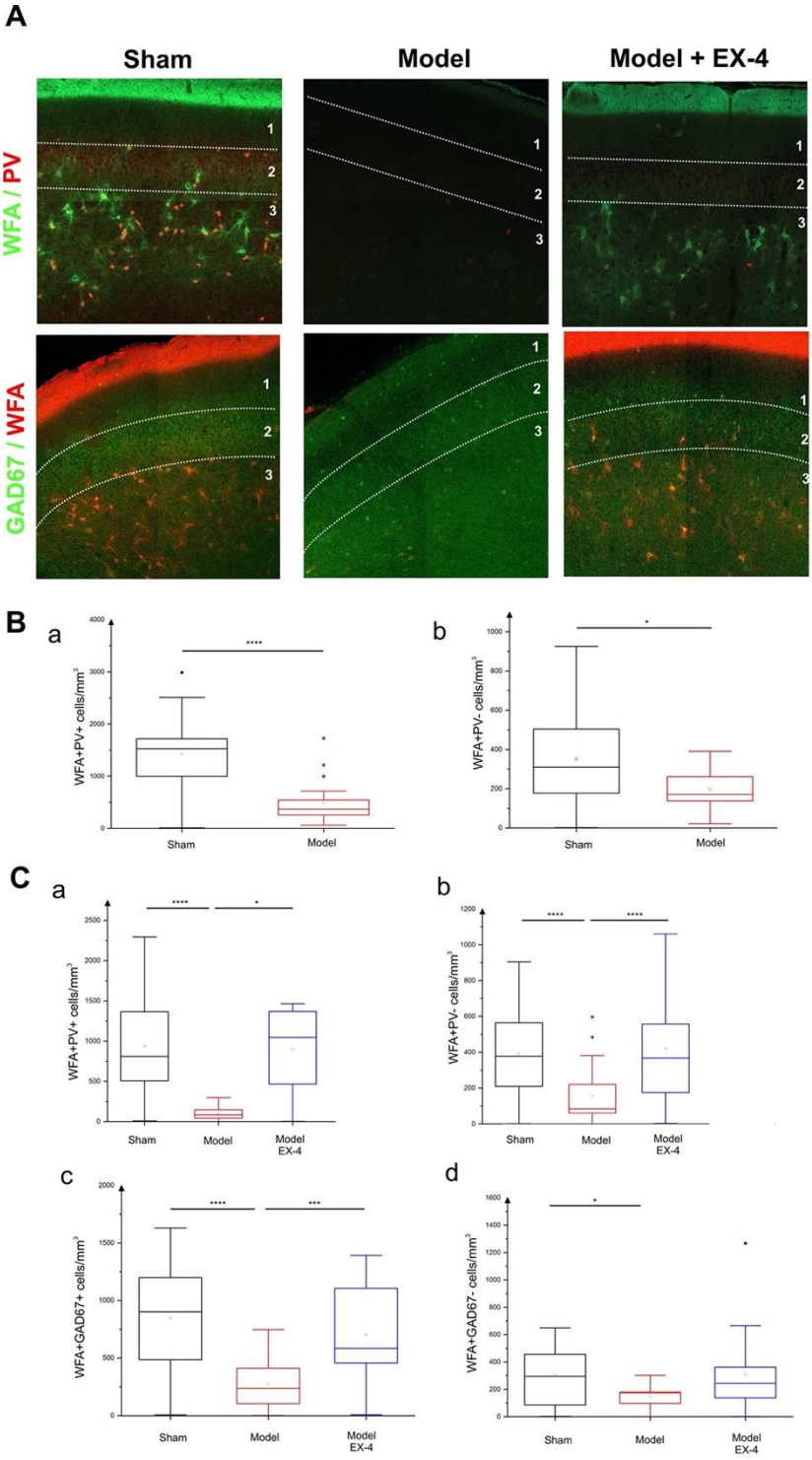

Figure 3.10 - Effect of EX-4 on the distribution of perineuronal nets (PNNs) in the PC of the early-stage model. A *Slices of the anterior PC of sham animals, model treated either with saline or EX-4 were double stained for PNNs (WFA in green) and PV (in red) [top row] and for PNNs (WFA in red) and GAD-67 (in green) [bottom row].* ***B Ba*** *Densities of WFA+/PV+ neurones in the PC of the early-stage model were decreased compared with that in sham animals.* ***Bb*** *Densities of WFA+/PV- neurones in the PC of the model were also decreased compared with that in sham animals. EX-4 prevented the decrease in the number of WFA+/PV- cells. Densities of WFA+/GAD+ neurones (Bc) and WFA+/GAD- neurones (Bd) in the PC were decreased in the model compared with that in the sham animals. Treatment with EX-4 prevented the decrease of WFA+/GAD+ neurones only. [Kruskall Wallis test *P < 0.05, *** P < 0.001, **** P < 0.0001].*

3.3.7 Increased number of dopaminergic interneurones in the OB GL of the early-stage PD model

Figure 3.11A shows representative examples of TH-immunopositive cells in the GL of the OB in each experimental group, sham, early-stage PD model, model + EX-4. Following the administration of the two toxins, unbiased stereology revealed that the average number of TH-immunopositive cells in the OB of the early-stage PD model was significantly *increased* by 41.1% compared with that in sham animals ($P < 0.01$, Figure 3.11B). Animals treated with EX-4 showed a smaller rise in TH-immunopositive cells (26.6%), and this was not significantly increased compared with the average number observed in sham animals ($P > 0.05$, Figure 3.11B), indicating a partial prevention of the toxins effect.

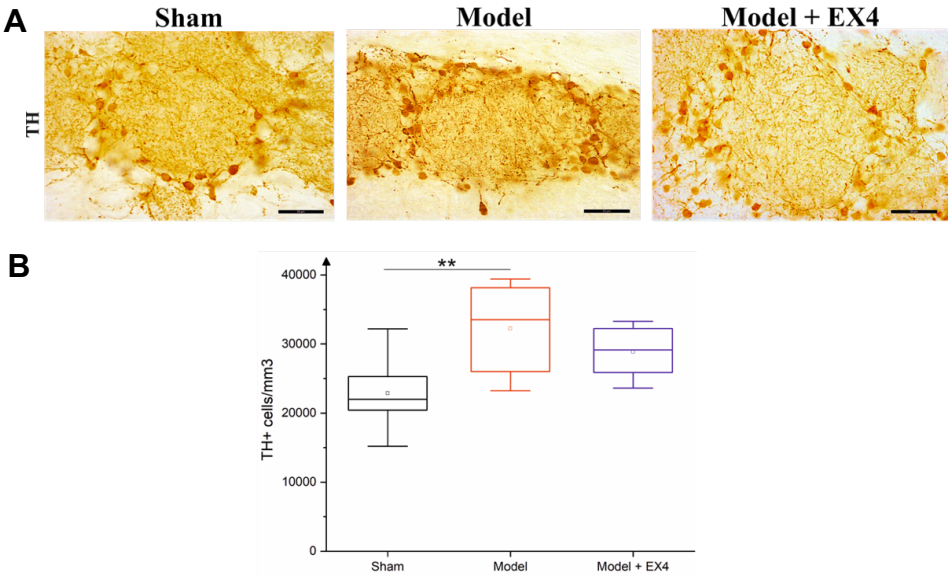

Figure 3.11 – Increase in TH-immunopositive cells in the glomerular layer (GL) of the olfactory bulb (OB) following 6-OHDA/DSP-4 administration. A *Representative images of a TH -staining in GL in sham animals (left panel), early-stage PD model (middle panel) and model treated with EX-4 (right panel). Scale bars represent 50 µm.* ***B*** *Number of TH-positive cells in the SN expressed as cell density per mm^3 (n=8 animals per experimental group). An increase in the number of TH-positive cells was observed in the GL of the early-stage model compared with sham (One way ANOVA ** $P < 0.01$). Treatment with EX-4 prevented the observed increase.*

3.3.8 Increased CBP interneurones in the OB of the early-stage PD model

Similarly to the PC, interneurones expressing CBP were analysed in the OB of the early-stage PD model and compared with those in the OB of sham animals. However, contrary to what was observed in the PC, expression of CBPs were significantly *increased* in the OB of the early-stage model compared with that in sham animals. Figures 3.12A-3.12C show representative examples of the distributions of PV-, CB- and CR-immunopositive interneurones of all experimental groups. CR- and PV-containing interneurones in the OB of the early-stage model were significantly increased compared with those in sham animals (Figures 3.12Bb-3.12Cb, One-way ANOVA, $P < 0.05$). While CR-expressing interneurones were found across all four layers, PV+ cells were mostly localised in EPL. Treatment with EX-4 prevented the observed increase (One-way ANOVA, $P > 0.05$). The average number of CB-expressing interneurones, which were located in the GL, did not significantly differ between groups (Figure 3.12Ab).

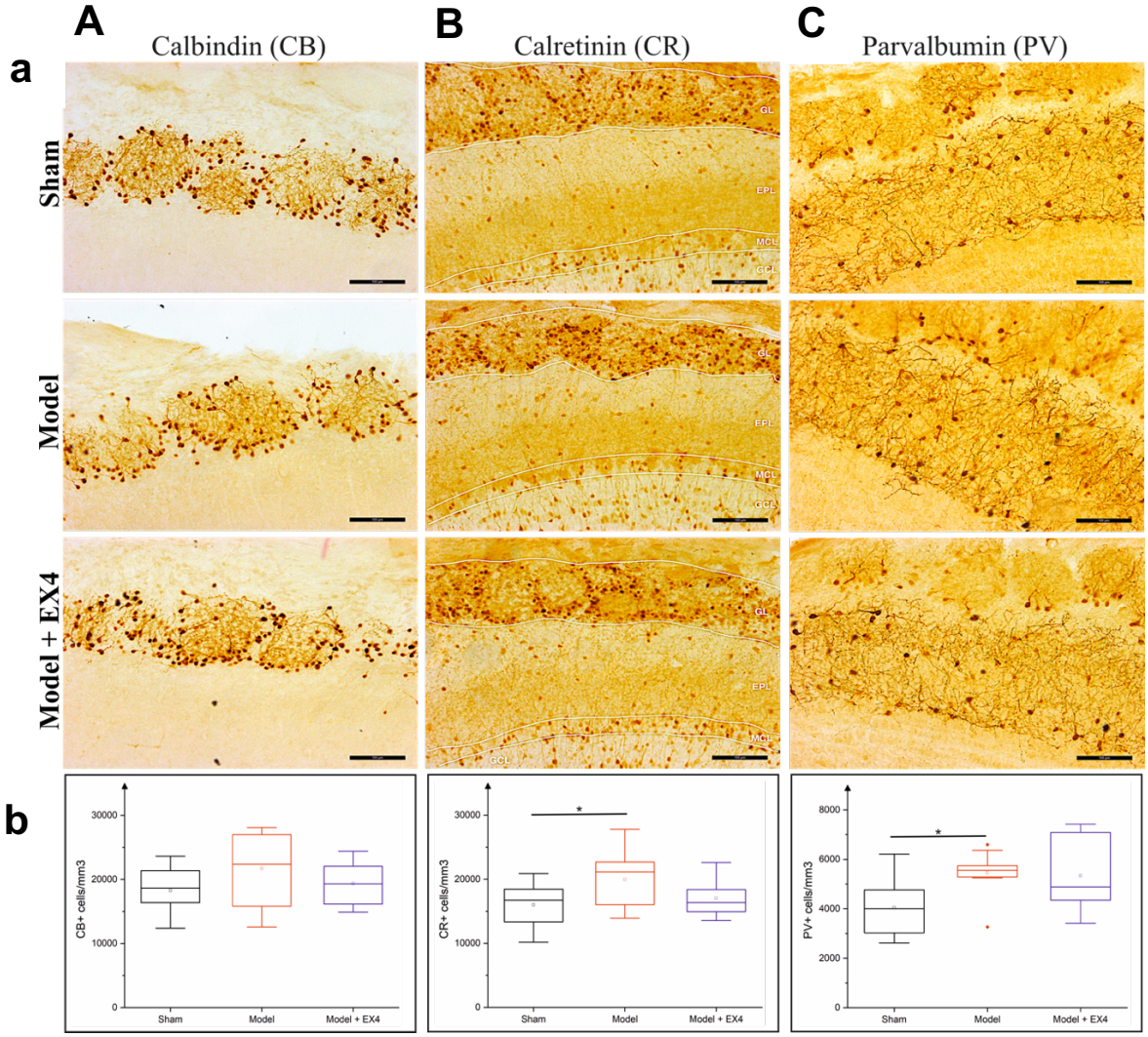

Figure 3.12 - Distribution of interneuronal CBPs in the early-stage PD model. A *Distribution of CB+ interneurones in the GL of the OB.* ***Aa*** *Examples of CB-immunostaining in the GL of the OB in each experimental group.* ***Ab*** *Cell densities in the GL of all experimental groups. The number of CB-positive interneurones was not significantly different across experimental groups.* ***B*** *Distribution of CR+ interneurones in the OB.* ***Ba*** *Examples of CR-immunostaining in all four layers of the OB for all experimental groups.* ***Bb*** *Cell densities in the OB of all experimental groups. A significant increase in the number of CB-positive interneurones was observed in the early-stage model compared with the sham animals (One-way ANOVA, P<0.05). Treatment with EX-4 prevented this increase.* ***C*** *Distribution of PV+ interneurones in the EPL of the OB.* ***Ca*** *Examples of PV-immunostaining in the EPL of the OB for all experimental groups.* ***Cb*** *Cell densities in the OB of all experimental groups. A significant increase in the number of PV-positive interneurones was observed in the early-stage model compared with the sham animals (One-way ANOVA, P<0.05). Treatment with EX-4 prevented this increase. All data are presented as mean ± SEM. Scale bars represent 100 µm. *P < 0.05. GL=Glomeruli layer, EPL=External plexiform layer, MCL=Mitral cell layer, GCL=Granule cell layer. Sham N=10; Model N=10; Model + EX-4 N=10.*

3.3.9 Cellular densities of GAD- and NeuN-immunopositive cells in the OB of the early-stage PD model.

As investigated in the PC, the number of GAD-67-immunopositive neurones was analysed to determine whether the results observed in the OB of the early-stage model was due to an up-regulation of the CBPs or to an increase in the number of neurones. Similarly to the PC, the density and distribution of GAD-67-positive cells did not differ among the experimental groups, suggesting an up-regulation of the expression of CBPs in the model rather than an increase in the number of cells (Figures 3.13Aa-Ab). The overall number of neurones in the OB was also studied, and the number of NeuN-immunopositive cells was found to be similar in the three experimental groups (Figures 3.13Ba-Bb).

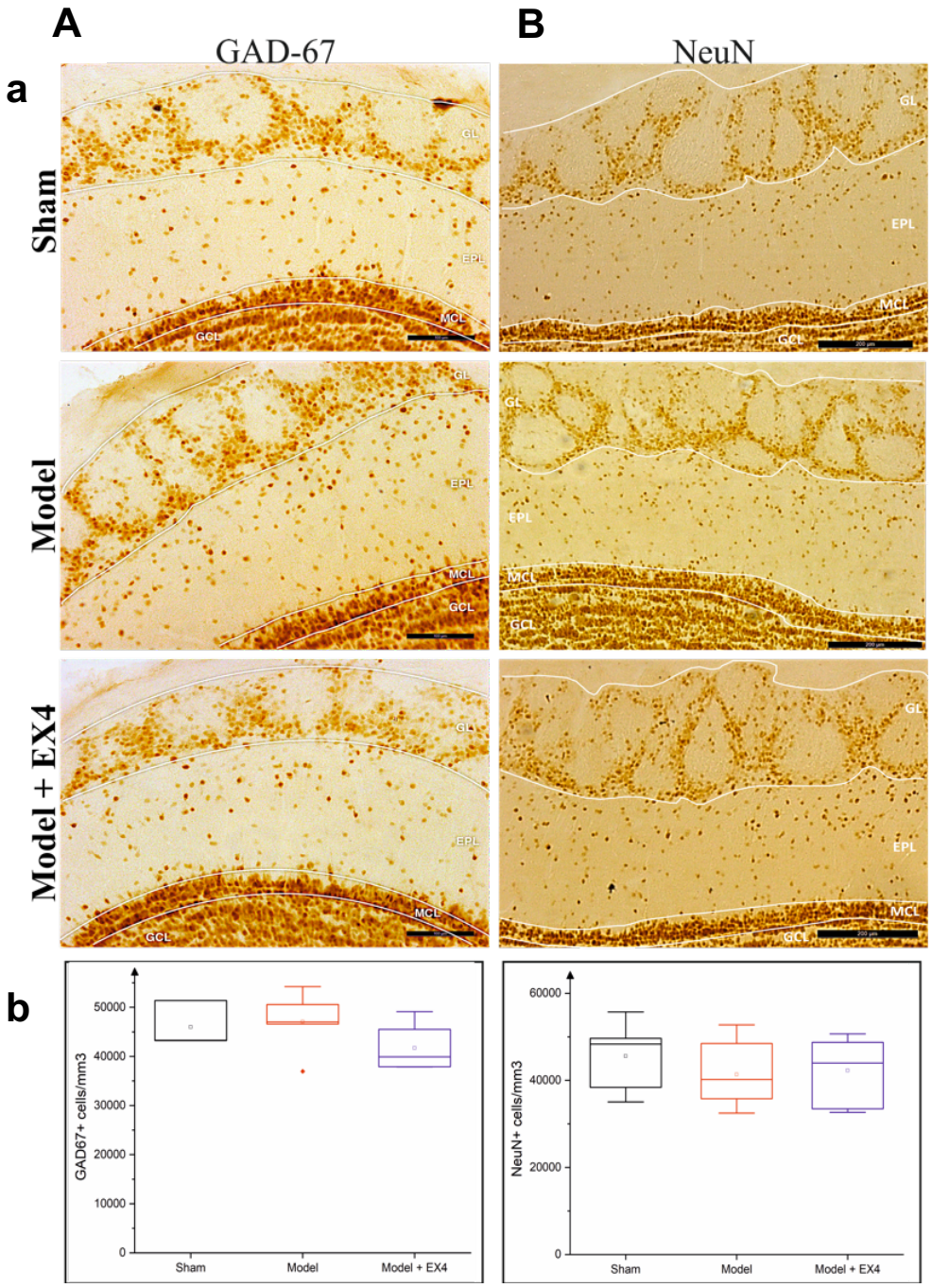

Figure 3.13 - Distribution of NeuN- and GAD-immunopositive neurones in the OB of all experimental groups. A *Distribution of GAD-67-immunopositive interneurones in the OB of the early-stage PD model.* ***Aa*** *Examples of GAD-67-immunostaining in the OB of all experimental groups.* ***Ab*** *GAD-67+ cell densities in the OB. The distributions were similar in all experimental groups.* ***B*** *Distribution of NeuN-immunopositive interneurones in the OB of the early-stage model.* ***Ba*** *Examples of NeuN-immunostaining in the OB of all experimental groups.* ***Bb*** *NeuN+ cell densities in the OB. The distributions were similar in all experimental groups. All data are presented as mean ± SEM. Scale bars represent 100 µm. GL=Glomeruli layer, EPL=External plexiform layer, MCL=Mitral cell layer, GCL=Granule cell layer. . Sham N=10; Model N=10; Model + EX-4 N=10.*

3.4 Discussion

The present study was aimed at validating and characterising an early-stage model of PD. Animals displayed hyposmia in absence of motor symptoms and a dopaminergic loss around 50% compared with sham animals (Sancandi *et al.*, 2018), validating the PD model as representative of the early-stage and allowing the study of the deficits that characterise this stage. Studying the aetiology of hyposmia is of a particular importance since it may facilitate the clinical diagnosis of PD years before the appearance of motor symptoms, allowing intervention from at a very early stage and possibly delay the onset of the more debilitating PD symptoms. Nevertheless, to date, the majority of the studies on the olfactory circuitry have been performed on either post-mortem tissues from PD patients or animals showing motor impairments. Thus, at present, the structural changes in the olfactory circuitry underlying hyposmia in the early stage of the disease are still largely uncovered (Doty, 2017). In this study, hyposmia in our early-stage PD model was shown to be associated with neuroinflammation, a decrease in interneuronal CBP and PNN expression in the PC, an *increase* in the overall number of neurones in the PC, and an *increase* in interneuronal CBP expression in the OB. Additionally, an *increase* in the number of dopaminergic interneurones in the OB GL was observed. Importantly, all these changes were prevented by EX-4 treatment (Sancandi *et al.*, 2018).

3.4.1 Monoaminergic loss in the midbrain results in dopaminergic and noradrenergic denervation in the PC

Dopaminergic and noradrenergic projections, starting from the SNpc and the LC respectively, innervate several areas of the forebrain including the PC, motor cortex, and other neocortical regions (Sara, 2009). A decrease in the number of cells producing either of the above neurotransmitters results in a perturbed innervation of such areas (Becker *et al.*, 2017), that has been linked to many psychiatric disorders, including schizophrenia (Krause, Márquez-Ruiz and Kadosh, 2013; Kesby *et al.*, 2018), ADHD (Engert and Pruessner, 2008; del Campo *et al.*, 2011), post-traumatic stress disorder and drug addiction (Arnsten *et al.*, 2015; Furini *et al.*, 2017; Le Dorze *et al.*, 2018), and neurodegeneration (Levite, Marino and Cosentino, 2017; Sitte *et al.*, 2017; D'Amelio, Puglisi-Allegra and Mercuri, 2018). In this study, neuronal loss in the SNpc and LC was also shown to be associated with decreased dopaminergic and noradrenergic levels in the PC of the early-stage PD model compared with those in the controls, as shown by unbiased stereology and HPLC quantification.

Dopaminergic and noradrenergic neurotransmission have been shown to modulate neuronal responses both in sensory and association areas (Jacob and Nienborg, 2018). For example, a study conducted by Bouret and Sara (2002) showed that stimulating the LC during odour tasks improved both the response engagement and the temporal organisation of the response to odour. Similarly, injections of dopaminergic agonists in the PC resulted in an increased *c-fos* expression (Steiner and Kitai, 2000), indicative of a greater neuronal activation (Bullitt, 1990). In addition, the PC has been found to

express a similar quantity of DA receptor mRNA as the prefrontal cortex (Santana, Mengod and Artigas, 2009), an area known for its dopaminergic modulation. Indeed, several studies have shown DA and NA involvement in the modulation of PC activity, with both neurotransmitters contributing to odour learning and perception through both suppression of excitatory synapses (DA: Malenka and Nicoll, 1986; NA: Hasselmo *et al.*, 1997) and enhancement of the activity of inhibitory interneurones by increasing their spontaneous inhibitory potentials (DA: Gellman and Aghajanian, 1993; NA: Kawaguchi and Shindou, 1998). These observations may explain the hyperactivation found by Moessnang and colleagues (2011) in the PC of hyposmic PD patients when subjected to an odour detection task. However, this dampening effect may not be cross-species, since a study conducted on the zebrafish found that bath-application of DA consistently reduced inhibitory post-synaptic currents in principal neurones of the homolog of mammalian olfactory cortex, therefore reducing inhibitory synaptic transmission (Schärer *et al.*, 2012).

In addition to these synaptic modulatory effects, growing evidence supports a neuroprotective role for these neurotransmitters in the brain with NA inhibiting microglial activation and suppressing pro-inflammatory mediator production (O'Neill and Harkin, 2018a) and DA counteracting the formation of α-synuclein aggregates (Luo *et al.*, 2016; Yedlapudi *et al.*, 2016). Taken together, evidence suggests that the changes observed in the early-stage PD model following the administration of the toxins may be the direct result of dopaminergic and noradrenergic denervation within the PC. Interestingly, single-toxin administrations failed to induce the same deficits (data not shown)

suggesting that a combination of toxins is required to induce hyposmia and the observed structural changes.

3.4.2 The model of early-stage PD displayed olfactory deficits in the absence of motor dysfunctions.

Hyposmia, defined as the inability to perceive, recognise, and discriminate or memorise odours, occurs in ~ 90% of PD patients (Berendse *et al.*, 2011; Roos *et al.*, 2019; Saiz-Sanchez *et al.*, 2020), and it is thought to be an early event occurring several years prior to clinical diagnosis, making it a good predictor of cognitive decline in neurodegenerative diseases and a reliable indicator for an early PD diagnosis (Poewe, 2008; Doty, 2012a, 2017; Sui *et al.*, 2019). The dual toxin-based model of early-stage PD was developed as a tool to mimic the deficits observed in PD patients in the early stages of the disease and, therefore, allow us to investigate the deficits underlying early onset of non-motor symptoms in the absence of motor signs. Behavioural assessments of the early-stage model revealed that significant olfactory deficits were displayed by the model without motor symptoms. This suggests that this model is indeed representative of an earlier stage of the disease than in previously described animal models in which NMSs, such as cognitive deficits or olfactory dysfunctions, were reported in the presence of motor dysfunctions (Tadaiesky *et al.*, 2008a; Santiago *et al.*, 2010; Carvalho *et al.*, 2013; Faggiani, Delaville and Benazzouz, 2015; Ledreux *et al.*, 2016; Nezhadi *et al.*, 2016). Notably, the animals were hyposmic, but not anosmic, as they found the treat in the hidden food test and displayed exploratory activity in the habituation/dishabituation test. The latter assesses whether an animal is able

to both smell and distinguish between same and different odours (Yang and Crawley, 2009). So far, animal models of PD have focussed mainly on the nigrostriatal dopaminergic system, providing important insight into the aetiology of MSs of PD for many decades; however, each model has its own strengths and limitations and none of them represents the disease in its entirety (Taylor, Greene and Miller, 2010; Jagmag *et al.*, 2016; Vingill, Connor-Robson and Wade-Martins, 2018). Indeed, while MSs can be achieved by depleting only dopaminergic neurones, NMSs cannot be accounted for by the DA depletion alone, suggesting an implication of other neurotransmitters, such as NA, in the aetiology of these deficits (Balestrino and Schapira, 2020). Despite the evidence of a co-degeneration of the dopaminergic and noradrenergic systems in PD patients (Buddhala *et al.*, 2015; Nahimi *et al.*, 2018; Peterson and Li, 2018), the role of NA depletion has often been overlooked, with most studies treating the animals with addition of uptake inhibitors to prevent the effect of 6-OHDA on NAergic cells (Schober, 2004; Blesa *et al.*, 2012a). Here, the combination of partial depletion of both DA and NA neurones resulted in the appearance of hyposmia in the absence of motor dysfunctions. Additionally, the combination of the depletions was essential for the appearance of olfactory deficits in the early-stage PD model, as depletion of either DA alone or NA alone was not sufficient to elicit hyposmia in either of the tests used to assess animals smell (data not shown). Lastly, the early-stage PD model displayed also cognitive deficits, suggesting a correlation between the loss of sense of smell and memory impairment.

3.4.3 Neuroinflammation was observed in the PC of the early-stage PD model.

Although the aetiology of hyposmia has yet to be elucidated, evidence has suggested that it is sustained by the presence of neuroinflammation (Doty, 2012a, 2017; Roos *et al.*, 2019; Song *et al.*, 2019), which is also considered to be one of the major components of PD pathogenesis (McGeer *et al.*, 1988). Microglial and astrocytic activations in the SNpc and striatum are a well-established response following injection of 6-OHDA (Gelders, Baekelandt and Van der Perren, 2018). Consequently, microglia cells take a M1 phenotype that is characterised by secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-12, and other cytotoxic molecules such as superoxide, nitric oxide (NO) and reactive oxygen species (ROS), enhancing pro-inflammatory responses during injuries and infections (Wang, Liu and Zhou, 2015). Specifically, these pro-inflammatory cytokines are released following the binding of ATP to the P2Y receptors (P2YRs), which are mainly expressed by microglia (Davalos *et al.*, 2005). PD patients were found to express higher levels of P2Y6Rs, a P2YR subtype linked to enhanced pro-inflammatory responses in macrophages (Garcia *et al.*, 2014), when compared with healthy subjects (Yang *et al.*, 2017). In healthy brains, microglial cells are characterised by thin, ramified processes and a relatively small cell body, features that mutate into shorter and less complex processes upon activation (Becker *et al.*, 2017). In this study, microglial activation was observed for the first time in the PC of an early-stage model of PD. Changes in cell morphology, including a decreased number of protruding branches and shorter processes,

resulted in a reduction of the overall intensity of the staining (Sancandi *et al.*, 2018).

Several studies have suggested that astrocytes also play a key role in the neuroinflammation observed in PD patients as well as animal models (Cicchetti *et al.*, 2002; Whitton, 2007; Wang, Liu and Zhou, 2015b; Gelders, Baekelandt and Van der Perren, 2018b). Astrocytes are generally slower in responding than microglial cells, probably due to the fact that part of the astrocytic response is elicited by pro-inflammatory mediators released from activated microglia, which are then further amplified by astrocytes (Saijo *et al.*, 2009). In addition, activation of microglia has been shown to induce activation of a subclass of astrocytes that are highly neurotoxic (Liddelow *et al.*, 2017). The activation of astrocytes is characterised by increased expression of glial fibrillary acidic protein (GFAP), hypertrophy of cell bodies and, in contrast to microglial cells, ramified processes (Wang, Liu and Zhou, 2015). Activated astrocytes were observed in the PC of the model of early-stage PD. Activation of astrocytes was progressive in nature and followed a rostro-caudal gradient (Suppl. Sancandi *et al.*, 2018) suggesting that glial cells in the anterior PC may therefore activate neighbouring cells possibly via gap junctions, with the release of toxic pro-inflammatory factors as previously suggested (Lucin et al., 2009) leading to the progression of neuroinflammation across the PC region.

3.4.4 Reduced expression of CBPs in the PC following dual-toxin treatment

Alongside with neuroinflammation, hyposmia in PD was also shown to be sustained by GABAergic deficits (Doty, 2012a, 2017). Recently, a “GABA-

collapse hypothesis" has been proposed due to the regulatory role of a Ca^{2+}/GABA mechanism that stabilises neuronal activity both at cellular and systemic levels (Błaszczyk, 2016). Briefly, this hypothesis suggests that a decline in the Ca^{2+}/GABA control leads to both weakened protective barriers, such as the BBB, and accumulations of intracellular deposits of calcium and α-synuclein, resulting in neuronal death (Błaszczyk, 2016). The presence of GABAergic deficits in PD patients and animal models is now well established (Abbott, Pye and Nahorski, 1982; Ubeda-Bañon *et al.*, 2010; Doty, 2012a; Hurley *et al.*, 2013; Surmeier and Schumacker, 2013). In addition, CBP-expressing interneurones, have been found to be affected in PD (Ubeda-Bañon *et al.*, 2017; Cave *et al.*, 2016; Petryszyn *et al.*, 2016; Hurley *et al.*, 2013). In agreement with these studies, a decrease in the density of CBP-expressing interneurones in the PC of the early-stage model was observed compared with that in sham animals.

CBPs, such as calretinin, calbindin, and parvalbumin, are buffer proteins that bind intracellular Ca^{2+} as its concentration increases within the cell and modulate the cytosolic calcium level, resulting in the control of neuronal firing (Bischop *et al.*, 2012). CBPs have been shown to regulate Ca^{2+} signals in neurones based on their Ca^{2+} affinity, cytosolic concentrations, their kinetics for Ca^{2+} binding and release, and their mobility inside the neurones (Schwaller, 2010). Owing to their Ca^{2+} regulatory properties, CBPs are thought to play a neuroprotective role as they are able to buffer potentially toxic fluctuations in intracellular Ca^{2+} concentrations, protecting neurones against cell death (Van Den Bosch *et al.*, 2002; Zündorf and Reiser, 2011; Nikoletopoulou and Tavernarakis, 2012). Thus, it was reasonable to assume that the decrease in

CBPs observed in this study was correlated with a decrease in the number of GABAergic interneurones in the PC of the model. In order to test this hypothesis, the density of GAD-67-immunopositive cells in the PC of each experimental group was quantified. Interestingly, the total number of GAD-67-positive cells was unaltered in the early-stage model compared with that in sham animals, suggesting that the observed reduction of CBPs was caused by a *downregulation* of CBPs rather than interneuronal death. Although the cause of this decrease in the expression of CBPs remains unknown, CBPs in GABAergic neurones can play the role of endogenous neuroprotectors, which prevent Ca^{2+} overload and subsequent neuronal death. Indeed, it was recently shown that PV and CB reduced the rate of Ca^{2+} increase, preventing the death of GABAergic neurones under ischemia-like conditions (Turovsky *et al.*, 2018). Another possible explanation may involve compensatory mechanisms in response to the cortical hyperactive state induced by monoaminergic loss. Indeed, PV deficiency has been shown to boost GABAergic release (Vreugdenhil *et al.*, 2003), and dysfunctions in glutamatergic transmission were found to alter PV expression (van Lier, Hierlemann and Knoflach, 2018), suggesting that the decrease in interneuronal CBP expression may be a consequence of the altered excitatory/inhibitory balance due to PC monoaminergic denervation. The cell bodies and proximal dendrites of most PV+ GABAergic interneurones are surrounded by condensed extracellular matrix aggregates, aka PNNs (Bozzelli *et al.*, 2018). The latter are mainly involved in the control of CNS plasticity and they have been observed to be coupled to highly active neurones suggesting a supporting role to the neural function (Sorg *et al.*, 2016). Moreover, experimental evidence suggests that

PNNs are linked to synapse formation (Pyka *et al.*, 2011; Yamada and Jinno, 2013; Xue *et al.*, 2014; Berretta *et al.*, 2015b) and they are thought to exert a neuroprotective role from oxidative stress by forming a protective shield around PV-positive interneurones (Cabungcal *et al.*, 2013; Suttkus *et al.*, 2014; Morishita *et al.*, 2015; Wen *et al.*, 2018). PNN reorganisation has been shown to result in abnormal CNS development and altered neural excitability (Liu *et al.*, 2013; Balmer, 2016; Lensjø *et al.*, 2017), whilst PNN degradation has been linked to several diseases, such as schizophrenia (Berretta, 2012; Berretta *et al.*, 2015b), epilepsy (Pollock *et al.*, 2014), and Alzheimer's disease (Baig, Wilcock and Love, 2005b; Lendvai *et al.*, 2013). Similarly, in this study, a decrease in the density of PNNs surrounding PV-positive cells as well as PNNs *per se* was observed in the PC of the early-stage PD model compared with sham animals. Although the cause of the PNN loss has yet to be established, it could be speculated that neuroinflammation plays a vital role as previously shown in epileptic (Kim *et al.*, 2017) and cerebral ischemic (Dzyubenko *et al.*, 2018) models. Additionally, it was recently reported that microglia activation is associated with PNN loss in an AD mouse model (Crapser *et al.*, 2020). Relatedly, using a mouse model of schizophrenia, oxidative stress-induced microglia activation was shown to trigger a cascade of event culminating in nuclear factor-kB (NF-kB) activation and secretion of various cytokines, which then resulted in PV and PNNs loss (Dwir *et al.*, 2020). Lastly, the overall density of neurones in the PC was investigated using the Neu-N marker. Remarkably, the number of Neu-N-positive cells was found to be *increased* in the PC of the early-stage PD model compared with that in the PC of sham. The cause behind this increase is unknown, however, it may be

associated with neurogenesis that occurs within the PC (Yuan, Liang and So, 2015). There is experimental evidence that the PC contains neurones expressing immature neuronal markers, such as doublecortin (Nacher, Crespo and McEwen, 2001), that have the potential to become mature neurones later in life (Pekcec, Löscher and Potschka, 2006). The fate of these newly generated neurones has yet to be elucidated, however, some doublecortin-positive cells have been found to co-express CBPs (Bédard and Parent, 2004; Klempin *et al.*, 2011), suggesting that the increased in Neu-N^+ cells detected in our early-stage model might be a compensatory mechanism for the downregulation of CBPs. However, further experiments are needed to validate this hypothesis. In addition, a disruption of PNNs has been shown to be associated with increased plasticity (Pizzorusso *et al.*, 2002; Nowicka *et al.*, 2009) and synaptogenesis (Wen *et al.*, 2018). Thus, the observed increase in Neu-N-positive cells may also be a consequence of the reduction in the density of PNNs.

3.4.5 Dual-toxin treatment upregulates dopaminergic and CBPs expression in the OB

The olfactory system is one of the oldest sensory modalities in the phylogenetic history of mammals, and it comprises the synergy of several brain regions, such as the OB. Therefore, the present study examined also the fate of dopaminergic and GABAergic interneurones in the OB following DA and NA insults to determine if a similar pattern of deficits as the one observed in the PC could also be established in the OB. Despite the fact that OBs present the most abundant and varied interneuronal populations in the brain,

with higher proportion of interneurones to excitatory neurones (100:1 ratio) than in other brain regions (1:5 ratio), they have received significantly less attention in normal and pathophysiological conditions than cortical interneurones (Kim, Choe and Moon, 2020).

Interestingly, the density of TH-immunopositive interneurones in the GL of the early-stage model was found to be increased compared with that in sham animals. Dopaminergic neurones in the olfactory bulbs were discovered more than 30 years ago (Halász *et al.*, 1981) and have been shown to co-release GABA and DA (Gall *et al.*, 1987). While GABA has been observed following a classical fast release pattern, DA release has been shown to increase gradually for many seconds following stimulation, suggesting that these cells may control synaptic timing in both intraglomerular and interglomerular circuits (Borisovska *et al.*, 2013). Indeed, these interneurones presynaptically inhibit glutamate release from olfactory nerve terminals by activating presynaptic $GABA_B$ receptors (Aroniadou-Anderjaska *et al.*, 2000; Murphy, Darcy and Isaacson, 2005). Similarly, activation of dopaminergic D2 receptors was found to inhibit transmission at the olfactory nerve-mitral cell synapse, whilst pre-treatment with a D2 antagonist completely prevented this effect (Hsia, Vincent and Lledo, 1999), suggesting that DA may play a role in controlling the inputs to the main olfactory bulb (Berkowicz and Trombley, 2000). OB involvement in the pathophysiology of PD is thought to start from the very early stage (Braak *et al.*, 2003; Altinayar *et al.*, 2014; Del Tredici and Braak, 2016; Engelender and Isacson, 2017; Gardner *et al.*, 2017; Rey *et al.,* 2018) with a key role in the aetiology of hyposmia (Doty, 2012; 2017; Niu *et al.*, 2018). Several PD studies showed an increase in the number of dopaminergic-

GABAergic interneurones in the OB of PD animal models (Yamada *et al.*, 2004; Winner *et al.*, 2006; Chiu *et al.*, 2014), primates (Belzunegui *et al.*, 2007; Pifl *et al.*, 2017), and humans (Huisman, Uylings and Hoogland, 2004; Mundiñano *et al.*, 2011). The observed increase in TH-positive cells in the OB of the early-stage model may therefore be a key pathophysiological change underlying hyposmia. In support of this hypothesis, Ilkiw and colleagues (2018) recently showed that injecting 6-OHDA directly in the OB of a rat PD model and therefore damaging the DA neurones in this region, prevents the occurrence of hyposmia.

Surprisingly, and in contrast to what was observed in the PC, CBPs expression in the OB was also found to be upregulated, despite an unchanged overall number of GABAergic interneurones. Whilst CB and PV expressions were restricted to a single layer, PG and EPL respectively, CR+ interneurones were found across all four layers. Although CB expression was not significantly different, a trend in upregulation was observed, suggesting that an increase in the number of animals may be needed to reach statistical significance. CBP-expressing interneurones have received markedly less attention than the dopaminergic class, however, an increase in CBPs was found in different areas of the anterior olfactory nucleus, which also correlated with α-synuclein co-localization (Ubeda-Bañon *et al.*, 2017). Although the significance of this interneuronal increase is yet to be understood, some authors have suggested a possible compensatory mechanism in response to neurodegenerative processes (Winner *et al.*, 2006; Paß *et al.*, 2020). Indeed, neurogenesis in the OB has been shown to occur during adulthood (Pignatelli and Belluzzi, 2017). Progenitors of OB interneurones were shown to arise from neural stem cells

in the subventricular zone (SVZ) (Lledo and Valley, 2016; Malvaut and Saghatelyan, 2016), specifically from the dorsolateral region (Fiorelli *et al.*, 2015), with the rate of bulbar dopaminergic interneurones reported to increase in adulthood (De Marchis *et al.*, 2007). Recently, in *MitoPark* mice, a genetic model for PD with severe mitochondrial dysfunctions and a severely impaired odour detection, an enhanced mobilisation of progenitor cells was found in the SVZ, supporting the theory of a compensatory response (Paß *et al.*, 2020). The observed increase may therefore be ascribed to the presence of newly generated interneurones. The SVZ projects also to the SNpc (He and Nakayama, 2009), however, data regarding newly born neurones in the latter is contradictory. Zhao *et al.* (2003) observed that new neurones travel from the SVZ to the SNpc and that, assuming a constant rate of neuronal turnover, it would take the lifespan of a mouse to replace the entire population of dopaminergic neurones in this area. In contrast, Frielingsdorf *et al.* (2004) reported that among the new neurones observed in the SNpc, none of them expressed a dopaminergic phenotype. The disruption of the system's homeostasis occurring during PD, together with the body's innate tendency to compensate for loss, may lead the SVZ to produce new dopaminergic cells that are then directed to the OB rather than to the SNpc, contributing to an enhanced local dopaminergic inhibition, which in turn sustains hyposmia. Nevertheless, this theory has yet to be fully investigated.

3.4.6 The changes observed in the olfactory system of the early-stage PD model are partially prevented by EX-4

EX-4 is a GLP-1 analogue that was shown to improve motor functions, sleep quality, and cognition, while reducing depression in several clinical trials on PD patients (Aviles-Olmos *et al.*, 2013, 2014; Athauda *et al.*, 2018). However, the effects of EX-4 on the underlying pathophysiology of PD are yet to be fully elucidated. GLP-1R activation in the brain is thought to trigger neurotrophic effects, neuroprotection, synaptic plasticity and to reduce neuroinflammation (Kim *et al.*, 2017; Grieco *et al.*, 2019). Relatedly, EX-4 has been shown to influence several cellular pathways, such as neuroinflammation, mitochondrial function, and cellular proliferation, within the CNS (Athauda and Foltynie, 2018). Indeed, GLP-1Rs, that are expressed by astrocytes and glial cells, were shown to be upregulated in response to inflammatory stimuli as a physiological response to harmful stimuli (Chowen *et al.*, 1999). However, GLP-1Rs are not only involved in physiological regulatory mechanisms, but also in energy balance control (Reiner *et al.*, 2016, 2018), counteracting drug addiction (Schmidt *et al.*, 2016; Hernandez *et al.*, 2017, 2018), and preventing neuronal death (Xie *et al.*, 2018). EX-4 was found to restore DA imbalance, resulting in significant improvements in behaviour and motor function in PD animal models (Harkavyi *et al.*, 2008; Kim, Moon and Park, 2009; Li *et al.*, 2009). In this study, treatment with EX-4 prevented both dopaminergic and noradrenergic loss. HPLC quantification of both monoamines in SNpc, striatum and PC showed that animals treated with EX-4 had comparable levels of neurotransmitters compared with those in sham animals. This neuroprotective effect may be ascribed to the ability of EX-4 to preserve mitochondrial function in

dopaminergic neurones by increasing expression of complex I, the main target of 6-OHDA, and anti-apoptotic proteins (Chen *et al.*, 2015; Nassar *et al.*, 2015). Alongside, GLP-1R stimulation has been known to increase intracellular cAMP (Drucker et al., 1987), that in turn has been shown to upregulate both expression and activity of the TH enzyme (Kim et al., 1993), possibly providing an additional compensatory mechanism for the dopaminergic loss (Athauda and Foltynie, 2018). EX-4 also prevented astrocyte activation within the PC of the early-stage model, however, it did not halt Iba1-immunopositive cell activation. Additionally, EX-4 was also able to partially prevent the downregulation of CBPs and PNNs loss as well as the increase in NeuN-immunopositive neurones. Lastly, EX-4 partially prevented the increase of GABAergic interneurones in the OB of the early-stage model. EX-4 beneficial effects were exerted by activating GLP1-Rs, as shown by the complete blockage of neuroprotective effects in the presence of the GLP1-R antagonist EX-39 (Sancandi *et al.*, 2018). The fact that EX-4 in our experiments, only partially prevented the observed changes following dual toxin treatment, allows us to speculate that a longer EX-4 treatment, or a change in the dose, may be needed to reveal a larger effect of the drug. Nevertheless, further experiments are required to test this hypothesis.

Although the mechanisms behind these effects remain unknown and require further investigation, increasing evidence suggests that anti-inflammatory treatments may be used as new strategies to counteract and slow down PD progression and eliminate L-DOPA side effects (Hirsch and Hunot, 2009; Rees *et al.*, 2011; Wang, Liu and Zhou, 2015b; Flood, Arbabzada and Sharma, 2016; Mehta and Tanner, 2016; Gelders, Baekelandt and Van der Perren,

2018b; Hassanzadeh and Rahimmi, 2019). Based on the present findings, the use of EX-4 may therefore be listed alongside with this alternative treatment line, with the potential to improve the long-term prognosis for millions of PD patients world-wide. Relatedly, other GLP1-R agonists are currently under investigation as potential treatments for PD (ClinicalTrials.gov Identifier: NCT03659682, ClinicalTrials.gov Identifier: NCT03439943), highlighting the potential therapeutic applications for GLP1 agonists in PD treatment.

3.4.7 Summary and future directions

To our knowledge, this is the first study showing significant cellular and structural changes in the PC of a early-stage model of PD. Hyposmia exhibited by the early-stage PD model (Sancandi *et al.*, 2018) was associated with a loss of monoaminergic transmission in the PC, a downregulation of CBPs and loss of PNNs, alongside a presence of ongoing neuroinflammation and a possible compensatory increase in expression of dopamine and CBPs in interneurones in the OB. Overall, the increased dopaminergic synthesis in the OB coupled with a downregulation of CBP in the PC are the most likely major changes driving hyposmia, with the former inhibiting the whole olfactory pathway whilst the latter disrupts the electrophysiological properties of the cortex. Additionally, all the observed deficits, but microglia activation, were partially prevented by EX-4 treatment (at the tested dose), suggesting that the observed changes may be initiated by a common denominator targeted by the drug or that EX-4 acts on multiple sites/levels. Nevertheless, additional experiments on the cellular mechanisms of action of EX-4 and other GLP-1 agonists are urgently required. Similarly, further experiments should be aimed

at determining whether other deficits and changes occur in the PC, such as a modification of the electrophysiological properties of pyramidal cells and interneurones in the PC and LOT neurotransmission *per se*, following the decrease in dopaminergic and noradrenergic inputs. Moreover, the bulbar increase of dopaminergic-GABAergic interneurones (and its significance) has yet to be explored extensively. For example, the increase in inhibitory transmission within the OB may be responsible for a reduced information processing; however, there is currently limited experimental evidence to confirm this theory. Lastly, other brain regions receiving inputs from the PC and involved in PD pathophysiology, such as the neocortex and hippocampus, will be investigated to determine whether they display the same deficits as the PC following dual toxin treatment in the next Chapter.

Chapter 4 – Along the olfactory pathway: PFC and Hippocampus

4.1 Introduction

The olfactory pathway comprises several other regions in addition to the previously studied olfactory bulbs (OB) and the piriform cortex (PC), including neocortical areas and the hippocampus (Franks et al., 2015). Among various neocortical areas, the prefrontal cortex (PFC), in particular, has been reported to have functional and structural connectivity with the olfactory pathway (Salimi *et al.*, 2019), with inputs directly from the PC (Pitkänen *et al.*, 2000; Kadohisa, 2013). The PFC associates olfactory inputs with taste, oral texture, and visual inputs, creating a multimodal representation of reward value and economic value of goods, such as the reward value of food (Padoa-Schioppa, 2011; Rolls, 2012). Similarly, the PC is also connected with the hippocampus, where olfactory inputs can be coded into episodic long-term memory (Rolls, 2010; Kadohisa, 2013). Indeed, damage to the hippocampus was shown to impair odour-place associative learning and temporal order memory for odour information (Kesner, Gilbert and Barua, 2002; Rolls and Kesner, 2006). Additionally, specific hippocampal neurones were found to encode the place of odour-related rewards (Kadohisa, 2013). Overall, both the PFC and the hippocampus have a role in the process of odour-elicited emotions and odour-associated emotional memory. Indeed, human imaging studies have observed that the PFC is activated by both unpleasant or pleasant odours, depending on the subjective value ascribed to the odours by participants (Anderson *et al.*, 2003; Grabenhorst *et al.*, 2007). Lastly, when subjects with post-traumatic stress disorder are exposed to the odour related to the traumatic memory, their PFC was shown to be active (Vermetten *et al.*, 2007). The hippocampus and

PFC have also been shown to be activated by odour cues during the autobiographical memory retrieval process (Larsson and Willander, 2009).

4.1.1 Cognitive decline in PD

Cognitive dysfunction is one of the NMSs of PD with the highest morbidity and mortality (Poewe, 2008; Narayanan, Rodnitzky and Uc, 2013; Radhakrishnan and Goyal, 2018; Seppi *et al.*, 2019), which is usually present in around 20% of patients at the time of PD diagnosis (Watson and Leverenz, 2010). Additionally, cognitive impairments were shown to correlate with hyposmia, with the latter being a good predictor of the former (Roberts *et al.*, 2016; Kotecha *et al.*, 2018; Yahiaoui-Doktor *et al.*, 2019). Cognitive decline varies in its severity across PD patients, from subtle or mild cognitive impairment that does not significantly disrupt daily living, to dementia and rate of progression, with longitudinal studies observing dementia onset in around 80% of patients after 20 years (Goldman *et al.*, 2018). To date, the mechanisms and processes underlying cognitive dysfunction in PD are poorly understood, and only few effective treatments are available to manage these debilitating symptoms (Balestrino and Schapira, 2020). For example, levodopa was found to inconsistently improve cognitive impairments in PD, depending on disease state and the integrity of striatal dopamine signalling (Pascual-Sedano *et al.*, 2008). Indeed, it is thought that the mesocortical dopaminergic projections to the prefrontal cortex may be involved in the aetiology of these symptoms (Narayanan, Rodnitzky and Uc, 2013). However, Vaillancourt and colleagues (2013) described the “dopamine overdose hypothesis” in which therapies that aimed at replenishing dopamine in the brain have the dual effect of: 1)

increasing performances on tasks related to the nigrostriatal-pathway, such as motor skills, whilst concomitantly 2) decreasing efficacy on tasks associated with the mesocorticolimbic-pathway, such as executive functions. Additionally, cholinergic, serotonergic, and noradrenergic neurodegeneration, in association with the lack of dopamine, have been shown to be involved in cognitive dysfunctions (Goldman *et al.*, 2018). Lastly, Brück *et al.* (2004) showed that the PFC and the hippocampus of non-demented patients in the early stage of the disease begin to atrophy.

4.1.2 Executive functions, PFC, and PD

Cognitive impairments in PD usually include deficits with working memory, planning, attention, and impulse control disorder, which are all cognitive processes associated with PFC activation (Schapira, Ray Chaudhuri and Jenner, 2017). Indeed, the PFC regulates the focus of the attention on one event or phenomenon to the exclusion of others as well as switching between them, allowing the individual to act automatically or even override automatic actions, a set of abilities frequently referred to as executive functions (Watson and Leverenz, 2010). Neuropsychological and imaging studies conducted on PD patients found that poor performances in all tests related to executive functions, including the Wisconsin Card-Sorting Task, attentional set shifting, and spatial working memory, correlated with a reduced metabolic activity in the medial frontal network (Cools *et al.*, 2001; Zgaljardic *et al.*, 2006; Huang *et al.*, 2007). Indeed, imaging studies have consistently demonstrated hypoactivation of prefrontal areas in combination with a marked decrease in lateral and medial prefrontal dopamine in late stage PD patients (Narayanan,

Rodnitzky and Uc, 2013). Nevertheless, there are instances during which the PFC of PD patients has been found to be hyperactive instead. Specifically, some studies have shown that PFC activation is higher among patients with PD than in healthy controls during walking, suggesting that motor control may become cortically mediated by executive-attentional network activation to compensate for deficits in the subcortical automatic locomotor control (Stuart *et al.*, 2018, 2019). In support of this idea, behavioural therapies designed to improve gait and enhance automaticity in patients with PD, have shown that PFC activation reduces following the intervention, further strengthening the above theory (Maidan *et al.*, 2018; Thumm *et al.*, 2018). However, the exact role of PFC dysfunctions in PD progression and manifestation has yet to be fully understood.

4.1.3 Explicit memory, hippocampus, and PD

The hippocampus is the key structure responsible for the spatial representation of environments as well as for "explicit" memory (Lisman *et al.*, 2017). The latter can be subdivided into episodic and semantic memories, that are the conscious recollection of an episode or concepts/meanings respectively (Jawabri and Cascella, 2020). Explicit memory, both in its verbal and non-verbal forms, is known to be affected in PD patients without dementia (Watson and Leverenz, 2010). Additionally, some evidence suggests that early changes in episodic memory may precede further cognitive decline in PD patients (Hoogland *et al.*, 2017; Perello and Andres, 2020). Deficits in both immediate and delayed story recall were reported in patients with PD (Fama *et al.*, 2000; Muslimović *et al.*, 2007), as well as impairments in recognition,

figure copy, and visual retention (Janvin *et al.*, 2006; Uc *et al.*, 2006; Pereira *et al.*, 2009), suggesting that PD patients without dementia have an increased risk for memory impairments. However, when the learning skills level of PD patients was compared with controls, there was no difference in their performance in delayed free recall and recognition tasks, indicating that it might be the encoding process that is impaired in PD (Chiaravalloti *et al.*, 2014). Human imaging studies on PD patients observed morphological and functional changes in the entorhinal cortex, hippocampus, and surrounding temporal areas, with some of them starting in the early stages (Pirogovsky-Turk *et al.*, 2015; Tanner *et al.*, 2015; Biundo, Weis and Antonini, 2016). For example, hippocampal atrophy was shown to function as a biomarker of initial cognitive decline in PD, leading to impaired memory encoding and storage (Weintraub *et al.*, 2011). Accordingly, during the initial stages of cognitive decline in PD, especially when impairments in memory encoding and storage arise, an ongoing hippocampal neurodegeneration, mainly involving the Cornu Ammonis (CA) 2-3 subfields was observed (Foo *et al.*, 2016; Novellino *et al.*, 2018). Similarly, a recent study reported that the thickness of the cell body layer of hippocampal CA1 is the most reliable predictor of episodic memory impairment (La *et al.*, 2019). Additionally, memory deficits in PD patients were recently shown to be related to a reduced connectivity between the hippocampus and the precuneus and superior parietal cortex that are regions related to associative memory and attentional control respectively (Bezdicek *et al.*, 2019). Overall, memory deficits in PD appear to be sustained by both encoding and retrieval deficits. Lastly, a human imaging study using DAT

binding found that selective hyposmia in PD correlated with reduced hippocampal striatal dopamine innervation (Nicolaas I Bohnen *et al.*, 2008).

4.1.4 Aims of the present chapter

Given the established roles of the PFC and hippocampus in PD progression, this chapter follows up on the deficits observed in the PC and OB of the early-stage PD model, such as altered expression of calcium binding proteins (CBPs) and increased neuronal density in the PC. Specifically, we hypothesised that these regions would show similar deficits to the ones observed elsewhere. Therefore, CBP-expressing interneurones in both PFC and hippocampus were analysed and quantified through unbiased stereology together with the overall number of both GABAergic interneurones and neurones. The PFC was analysed as a whole, whilst the hippocampus was subdivided in the subfields CA1-2-3 and each region was analysed separately. Lastly, as in the previous chapter, EX-4's effectiveness in preventing or inhibiting the deficits in the early-stage PD model was also investigated.

4.2 Materials and Methods

4.2.1 Experimental Groups

Animals were divided into three groups: sham, DSP-4 + 6-OHDA (early-stage model), and DSP-4 + 6-OHDA + EX-4. Figure 4.1 shows the experimental timeline. On day 1, animals were injected intraperitoneally either with saline (sham) or DSP-4 (model). Three days later, they underwent surgeries during which either saline (sham) or 6-OHDA (model) were delivered directly into the striatum (bilaterally). Two weeks following the surgeries, animals were injected intraperitoneally twice daily with either EX-4 (Sigma Aldrich) or saline for 7 days (Figure 4.1). Finally, three weeks after the surgeries, animals were culled and the brains were collected.

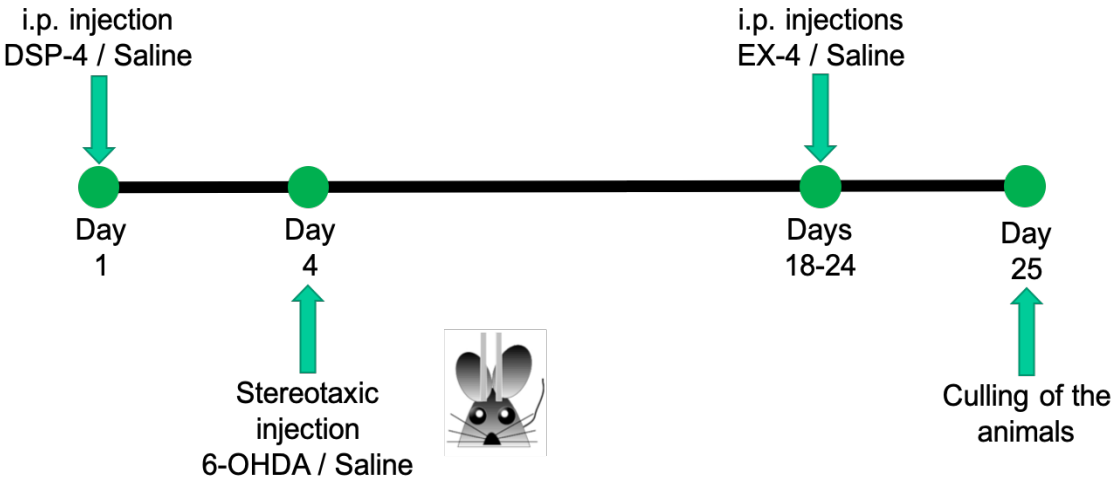

Figure 4.1 – Experimental timeline. *On day 1, animals received the injection of either DSP-4 (early-stage model, 25 mg/Kg) or saline (sham). 3 days later, stereotaxic surgeries were performed to bilaterally deliver in the striatum either 6-OHDA (early-stage model, 15 μg) or saline (sham). Next, from day 18, animals were treated daily for a week with either EX-4 early-stage model) or saline (sham and early-stage model). On day 25, animals were culled and the brains collected for further analyses.*

4.2.2 Drugs

N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4), a selective noradrenaline neurotoxin, and 6-hydroxydopamine (6-OHDA), a neurotoxin used to deplete both noradrenergic and dopaminergic neurones respectively, were purchased from Sigma (Sigma Aldrich, Gillingham, UK). DSP-4 was delivered at a concentration of 25 mg/kg in saline solution and injected intraperitoneally (i.p.) 4 days prior to the 6-OHDA injection, while 6-OHDA was dissolved at a concentration of 5 mg/ml in saline solution containing 0.9% ascorbic acid and delivered bilaterally in the striatum. The optimum doses for DSP-4 and 6-OHDA were chosen to induce *partial* reduction of NA and DA levels based on previous studies (Jonsson et al., 1981; Prezedborski et al., 1995), mimicking the early stage of the disease. EX-4 (Sigma Aldrich) was administered twice daily via intraperitoneal injection 12 days after surgery (with saline as a vehicle) at a dose of 0.5 µg/kg for a period of 7 days.

4.2.3 Immunohistochemistry

Briefly, 50 µm coronal sections containing either the PFC or the hippocampus were cut, one slice in every 12 was collected and 4-5 slices per animal were used for analysis. Sections were incubated first in 1% H_2O_2 for 30 minutes and then in 1% sodium borohydride ($NaBH_4$) for 30 minutes to decrease background staining and then in 10% normal goat serum (NGS) for all antibodies for another 60 min to block non-specific antibody binding. Sections were incubated overnight at 4^{o}C in a mixture of primary antibodies and triton X-100 (Sigma Aldrich) [1% Triton for GAD-67 - 0.1% for all other antibodies]

made up in phosphate buffer solution (Primary antibodies are listed in Chapter 2). CBP, GAD-67, and NeuN immunostainings were carried out on coronal sections containing either the PFC or the hippocampus according to the immunoperoxidase protocol described in Chapter 2. Sections were dehydrated and mounted using DPX. The optical fractionator probe was used to determine the number of immunopositive neurones in both regions (StereoInvestigator software, MicroBrightField) using a Nikon microscope coupled to a computer-controlled x-y-z motorized stage and an MBF video camera system. Unbiased stereology was carried out on 4-5 slices per animal (*n* = 5 per experimental group) with the following parameters: counting frame 60 µm x 60 µm; grid size 200 µm x 200 µm; section thickness 50 µm, dissector height 12 µm. Only neurones with visible nuclei and dendrites were counted. Lastly, the neuronal quantification in both brain areas was performed including all layers.

4.2.4 Determination of the CA1-2-3 borders

The borders between the different hippocampal CA regions were defined using α-actinin-2, which is expressed solely in CA2, staining as previously described (Mercer et al., 2007; Botcher et al., 2014). 1 in 12 slices per animal were collected and processed for α-actinin-2 staining (4 slices per animal for all three experimental groups) and images of the staining were taken with the DMR microscope and Leica Application Suite V4 (Leica Microsystems) at 2.5x and 5x magnification, with constant video camera settings, light intensity, and microscope calibration. An example of the staining in the three experimental groups is shown in Figure 4.2a. Images of GAD-67 and CBPs staining were

then superimposed on the digital images of the α-actinin-2 staining in a section from the same frontocaudal depth using Corel Photo Paint X6 software to clearly define the borders of the CA2 region in each slice. Examples of this superimposition are illustrated in Figure 4.2b,c,d,e.

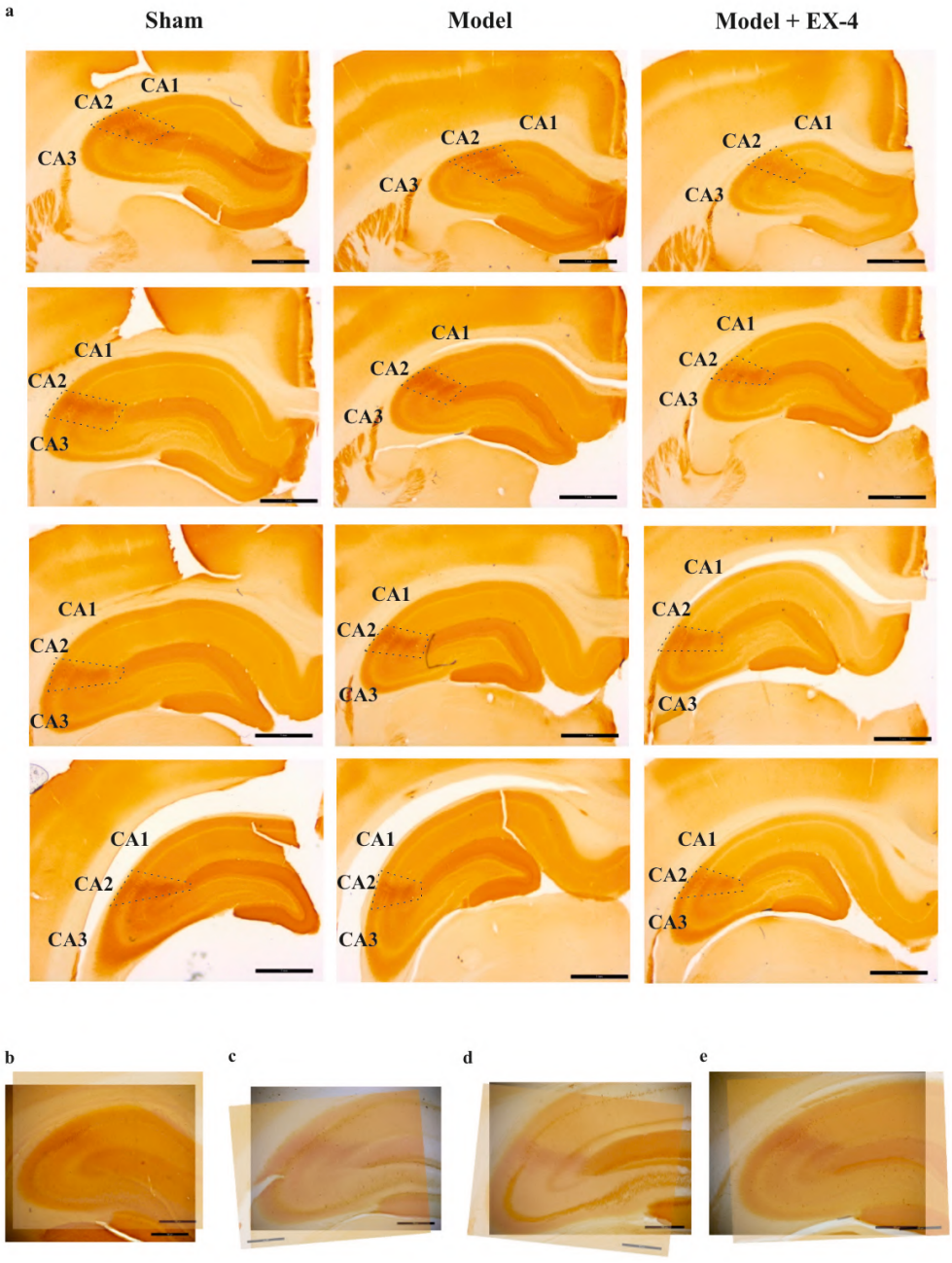

***Figure 4.2. Characterisation of the hippocampal CA2 borders**. **a)** Example of α-actinin-2 staining in each experimental group (1 in 12 slices - 4 slices per animal). The black dotted lines represent the CA2 region. Scale bars represent 1 mm. **b,c,d,e)** Examples of GAD-67 (b), PV (c), CB (d), and CR (e)-stained rat brain slices superimposed with corresponding α-actinin-2 stained rat brain slices, allowing to determine the borders of the CA2 area. Scale bars represent 500 µm.*

4.2.5 Statistical analysis

All data are presented as mean ± SEM. Results were analysed using One-way ANOVA and the Tukey post-hoc test was applied. Significance was set at $P < 0.05$.

4.3 Results

4.3.1 Decreased CBP interneurones in the PFC of the early-stage PD model.

Interneurones expressing the CBPs CB, CR, and PV were analysed in the PFC of the early-stage PD model and compared with those in control animals. Figures 4.3Aa-Ca show representative examples of the distributions of CBP-immunopositive interneurones for all experimental groups. The average numbers of both CB- and PV-positive interneurones in the PFC of the early-stage model were significantly decreased compared with controls (Figures 4.3Ab-Cb, One-way ANOVA $P < 0.05$). Treatment with EX-4 partially prevented the observed decreases compared with controls (One-way ANOVA, $P > 0.05$). In contrast, the average number of CR-expressing interneurones did not significantly differ between groups (Figure 4.3Bb).

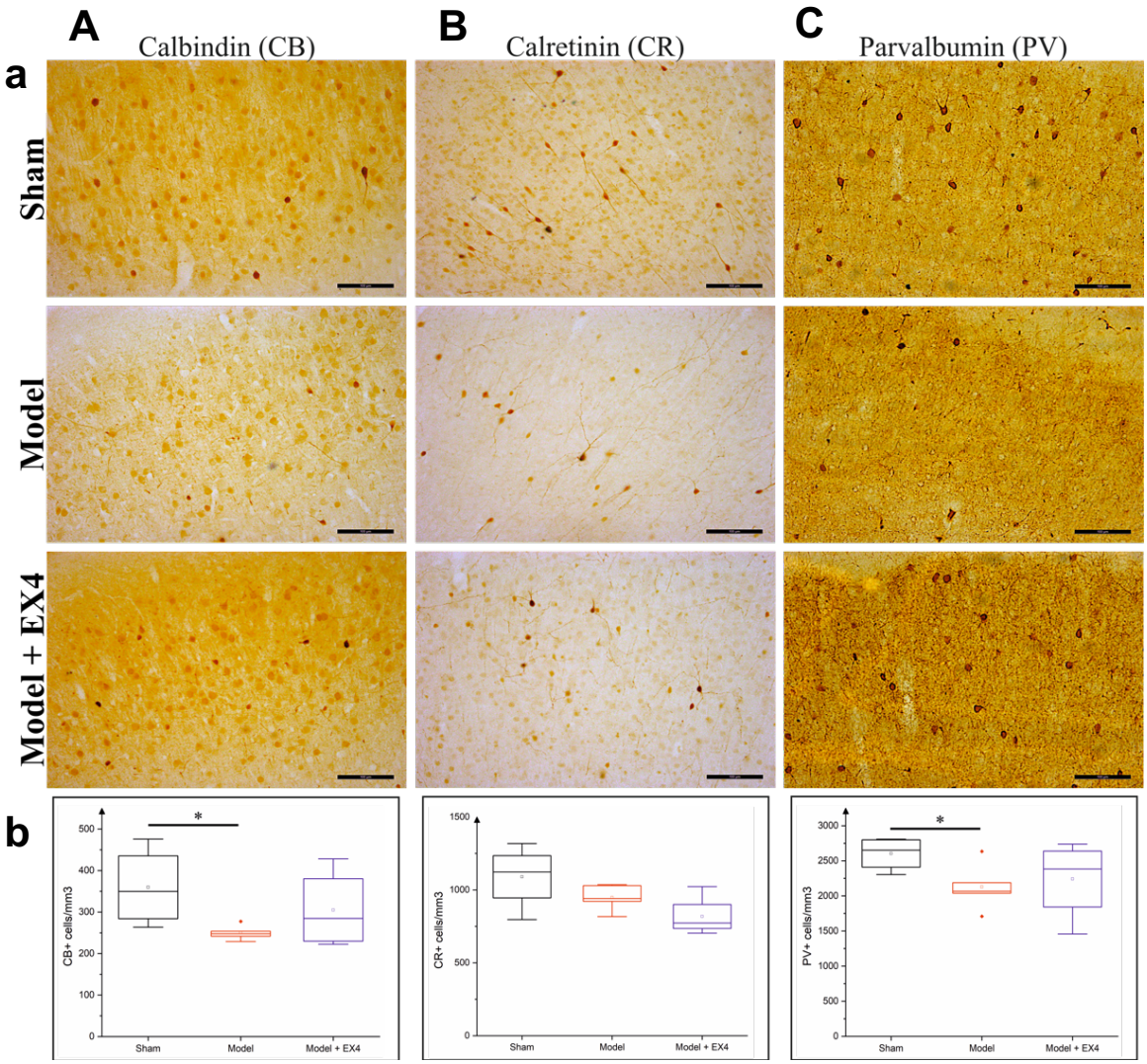

Figure 4.3 - Distribution of CBP-containing interneurones in the PFC. A *Distribution of calbindin-positive (CB+) interneurones, including examples of CB+ cells (**a**) and cell densities (**b**), in the PFC of all experimental groups. The number of CB-positive interneurones was significantly decreased in the early-stage model compared with sham animals.* ***B*** *Distribution of calretinin-positive (CR+) interneurones, including examples of CR+ cells (**a**) and cell densities (**b**), in the PFC of all experimental groups. The distributions were similar in all experimental groups.* ***C*** *Distribution of parvalbumin-positive (PV+) interneurones, including examples of PV+ cells (**a**) and cell densities (**b**), in the PFC of all experimental groups. A significant decrease in the number of PV-positive interneurones was observed in the early-stage model compared with sham animals. Treatment with EX-4 prevented both observed decreases. [One-way ANOVA, *P<0.05]. All data are presented as mean ± SEM. Scale bars represent 100 µm. Sham n=5, Model n=5, Model + EX-4 n=5.*

4.3.2 Cellular densities of GAD- and NeuN-immunopositive cells in the PFC of the early-stage PD model are unaltered.

The number of GAD-67-immunopositive interneurones was also analysed to determine whether the decrease in the number of CBP-containing interneurones in the PFC of the model was due to a down-regulation of the CBPs or to interneuronal death. The density and distribution of GAD-67-positive cells did not differ among the experimental groups, suggesting a down regulation of the CBPs in the model rather than a cellular loss (Figure 4.4Ab). The overall number of neurones in the PFC was also studied. The number of NeuN-immunopositive cells was not significantly affected in the model compared with controls (Figure 4.4Bb).

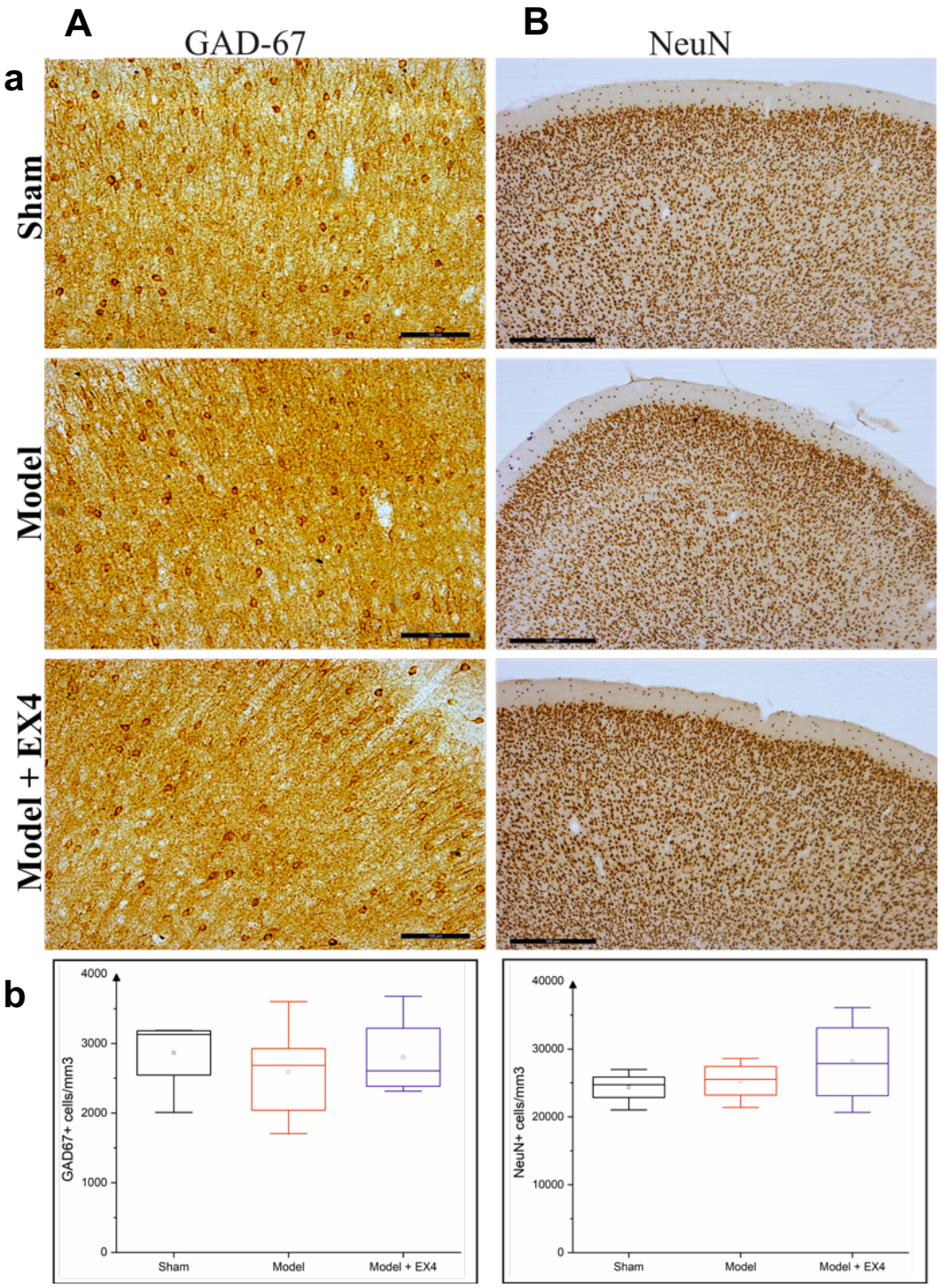

Figure 4.4 - Distribution of NeuN- and GAD67-immunopositive neurones in the PFC. A *Distribution of GAD-67-immunopositive interneurones in the PFC of the early-stage PD model.* ***Aa*** *Examples of GAD-67-immunostaining in the PFC of all experimental groups. Scale bars represent 50 µm.* ***Ab*** *Cell densities in the PFC. The distributions were similar in all experimental groups.* ***B*** *Distribution of NeuN-immunopositive interneurones in the PFC of the early-stage model.* ***Ba*** *Examples of NeuN-immunostaining in the PFC of all experimental groups. Scale bars represent 100 µm. Bb Cell densities in the PFC. The distributions were similar in all experimental groups. All data are presented as mean ± SEM. Sham n=5, Model n=5, Model + EX-4 n=5.*

4.3.3 Dual-toxin treatment led to an increase in CB-positive interneurones in CA1 of the early-stage PD model.

Interneurones expressing CBPs were analysed in each hippocampal CA region of the early-stage PD model and compared with those in control animals. Figures 4.5-7Aa-Ca show representative examples of the distributions of PV-, CB- and CR-immunopositive interneurones of all experimental groups in CA1, CA2, and CA3 respectively. Out of the three CBPs analysed, CB-positive interneurones in CA1 of theearly-stage model were the only CBP-positive interneurones in the CA1 region that showed a significant *increase* in number compared with shams (One-way $P < 0.05$, Figure 4.5Ab). Treatment with EX-4 partially prevented this increase (One-way ANOVA $P > 0.05$). Interneuronal densities in the CA2 region of the early-stage PD model were similar to those observed in sham animals (One-Way ANOVA $P > 0.05$, Figure 4.6Ab). However, treatment with EX-4 significantly *increased* the density of CR+ interneurones compared with the model (One-Way ANOVA $P < 0.01$) as well as of CB+ interneurones compared with both the model (One-Way ANOVA $P < 0.01$) and controls (One-Way ANOVA $P < 0.05$) (Figure 4.6Ab). Lastly, the distributions of CBP-expressing interneurones were similar in the CA3 region of the three experimental groups (Figure 4.7Ab).

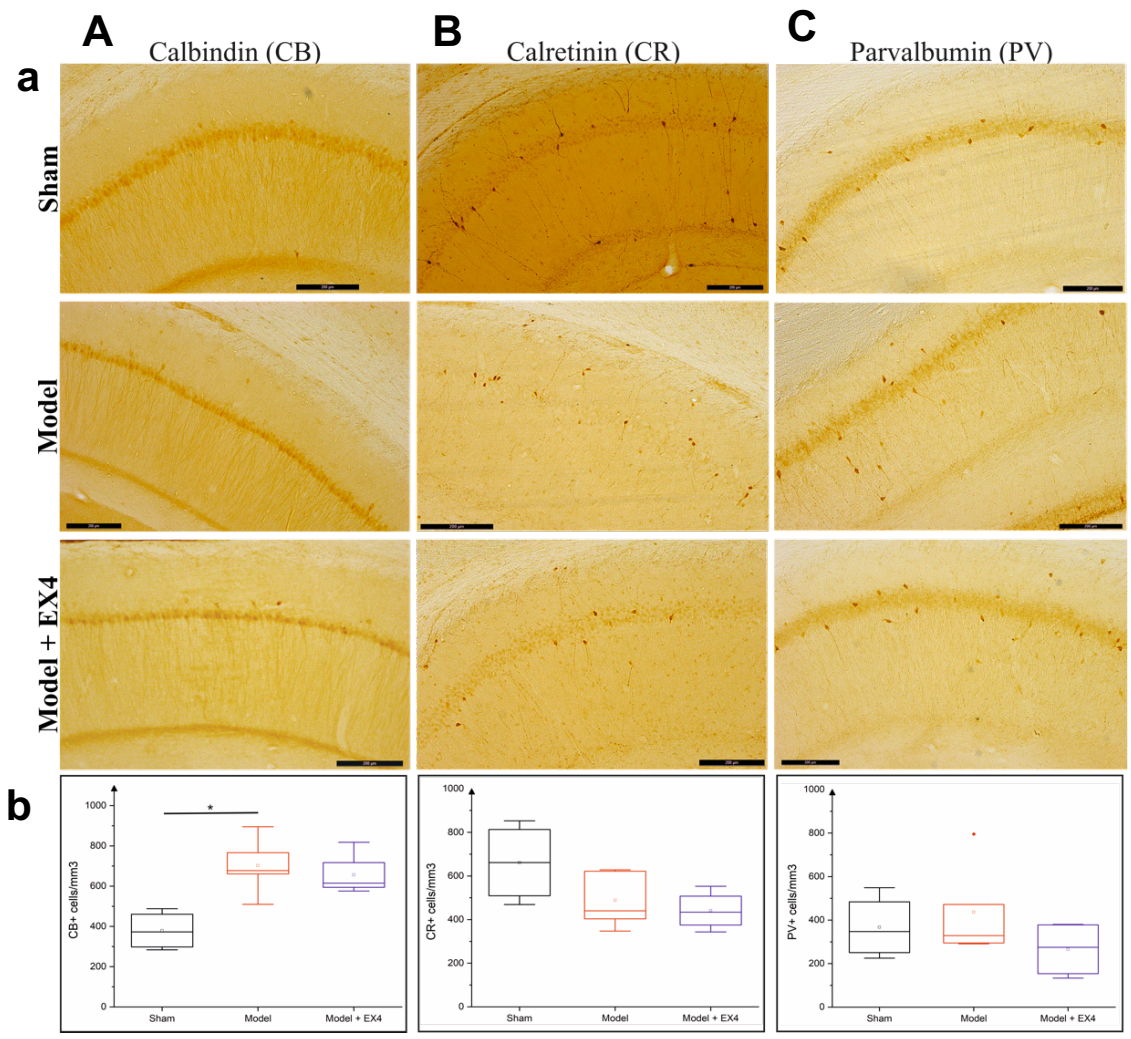

Figure 4.5 - Distribution of interneuronal CBPs in hippocampal CA1. A *Distribution of CB+ interneurones, including examples of CB+ cells (**a**) and cell densities (**b**), in CA1 of all experimental groups. The number of CB-positive interneurones was significantly increased in the early-stage model compared with sham animals. Treatment with EX-4 prevented the observed increase.* ***B*** *Distribution of CR+ interneurones, including examples of CR+ cells (**a**) and cell densities (**b**), in the CA1 of all experimental groups. The distributions were similar in all experimental groups.* ***C*** *Distribution of PV+ interneurones, including examples of PV+ cells (**a**) and cell densities (**b**), in CA1 of all experimental groups. *P<0.05. All data are presented as mean ± SEM. Scale bars represent 200 µm. Sham n=5, Model n=5, Model + EX-4 n=5.*

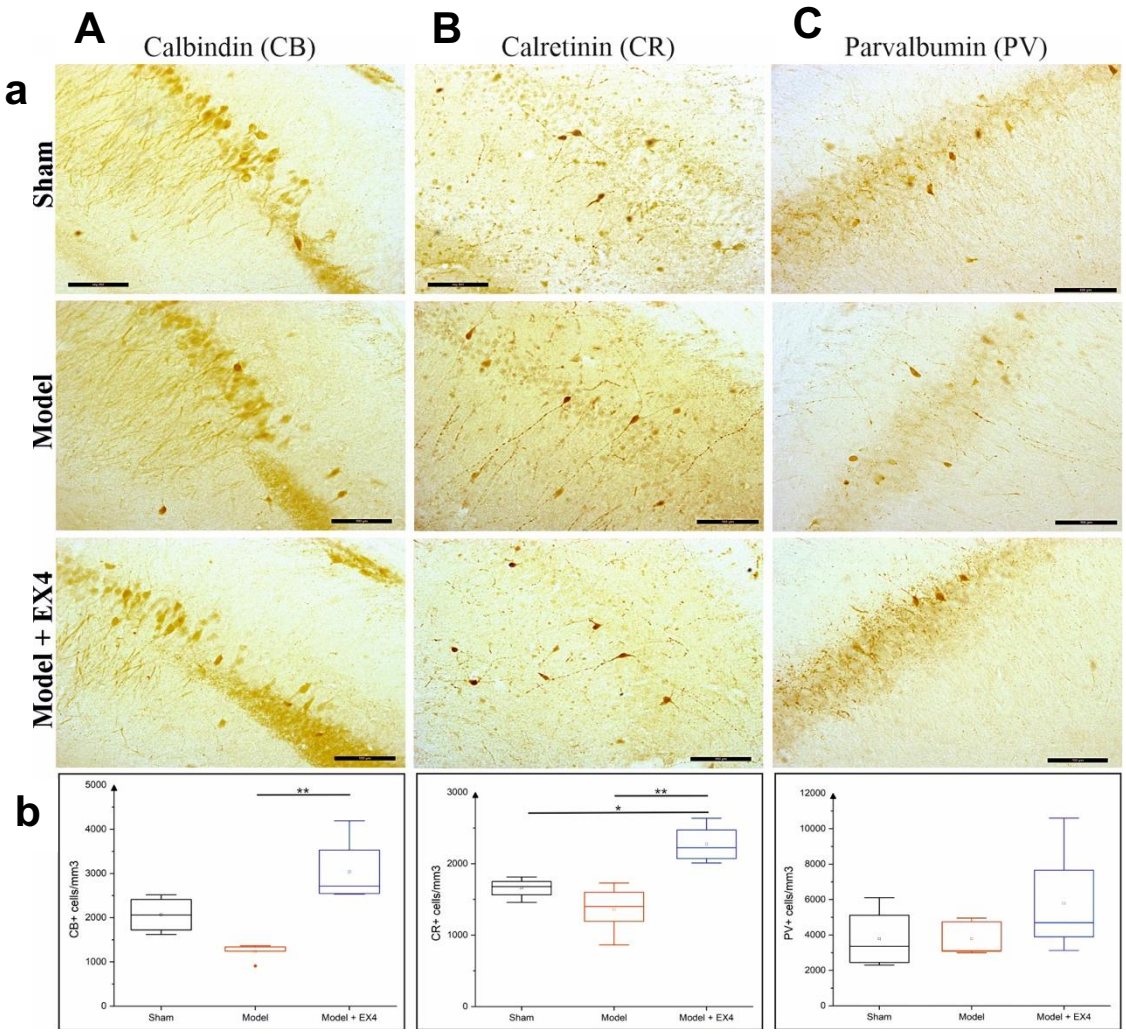

Figure 4.6 - Distribution of interneuronal CBPs in hippocampal CA2. A *Distribution of CB+ interneurones, including examples of CB+ cells (**a**) and cell densities (**b**), in CA2 of all experimental groups. An increase was observed in the model + EX-4 group compared with shams (One-Way ANOVA P < 0.01).* ***B*** *Distribution of CR+ interneurones, including examples of CR+ cells (**a**) and cell densities (**b**), in CA2 of all experimental groups. Treatment with EX-4 led to an increased density compared with both the model (One-Way ANOVA P < 0.01) and controls (One-Way ANOVA P < 0.05).* ***C*** *Distribution of PV+ interneurones, including examples of PV+ cells (**a**) and cell densities (**b**), in CA2 of all experimental groups. The distributions were similar in all experimental groups. All data are presented as mean ± SEM. Scale bars represent 100 µm. Sham n=5, Model n=5, Model + EX-4 n=5.*

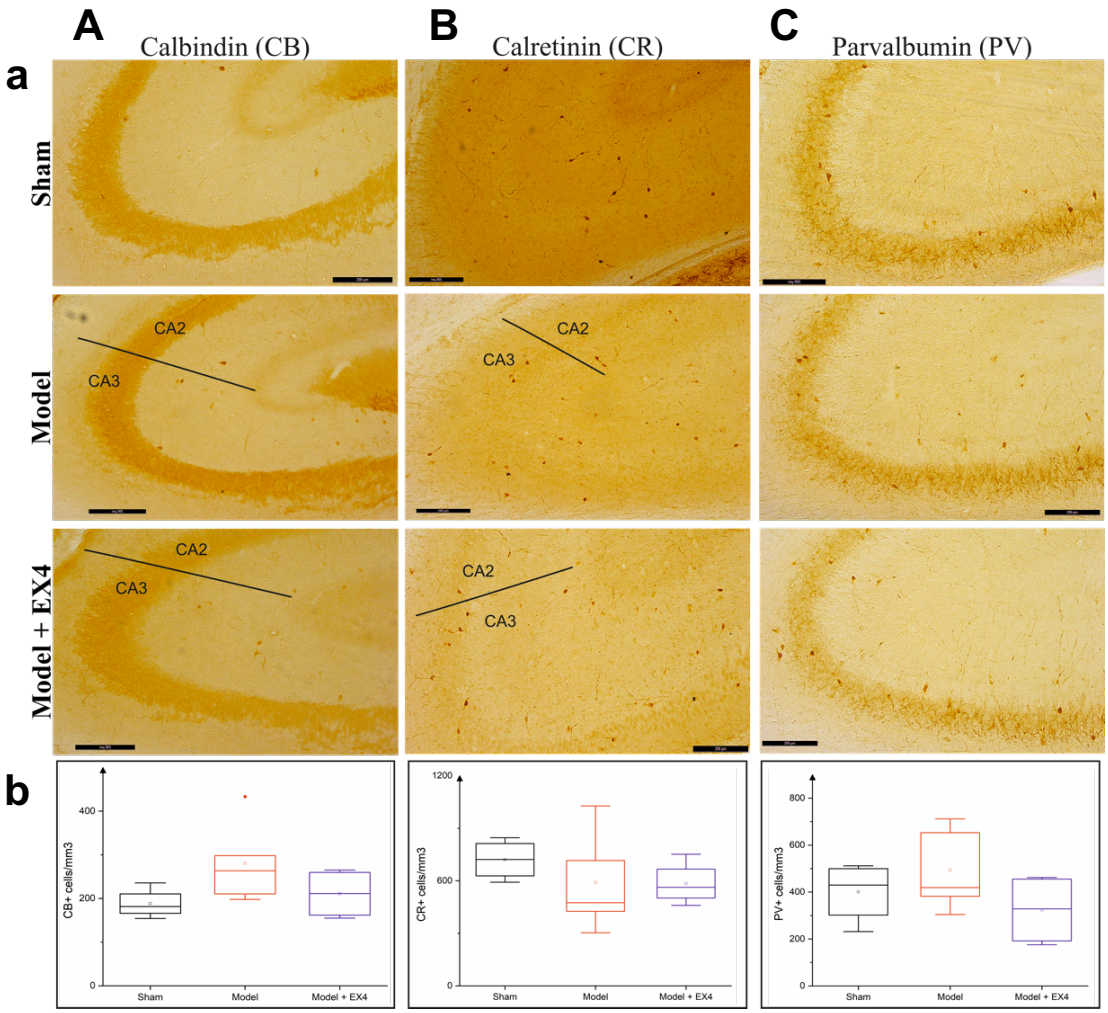

Figure 4.7 - Distribution of interneuronal CBPs in hippocampal CA3. A *Distribution of CB+ interneurones, including examples of CB+ cells (**a**) and cell densities (**b**), in CA3 of all experimental groups. No significant difference was observed between groups.* ***B*** *Distribution of CR+ interneurones, including examples of CR+ cells (**a**) and cell densities (**b**), in the CA3 of all experimental groups. Interneuronal cell densities were similar for the three groups.* ***C*** *Distribution of PV+ interneurones, including examples of PV+ cells (**a**) and cell densities (**b**), in CA3 of all experimental groups. The distributions were similar in all experimental groups. All data are presented as mean ± SEM. Scale bars represent 200 µm. Pictures in which borders are not highlighted show only the region of interest. Sham n=5, Model n=5, Model + EX-4 n=5.*

4.3.4 Reduced density of GABAergic interneurones in CA2 of the early-stage model.

Representative examples of GAD67-positive interneurones are shown in Figures 4.8-10Aa. The density and distribution of GAD-67-positive cells in both CA1 and CA3 did not differ among the experimental groups (Figures 4.8Ab and 4.10Ab). Surprisingly, CA2 was the only hippocampal region of the early-stage PD model in which GABAergic interneurones were significantly decreased compared with shams (One-Way ANOVA $P < 0.05$, Figure 4.9b). Treatment with EX-4 not only prevented the observed decrease in the model (One-Way ANOVA $P < 0.001$) but it induced a significant *increase* of GABAergic interneuronal density compared with sham animals (One-Way ANOVA $P < 0.05$, Figure 4.9b).

Similarly to the PFC, the overall number of neurones in CA1 and CA3 hippocampal regions was similar in the three experimental groups (Figures 4.8Bb and 4.10Bb). Figures 4.8Ba and 4.10Ba display NeuN+ neurones in both CA1 and CA3 of all experimental groups.

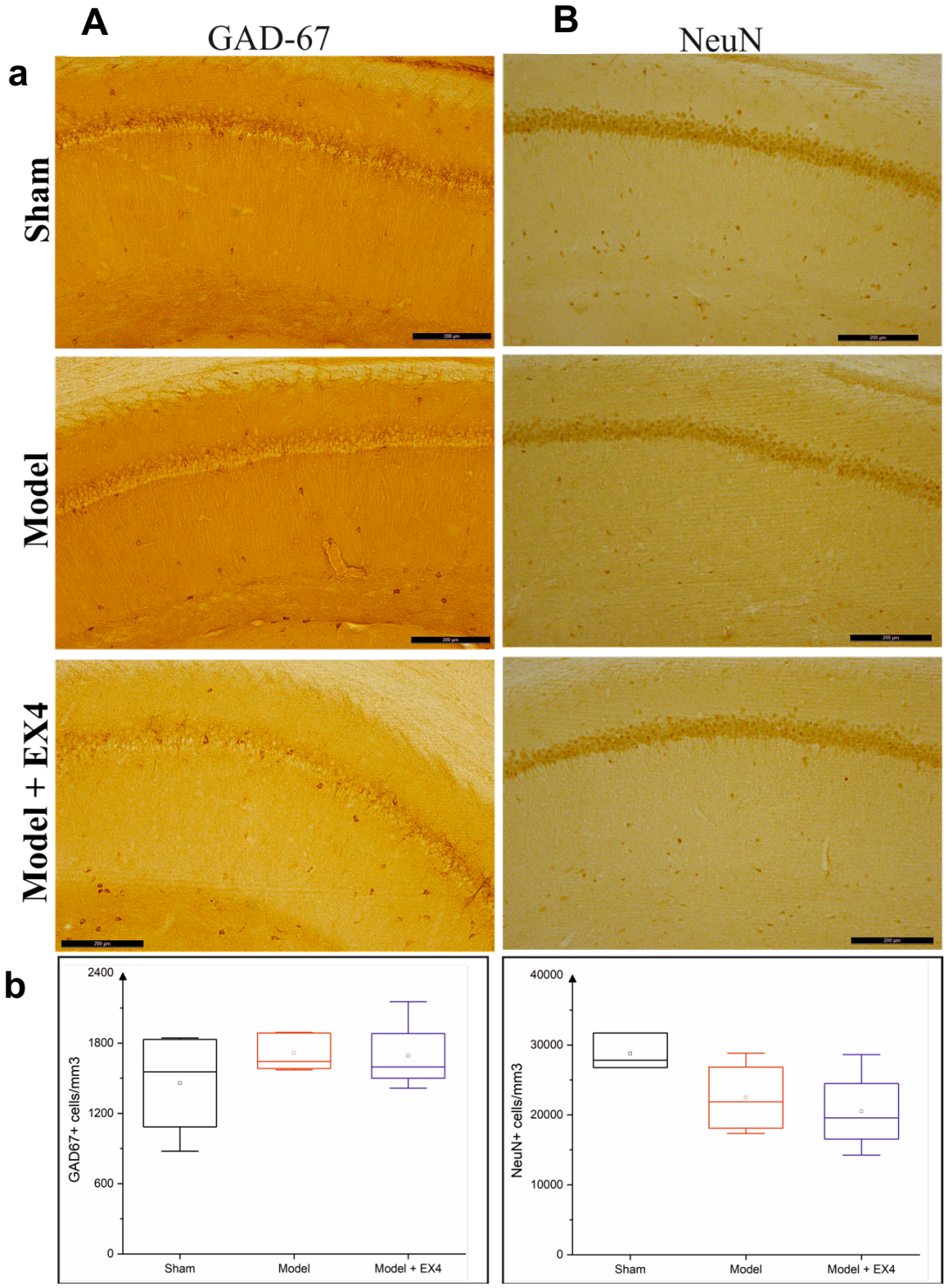

Figure 4.8 - Distribution of NeuN- and GAD-immunopositive neurones in hippocampal CA1. ***A*** *Distribution of GAD-67-immunopositive interneurones in CA1 of all experimental groups.* ***Aa*** *Examples of GAD-67-immunostaining.* ***Ab*** *Cell densities in CA1. The distributions were similar in all experimental groups.* ***B*** *Distribution of NeuN-immunopositive interneurones in CA1 all groups.* ***Ba*** *Examples of NeuN-immunostaining.* ***Bb*** *Cell densities in CA1. The density of NeuN-positive neurones in the early-stage PD model was similar compared with that in the sham animals. Scale bars represent 200 µm. Sham n=5, Model n=5, Model + EX-4 n=5.*

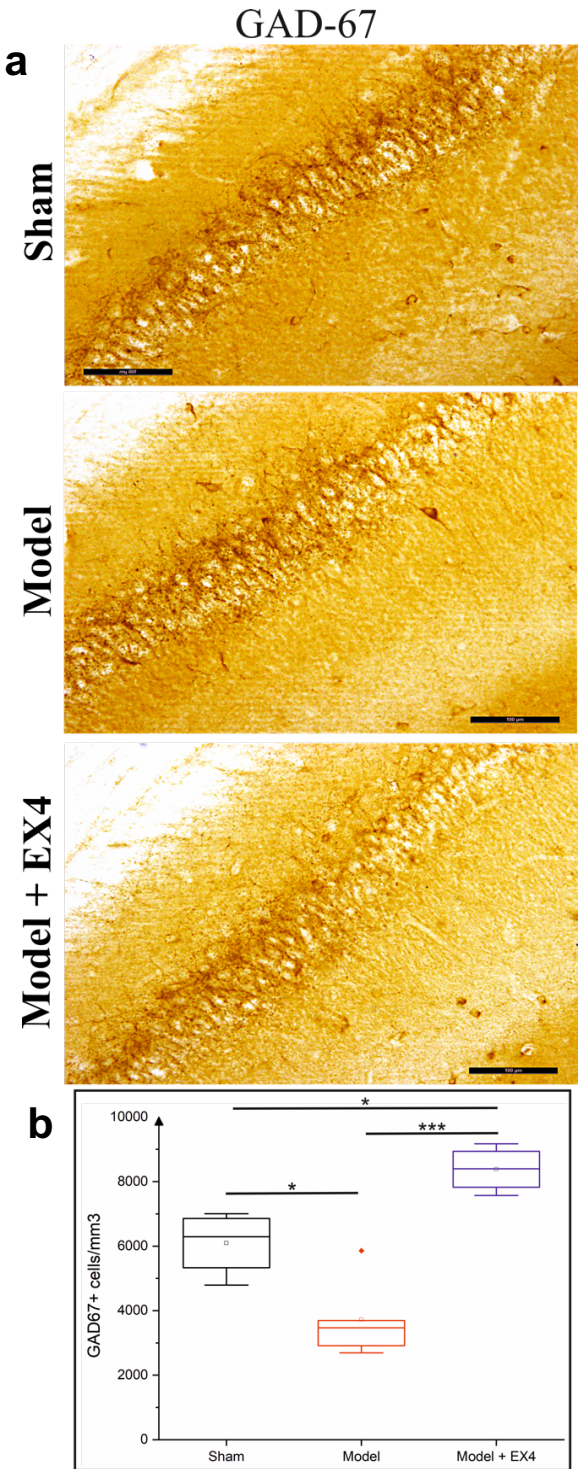

Figure 4.9 - Distribution of GAD-immunopositive neurones in hippocampal CA2 of all experimental groups. a *Examples of GAD-67-immunostaining.* ***b*** *Stereological quantification of GABAergic interneurones in CA2. A significant decrease in the early-stage PD model compared with controls was observed (One-Way ANOVA $P < 0.05$). The latter was prevented by treatment with EX-4 (One-Way ANOVA $P_{model\ vs\ model\ +\ EX\text{-}4} < 0.001$), which also produced a significant increase of GABAergic interneurones compared with shams (One-Way ANOVA $P < 0.05$). Scale bars represent 100 µm. Sham n=5, Model n=5, Model + EX-4 n=5.*

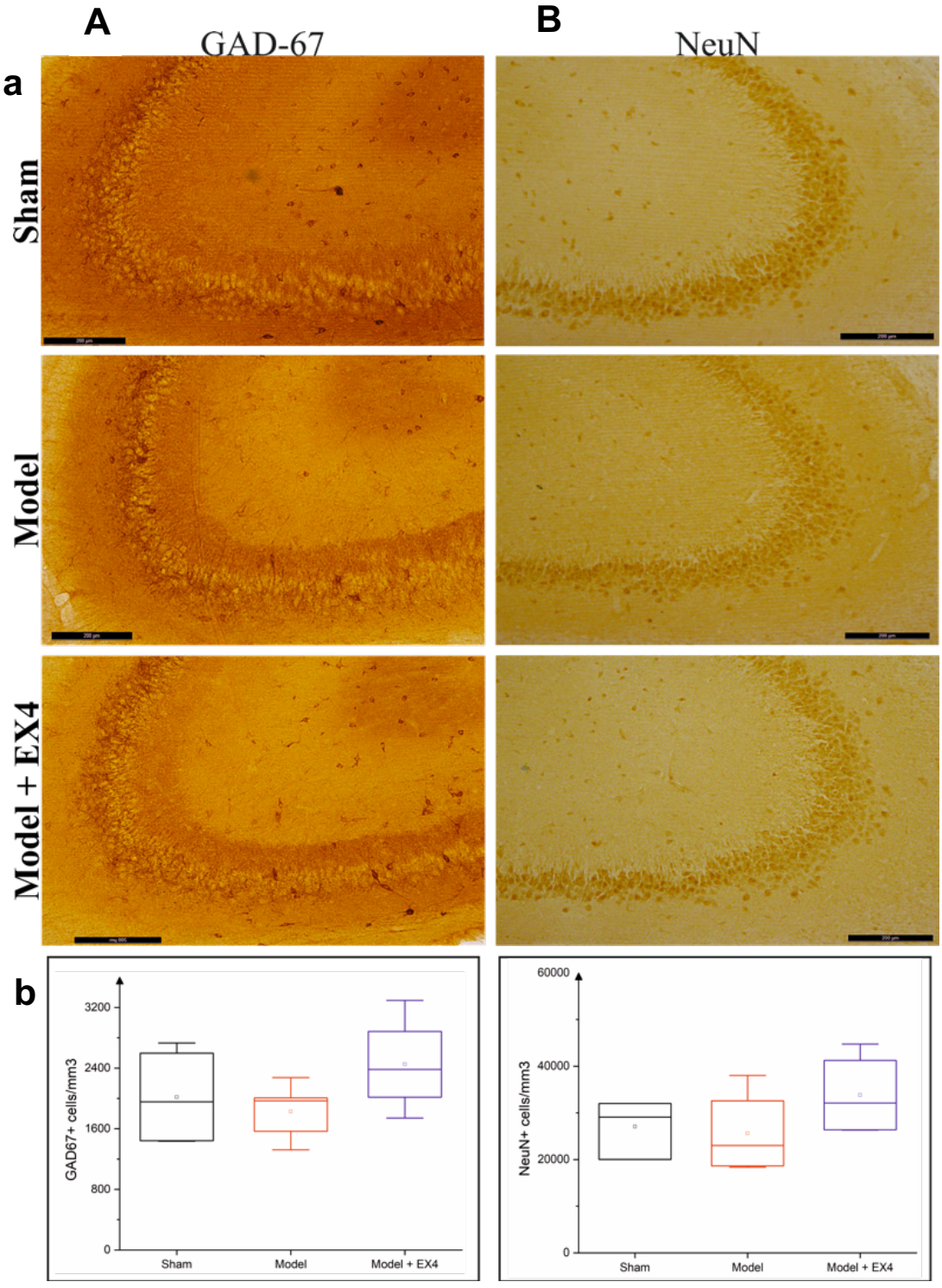

Figure 4.10 - Distribution of NeuN- and GAD-immunopositive neurones in hippocampal CA3. ***A*** *Distribution of GAD-67-immunopositive interneurones in CA3 of all experimental groups.* ***Aa*** *Examples of GAD-67-immunostaining.* ***Ab*** *Cell densities in CA3. The distributions were similar in all experimental groups.* ***B*** *Distribution of NeuN-immunopositive interneurones in CA3 all groups.* ***Ba*** *Examples of NeuN-immunostaining.* ***Bb*** *Cell densities in CA3. The density of NeuN-positive neurones in the early-stage PD model was similar compared with that in the sham animals. Scale bars represent 200 µm. Sham n=5, Model n=5, Model + EX-4 n=5.*

4.4 Discussion

The PFC and the hippocampus are key brain regions related to several higher cognitive functions as well as part of the extended olfactory circuitry. Given the deficits reported in the previous chapter in the PC and OB, and given the important roles these structures play in PD aetiology and progression, we investigated in this study whether both regions presented structural changes in the early-stage model. Similarly to the findings in the PC, we reported for the first time that the PFC of the early-stage PD model showed a significant decreased expression of CBPs despite an unchanged overall number of both GABAergic interneurones and neurones. These changes were partially prevented by treatment with EX-4. Additionally, a significant loss of GABAergic interneuronal population in the CA2 hippocampal region of the model was observed, whilst CBP expression was found to be unaffected by the dual-toxin treatment. EX-4 was able not only to prevent this loss in the early-stage model, but it led to a significant increase of GAD-67+ cells compared with shams.

4.4.1 Expression of CBPs is decreased in the PFC of the early-stage PD model.

The PFC is tightly linked to the olfactory pathway, receiving direct inputs from the primary olfactory cortex (Pitkänen *et al.*, 2000; Kadohisa, 2013). Similarly to the changes observed in the PC, a significant reduction in the expression of CBPs was reported in the PFC of the early-stage PD model for the first time. This result is in line with previous studies that investigated CBP-expressing interneuronal populations in neurodegenerative diseases (Satoh *et al.*, 1991; Lanoue, Blatt and Soghomonian, 2013). Specifically, PV mRNA expression

was shown to reduce without evidence of cell loss in the dorsolateral prefrontal cortex of PD patients, suggesting the downregulation of inhibitory neurotransmission in the frontal cortex (Lanoue, Blatt and Soghomonian, 2013). Similarly, Satoh *et al.* (1991) reported a significant decrease in both the number and size of PV-immunoreactive neurones in the CNS of Alzheimer's disease patients.

Despite the evidence of neuropathological abnormalities in frontal brain areas in PD, their molecular and cellular alterations are poorly understood (Murueta-Goyena *et al.*, 2019). As the PFC is responsible for higher cognitive functions, deficits in this region have been shown to lead to cognition impairments (Zgaljardic *et al.*, 2006), which are a predominant symptom of neuropsychiatric disorders. Hence, most of the pathogenetic mechanisms occurring in the PFC that may lead to cognitive dysfunctions, such as a decreased expression of CBPs, have been studied within the neuropsychiatric field, associating these deficits with disorders such as depression (Rajkowska *et al.*, 2007; Zadrozna *et al.*, 2011) and schizophrenia and bipolar disorder (Beasley *et al.*, 2002; Tooney and Chahl, 2004; Sakai *et al.*, 2008). Nevertheless, it was recently shown that cognitive dysfunctions in PD may be associated with a reduction in both expression and metabolism of dopamine D2 receptors (Christopher *et al.*, 2014; Li *et al.*, 2019; Fang *et al.*, 2020).

Although the reduction of CBPs expression in PFC and its functional implication has yet to be further investigated, some speculations may be proposed. For example, preliminary experiments showed that the reduction of CBP expression in the PC reported in the previous chapter is associated with neuronal hyperexcitability recorded in pyramidal neurones (data not shown).

Therefore, it is reasonable to assume that a similar disruption in neuronal excitability could characterise the PFC of our early-stage PD model. In agreement with this assumption, an enhanced GABAergic neurotransmission through zolpidem administration was observed following modulation of aberrant beta-frequency oscillations in early stage PD patients (Hall *et al.*, 2014), which is then thought to lead to the restoration of cognitive functions (Hall *et al.*, 2010). Additionally, PARK7 knockout mice, a genetic model of PD, displayed substantially higher PFC circuit activity recorded as spontaneous Ca^{2+} signals (Li *et al.*, 2019). In contrast, previous imaging studies conducted on late stage PD patients showed a hypoactivation of the PFC that was associated with poor performance on cognitive tests compared with controls (Cools *et al.*, 2002; Dirnberger, Frith and Jahanshahi, 2005; Jahanshahi *et al.*, 2010; Harrington *et al.*, 2011), highlighting the need for further research on potential alterations of the excitation/inhibition balance. Indeed, while these findings suggest a decreased GABAergic activity in PFC in early stage PD (Murueta-Goyena *et al.*, 2019), the presence of an abnormal GABAergic neurotransmission and its contribution to cognitive dysfunction in the early-stage stage has yet to been established.

4.4.2 CA2 of the early-stage PD model is the first hippocampal region to display structural deficits.

This chapter also focusses on another key brain structure involved in the cognitive aspect of smell processing: the hippocampus. This region was therefore analysed to investigate for similar deficits as in the PFC. In contrast to the data in the PC and PFC, injections of the toxins did not have any effect

on the distributions of PV- and CR-containing interneurones in CA1, CA2 and CA3. The number of CB+ interneurones in CA1 however, appeared to be increased compared with sham animals. Although the overall number of GAD67+ neurones in CA1 and CA3 was similar in the three experimental groups, a significant decrease in the number of interneurones in CA2 of the early-stage PD model was observed compared with shams. To our knowledge, this is the first study that investigated potential deficits associated with hyposmia in the hippocampus of an early-stage PD model.

Similarly to the PFC, experimental evidence on cellular and molecular deficits occurring in the hippocampus in early stage PD is elusive due to the key role played by this structure in both cognitive functions and spatial cognition and memory (Lisman *et al.*, 2017). Indeed, the majority of PD studies were performed on more advanced PD patients, evaluating these cognitive skills in a clinical setting, aiming to find a correlation between the disease stage and progression (Jordan, 2020). Nevertheless, studies conducted on PD animal models have reported an impaired hippocampal neurogenesis associated with both the presence of α-Synuclein aggregates (Desplats *et al.*, 2012) and an early serotonergic deficit (Kohl *et al.*, 2016). This reduction in neurogenesis is in turn thought to contribute to the pathogenesis of depression and cognitive decline in PD (Regensburger, Prots and Winner, 2014). However, it was recently observed that pharmacological agonism of D1 receptors exerted anxiolytic and antidepressant-like effects as well as enhanced neural-stem cell proliferation in a 6-OHDA PD animal model (Mishra *et al.*, 2019), suggesting that the impaired neurogenesis may also be a consequence of the dysfunctional dopaminergic transmission.

The finding that GABAergic cells in the early-stage PD model are affected only in CA2 is in agreement with the knowledge that CA2 is thought to be the first hippocampal area to be structurally affected in PD (Györfi *et al.*, 2017). Additionally, the CBPs analysed here were not significantly downregulated, suggesting that other GABAergic cells may be affected, such as somatostatin+ interneurones. Indeed, the mRNA level of somatostatin in GABAergic interneurones derived from iPSCs of PD patients were recently found to be significantly decreased (Iwasawa *et al.*, 2019). Nevertheless, more experiments are needed to explore the implications of this GABAergic loss and its implication in potential cognitive deficits in the early stage of PD.

4.4.3 Treatment with EX-4 partially prevents the observed deficits in the PFC and hippocampus

Similarly to the data reported in the previous Chapter, treatment with EX-4 was able to partially prevent the detected deficits in both the PFC and hippocampal regions. Specifically, treatment with the GLP1-R agonist resulted in a partial prevention of the CBP downregulation in the PFC and GABAergic loss in the CA2 hippocampal region. Although the mechanisms behind these protective effects remain unknown, the anti-inflammatory properties exerted by EX-4 are currently regarded as the primary mechanism of action for the observed beneficial effects on different diseases (Li *et al.*, 2015; Zhang *et al.*, 2016; Rocha-Ferreira *et al.*, 2018).

Interestingly, the density of GAD-67+ interneurones in CA2 of the early-stage PD model treated with EX-4 was significantly increased compared with controls. The treatment with EX-4, therefore, not only prevented neuronal loss,

but it also caused a significant increase in their number in this hippocampal region. This increase may be ascribed to the known effects of the GLP-1R agonist on neurogenesis in the adult brain (Hunter and Hölscher, 2012). Indeed, GLP-1R agonists are thought to activate the differentiation of neural stem cells into neurones (Kim, Kim and Song, 2020). EX-4, in particular, was shown to promote adult neurogenesis both *in vitro* and *in vivo*, while also normalizing dopamine imbalance in PD models (Bertilsson *et al.*, 2008). Additionally, a two-week treatment with EX-4 led to an increase in doublecortin, a key marker of hippocampal neurogenesis, in adult rodents (Isacson *et al.*, 2011). Lastly, the observed increase of GABAergic cells following EX-4 treatment is in line with the finding of an increased density of NeuN+ cells in the PC of the early-stage PD model treated with EX-4 reported in Chapter 3. However, further experiments are required to determine the full extent of potential effects exerted by EX-4 on neurogenesis in this PD model.

4.4.4 Summary and future directions

Continuing along the olfactory pathway, the present chapter investigated for potential deficits underlying hyposmia in two key regions involved in the cognitive aspect of smell processing: the PFC and the hippocampus. As these two regions receive inputs from the PC, focus was given to determine any potential changes in the early-stage model following toxin injections. We reported a decrease in the expression of CBPs in the PFC and a decrease in the density of GABAergic cells in the CA2 hippocampal region of an early-stage PD model for the first time. Treatment with EX-4 was not only able to prevent the observed deficits in both regions, in line with the results reported

in the previous Chapter, but it also led to a significant increase in GABAergic interneurones in CA2. The functional significance as well as the implications of these deficits/changes remain to be explored. Similarly, the potential mechanism underlying these changes, such as neuroinflammation, has yet to be investigated.

Chapter 5 - Gene therapy: a new potential treatment for PD

5.1 Introduction

Gene therapy is a modern technique that aims at treating or preventing serious diseases by inserting genes into patient's cells instead of using drugs or surgery (Deverman et al., 2018). This can be achieved in different ways. Firstly, a mutated gene that causes a disease may be replaced with a healthy copy of the interested gene. Secondly, a dysfunctional mutated gene may be inactivated or "knocked out". Lastly, a completely new gene may be introduced into the body to help fight a disease (Gonçalves and Paiva, 2017). Recently, gene therapy has been receiving growing interest since it aims to allow for durable clinical benefits with a single treatment compared with conventional drugs that may require repeated administrations (Kumar et al., 2016). Although the first clinical trials using gene therapy started in the early 1990s, it was not until the early 2010s that these trials resulted in substantial clinical progresses. This is due to maturation of the science behind gene therapy and a better understanding of target cells, alongside with development of new vectors, safety modifications, and improvements in gene transfer efficiency and delivery (Dunbar et al., 2018). Currently, retroviruses and adeno-associated viruses (AAVs) are the ones showing the greatest clinical success. The former was first used in clinical trials aimed at delivering a normal copy of a specific defective gene into the genome of T cells or hematopoietic stem cells of patients with immunodeficiencies or cancer (Dunbar, 1996). Indeed, retroviral vectors have the ability to integrate into the genome of the host cell making permanent genetic modification and long-term expression of the transgene (Matuskova and Durinikov, 2016). AAVs, on the other hand, are engineered from a non-pathogenic, non-enveloped parvovirus that does not

replicate, requiring the association with either an adenovirus or a herpesvirus in order to be able to replicate (Asokan et al., 2012). In addition, AAVs can be loaded with no more than ~5.0 kb of DNA, whilst retroviral vectors can carry up to 8 kb of DNA, allowing the insertion of larger molecules (Dunbar et al., 2018). Lastly, most of these vectors do not integrate with the DNA, however they become stable episomes that have the advantage of decreasing the risks associated with genetic integration and limiting long-term expression from AAV vectors to long-lived postmitotic cells at the same time (Deverman et al., 2018). Although gene therapy has vastly improved in safety and effectiveness over the past 10 years, and is currently a promising treatment option for a number of diseases, such as inherited disorders, cancer, and certain viral infections (Gonçalves and Paiva, 2017), the efficacy and potential long term side effects for a wider range of disease remains to be investigated. Thus, clinical trials based on novel gene therapy are currently run only for diseases with no effective cure (Kumar et al., 2016).

5.1.1 Gene therapy for PD

PD is a progressive, long-lasting neurodegenerative disease that has currently no cure, making it an ideal target for gene therapy. In addition, the representative symptoms of PD, like motor impairments, have been well characterised and linked to dysfunctions in brain areas that can be effectively targeted with viral vectors for gene transfer to brain cells (Coune, Schneider and Aebischer, 2012; Deverman *et al.*, 2018). Thus, part of the gene therapy strategy aims at increasing the efficacy and at reducing the side effects of the current pharmacological and surgical treatments (Jarraya *et al.*, 2009; Chen,

Lü and Li, 2014; Axelsen and Woldbye, 2018; Chakraborty and Diwan, 2020). However, one of the major challenges is designing an effective system that can deliver molecules to the brain and modify the disease by altering gene expression for prolonged periods, in such a way that the vectors are not impeded by the immune response (Izco *et al.*, 2019).

Recently, our knowledge of the genetics behind PD pathophysiology has greatly improved over the past years with the identification of familial forms of PD, identification of gene mutations responsible for these familial forms, and the mapping of risk variants for the disease (Poewe *et al.*, 2017). For example, five different missense mutations in the SNCA α-synuclein gene have been identified as potential causes of PD, which are associated with severe Parkinsonism at an early age and a rapid progression of the disease together with dementia, cognitive deficits, hallucinations and fluctuations of consciousness as common features (Ferreira and Massano, 2017). In addition, also duplications and triplications of the SNCA locus, which are more frequent than point mutations, have been linked to PD genesis (Nishioka et al., 2006). Mutations of the gene LRRK2, which produces the protein dardarin, have also been associated with PD pathogenesis (Zimprich et al., 2004), with an estimated frequency of LRRK2 mutations in hereditary PD of around 4% (Healy et al., 2008). Lastly, 79 different PRKN parkin mutations have been reported so far, which are not only involved in familial forms of PD, but also in sporadic cases (Ferreira and Massano, 2017). The clinical subtype associated with mutations of this gene ranges from early to late-onset, and dominant to sporadic, depending on the mutation point (Lohmann et al., 2003; Kay et al., 2007). Nevertheless, alterations of parkin are the most common cause of

autosomal-recessive early-onset parkinsonism, accounting for more than 60% of autosomal-recessive juvenile Parkinsonism (Ferreira and Massano, 2017). During the past few years, the field of gene therapies for PD has grown exponentially, with some therapies entering clinical trials (Jamebozorgi *et al.*, 2018; Merola *et al.*, 2020). Gene therapies for PD are classified according to their roles in targeting the disease progression or in treating symptoms. The former category aims at slowing down PD progression by targeting neurotrophic components such as neurturin, glial cell line-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF) (Cabezas et al., 2014, 2016, 2018), whilst the latter category focuses on restoring DA or GABA synthesis (Axelsen and Woldbye, 2018). Currently, there are four main types of gene therapy for PD that are being investigated and tested: AADC-TH-GCH gene therapy, RNA interference-based therapy, CRISPR-Cas9 gene editing system, and viral vectors-mediated gene delivery (Maiti, Manna and Dunbar, 2017; Axelsen and Woldbye, 2018). The AADC-TH-GCH gene therapy is based on the notion that in order to convert L-DOPA into DA, a three-enzyme system is required, involving aromatic amino acid decarboxylase (AADC), tyrosine hydroxylase (TH), and guanosine triphosphate cyclohydrolase (GTC). Specifically, TH and GCH catalyse tyrosine, converting it into L-DOPA, whilst AADC converts the L-DOPA into DA, allowing it to increase basal DA levels in advanced PD (Jarraya et al., 2009). In contrast, RNA interference is a physiological process responsible for the regulation of gene expressions that prevents viruses and other transposable elements to enter the genome (Agrawal et al., 2003). Therefore, gene therapies based on RNA interference operate by silencing defective genes through administration of small

interfering RNAs, preventing the expression of genes related to PD such as SNCA and parkin (Jamebozorgi et al., 2018). CRISPR-Cas9 is a gene editing system that is able to add, modify or degrade certain sequences of the nucleic acids at the genome level, making it a powerful tool to counteract PD pathogenesis and progression (Sander and Joung, 2014; Kolli et al., 2018). It was also recently employed to show that downregulation of PKCδ expression in DA neurones remarkably reduces apoptosis (Song et al., 2019). Lastly, viral vector-mediated gene delivery is based on the observation that several viral vectors, such as lentivirus, non-lentivirus, adeno-virus, and recombinant adeno-associated virus, can integrate within the host cell and induce specific gene expressions, promoting dopaminergic cell survival; hence, preventing degeneration of the dopaminergic system (Maiti et al., 2017; Jamebozorgi et al., 2018). Indeed, improvements in motor functions as well as increased dopaminergic terminals in the putamen were observed in a MPTP animal model following AAV2-GDNF vector injections (Eberling et al., 2009). Similarly, a phase I clinical trial on 12 PD patients demonstrated that AAV2-NRTN, an homologue to GDNF expressed in the substantia nigra and striatum, is safe and well-tolerated at 12 months (Marks Jr *et al.*, 2008). Moreover, the delivery of AAV-GAD (glutamic acid decarboxylase) vector in subthalamic nuclei was shown to increase GABA synthesis and restore balance of neuronal firing, resulting in a normalization of inhibitory signalling in PD patients (LeWitt et al., 2011). Additionally, the gene therapies reported above led to neuroprotection, suggesting they may be effective in halting or slowing down neurodegeneration in PD.

5.1.2 Background and aims of this chapter

PD is a progressive and debilitating neurological disease with no present cure, and, over the past years, several compounds have been suggested as potential new treatments, such as EX-4 which has been extensively discussed in the previous chapters. One disadvantage of the conventional EX-4 treatment is that EX-4 half-life is short, thus, in order to benefit from its neuroprotective effects, patients are required to undergo multiple injections throughout the treatment period. In order to overcome this issue, we hypothesised that a one-time delivery of a viral vector carrying the EX-4 gene would result in neuroprotective effects similar to what was observed following i.p. administration of EX-4. Therefore, by combining the known neuroprotective properties of this drug with long-term benefits of gene therapy, the present project aims at evaluating whether the injection of a novel AAV vector, that carries the gene for EX-4, can prevent neuronal loss following 6-OHDA administration. In contrast to the previous chapter, here only the 6-OHDA, and at a higher concentration, was used to induce neuronal loss. This is because the novel AAV vector has never been tested on an animal PD model before, hence, we first investigated its effects on the SNpc in a later stage of the disease than in the early-stage investigated previously. A GFP-tagged empty AAV vector was first injected stereotaxically in the striatum to visualise how the virus spread within the brain. Two routes of administration of the EX-4 gene loaded AAV vector were then tested: intravenously (i.v.) and stereotaxically in the brain. Additionally, in the former set of experiments, the viral vector was delivered two weeks prior 6-OHDA, to account for the time required by the viral vector to reach the brain and for neurones to start

expressing EX-4. In contrast, when delivered stereotaxically, the viral vector was co-injected with the 6-OHDA due to animal licence requirements. Both the effectiveness and the tolerability of the virus were investigated following both procedures via weight monitoring and quantification of the density of TH-positive cells in the SNpc through unbiased stereology.

5.2 Materials and Methods

5.2.1 Animals

Because the i.v. injection was performed two weeks prior to the stereotaxic surgery, two batches of Male albino Wistar rats, 100 g and 200 g respectively, were purchased from either Harlan Laboratories, Inc., UK or Charles River Laboratories, UK. Animals were housed in groups of 4 in the Biological Service Unit (BSU) of the UCL School of Pharmacy. Animals had *ad libitum* access to food (standard rodent diet) and water. Conditions of humidity (40-60%), temperature (18-22°C) and a 12 hr light-dark cycle (light phase from 7 am to 7 pm daily) were kept constant in the BSU in line with the Home Office regulations. All experiments were approved by the Bloomsbury AWERB and carried out in accordance with UK Home Office [and European Communities Council Directive of 24 November 1986 (86/609/EEC)] guidelines (PPL No. 70/8199 and PP3144142). Animals from the first batch were divided into 4 experimental groups: i.v. saline + saline (sham + i.v. sal; n=3), i.v. virus + saline (sham +i.v. virus; n=3), i.v. virus + 6-OHDA (model + i.v. virus; n=3), and i.v saline + 6-OHDA (model; n=5) (Figure 5.1A). The latter group was used to investigate the effects of 6-OHDA alone and will therefore be used as standard to compare the two routes of administration of the AAV vector. Animals from the second batch were divided into 3 groups: stereotaxic saline (sham; n=5), stereotaxic virus (sham + stereo virus; n=5), and stereotaxic 6-OHDA + virus (model + stereo virus; n=5) (Figure 5.1B). Also, two animals (200 g) were purchased and an empty GFP tagged viral vector was injected unilaterally in the striatum to note the spread of the virus. Only a small number of animals was used in this study due to the high costs of vector production as

well as because this study was intended to be a pilot study to assess the potential of the project.

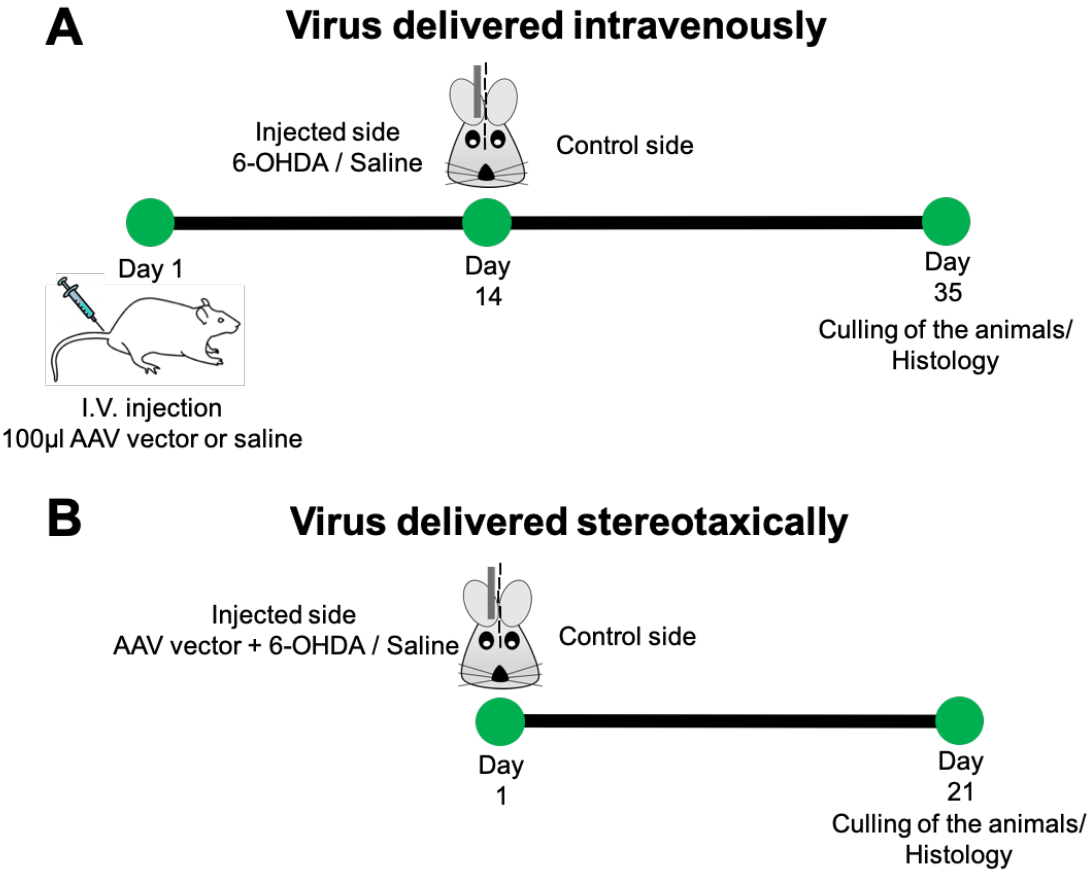

Figure 5.1 – Experimental timelines. A) The virus was delivered intravenously on day 1. After two weeks, animals underwent stereotaxic surgery to unilaterally receive either saline (Sham) or 6-OHDA (Model). Animals were culled 3 weeks following the surgeries. **B)** The viral vector was co-delivered unilaterally in the brain with either saline (Sham) or 6-OHDA (model) on day 1. Animals were culled 3 weeks later.

5.2.2 Compounds

The EX-4 viral vector was produced by Charité Universitätsmedizin Berlinand and the structure was cloned at the UCL School of Pharmacy by Prof Ahad Rahim. Based on previous studies (Tordo et al., 2018), 100 µl of the virus was administered intravenously, whilst 3 µl was injected into the striatum during

the stereotaxic surgery. 6-hydroxydopamine (6-OHDA) was purchased from Sigma (Sigma Aldrich, Gillingham, UK) and dissolved at a concentration of 10 mg/ml in saline solution containing 0.9% ascorbic acid and delivered unilaterally in the striatum. The optimum dose for 6-OHDA was chosen to induce partial reduction of DA levels based on previous studies (Sauer and Oertel, 1994).

5.2.3 Surgeries and drug administration

On day 1, animals from the first batch were anesthetised with isoflurane (5% v/v in O_2 for induction and 2% v/v in O_2 for maintenance delivered through a fitted anaesthetic nose mask) and then injected intravenously with either saline or the viral vector. After 14 days, rodents were anesthetised once again following the above-described procedure and were then placed in a stereotaxic frame to restrain the head and kept warm using a heating blanket. Note that Day 14 for this batch corresponds to Day 1 for the second batch of animals that received the virus stereotaxically. At this point, all animals received a subcutaneous injection of 0.2 ml of both non-steroidal anti-inflammatory drug Rimadyl (Zoetis) and the local anaesthetic bupivacaine hydrochloride (Marcain). The head was then shaved and the skull exposed to reveal the bregma, which is defined as the point of intersection of the sagittal suture with the curve of best fit along the coronal suture (Paxinos et al., 1985). One hole was then pierced on top of the striatum using the following coordinates: anterior-posterior (AP) +1.0 and medio-lateral (ML) + 3.0 from bregma; dorso-ventral (DV) -6.5), according to the atlas of Paxinos and Watson (Paxinos and Watson, 1982). The viral vector (3 µl) and either saline

solution or 6-OHDA-ascorbic acid solution (2 µl) were delivered in a total volume of 5 µl at a flow rate of 1 µl/min using a Hamilton 10 µl syringe which was slowly lowered to the correct depth with a micromanipulator. The needle was kept in place for a further 5 minutes to allow infusion and prevent efflux of the toxin. Additionally, 3 µl of the GFP-tagged empty viral vector were delivered using the same coordinates in two separate animals. The skin over the wound was then closed with sutures and 1 ml of saline solution was injected subcutaneously to compensate for potential fluid loss. The behaviour and weight of the animals were then monitored daily. Sutures were removed 7-10 days after surgery. Animals were culled 21 days after surgery and brains were harvested for further analysis.

5.2.4 GFP-tagged empty viral vector

Rats were anaesthetised by inhalation of isoflurane and intraperitoneal injection of Euthatal (Merial, Harlow, UK) (60 mg/kg) and then, as described previously in Chapter 2, perfused transcardially with ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing in mM: 124 NaCl, 25.5 $NaHCO_3$, 3.3 KCl, 1.2 KH_2PO_4, 1 $MgSO_4$, 2.5 $CaCl_2$, 15 mM D-Glucose equilibrated with 95% O_2/5% CO_2. Brains were then removed and fixed overnight (4% paraformaldehyde, 0.2% saturated picric acid solution, 0.025% glutaraldehyde solution in 0.1 M phosphate buffer). 50 µm coronal sections of different brain regions were cut. Slices containing the olfactory bulb, prefrontal cortex, striatum, motor and piriform cortices as well as SNpc were treated as described above and incubated overnight at 4°C in a mixture of rabbit anti-GFP antibody (1:2000, Sigma) and 0.1% triton X-100 (Sigma Aldrich) made

up in phosphate buffer solution. Next, sections were incubated overnight in an alexa-488 conjugated anti-rabbit secondary antibody (1:400). All fluorescently-labelled sections were then mounted on slides in Vectashield (Vector Laboratories) and studied using a DMR microscope and Leica Application Suite V4 (Leica Microsystems) at 20x magnification with constant light intensity, microscope calibration and video camera settings.

5.2.5 TH-immunohistochemistry and unbiased stereology

Briefly, animals were culled and brains were obtained as described above. 50 µm coronal sections containing the SNpc were cut from both hemispheres. One in every 6 sections was collected and 4-5 slices per animal were used per staining. Sections were incubated first in 1% H_2O_2 for 30 minutes and then in 1% sodium borohydride ($NaBH_4$) for 30 minutes to decrease background staining and then in 10% normal goat serum (NGS) for another 60 min to block nonspecific antibody binding. Sections were incubated overnight at 4^{o}C in rabbit anti-TH antibody (1:7500, Sigma) and 0.1% triton X-100 (Sigma Aldrich) made up in phosphate buffer solution. Following incubation in primary antibody, sections were incubated overnight in secondary antibody, biotinylated goat anti-rabbit antibody (1:500, Vector Laboratories) made up in PBS. To visualise the stained neurones, sections were washed in 0.1M PB and PBS, incubated in ABC (Vector Laboratories) for at least 2 hours, washed in TRIS buffer, and then in 3, 3' diaminobenzidine (DAB, Sigma Aldrich). The stained neurones were then revealed using H_2O_2. Sections from the different experimental groups were processed together using the same immunoreagents and the DAB reaction was stopped at the same time to allow

comparison between groups. Sections were then placed onto Superfrost slides, dehydrated, cleared with Histoclear and mounted using DPX (Sigma Aldrich). The optical fractionator probe was used to determine the number of TH-immunopositive neurones in both regions (StereoInvestigator software, MicroBrightField) using a Nikon microscope coupled to a computer-controlled x-y-z motorized stage and an MBF video camera system. Unbiased stereology was carried out on 5 slices per animal (n = 6 per experimental group) with the following parameters: counting frame 60 µm x 60 µm; grid size 200 µm x 200 µm; section thickness 50 µm, dissector height 12 µm. Only neurones with visible nuclei and dendrites were counted. Data are displayed as cell density per mm^3 ± SEM.

5.2.6 Statistical analysis

All data are presented as mean ± SEM. Results were analysed using One-way ANOVA and the Tukey post-hoc test was applied. Significance was set at $P<0.05$.

5.3 Results

5.3.1 Tolerability of the viral vector

Animals injected with the viral vector, both intravenously and stereotaxically, showed a weight gain comparable to the sham group and animals injected with empty vector (Figure 5.2). Animals injected i.v. with the viral vector started and ended at lower weights; however, the weight curve had a steepness comparable with the other experimental groups. Similarly, the steepness of the weight curve of the sham + stereo virus group was not significantly different (One-way ANOVA $P>0.05$) from the other experimental groups. Nevertheless, this group started as the heaviest and ended with an average weight lighter than sham.

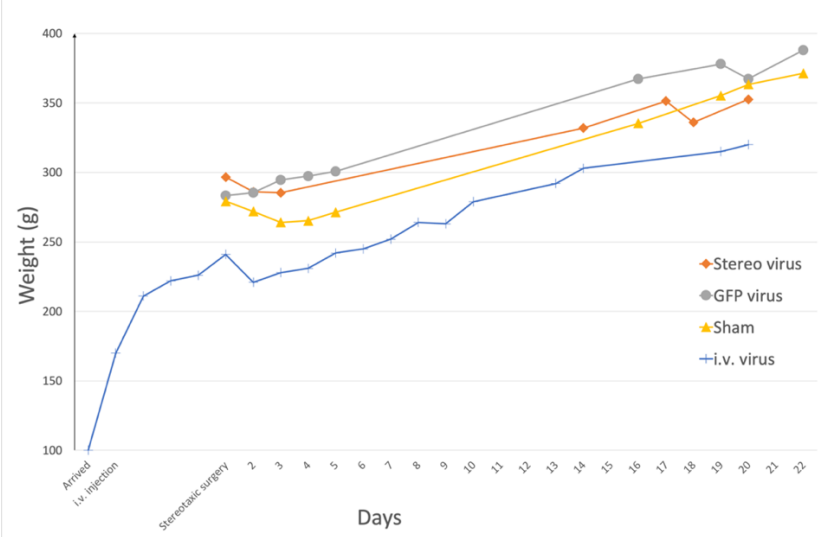

Figure 5.2 - Animal weight curves. Animals injected with the EX-4 loaded virus, both intravenously or stereotaxically, did not show any significant difference in weight gain over time compared with the other experimental groups. The i.v. virus group arrived in advance compared with the others due to the two weeks needed for the neurones to start producing EX-4 following the virus injection. A dip in weight can be noticed in the days following surgeries, this is normal and observed across all groups independently of the treatment received. Orange rhombuses: Sham + stereotaxic virus; Grey circles: Sham + GFP-tagged empty virus; Yellow triangles: Sham; Blue crosses: Sham + intravenous virus.

5.3.2 Virus spread following specific pathways

Figure 5.3 displays how the GFP-tagged empty viral vector spread within the brain following the unilateral stereotaxic injection in the striatum. The virus was found to be expressed in several regions of the injected hemisphere. Predictably, the striatum was the area with the highest concentration of the vector as shown by the high green fluorescence intensity. Interestingly, the vector travelled back from the striatum to the SNpc along the dopaminergic axons. In addition, the prefrontal cortex and the piriform cortex, which are areas rich in dopaminergic projections from the SNpc, were found to express the virus. Lastly, expression of the viral vector was also observed in the olfactory bulb, a region that is both anatomically and functionally connected to the piriform cortex. The virus was also expressed in the prefrontal cortex of the contralateral side suggesting that the viral vector was able to cross the corpus callosum.

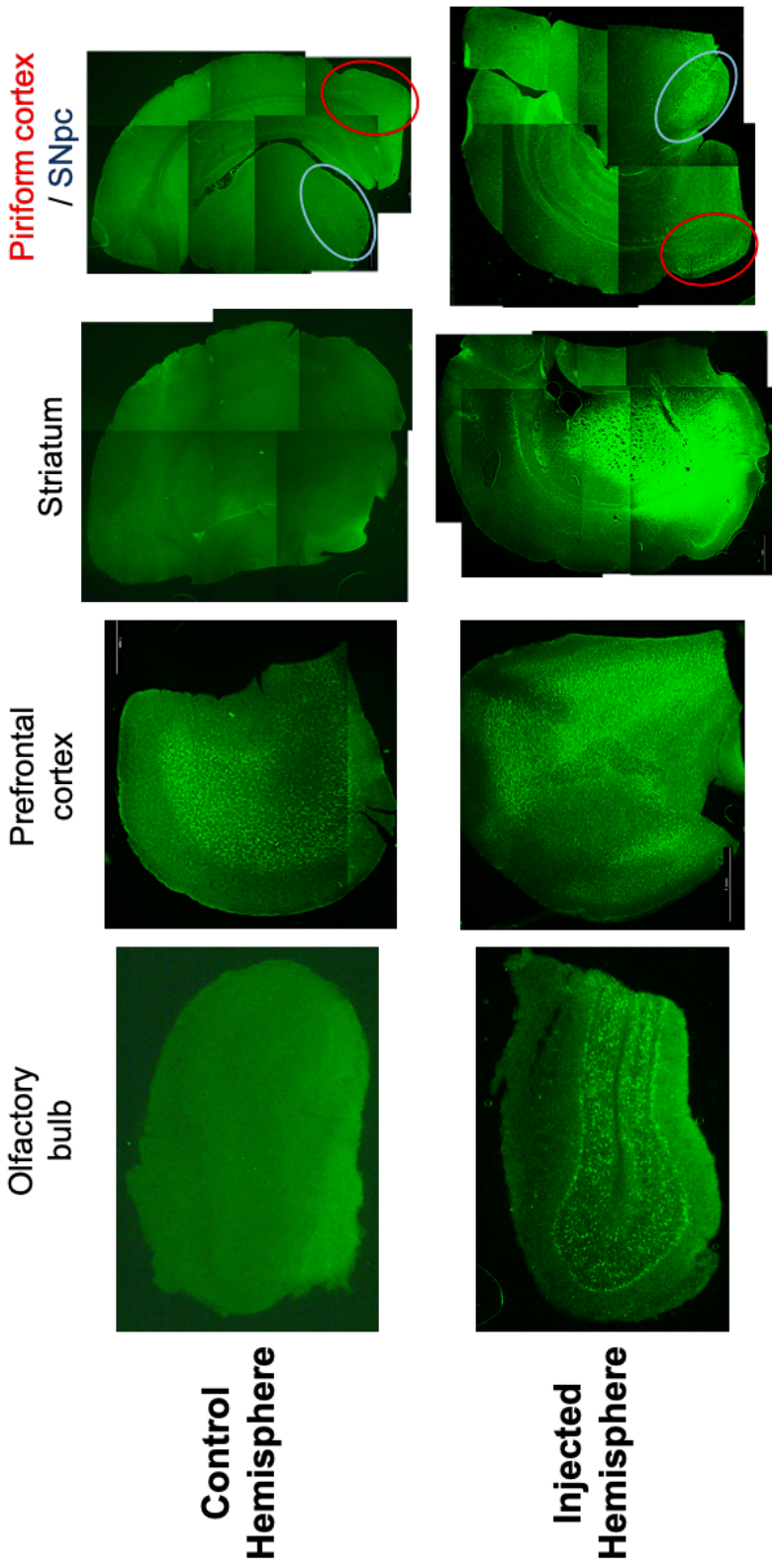

Figure 5.3 – GFP-tagged empty AAV vector spreading within the brain. In the control hemisphere, the virus is only expressed in the prefrontal cortex (Top panels). In contrast, in the injected hemisphere, expression of the virus was observed in the olfactory bulb, prefrontal cortex, striatum, piriform cortex (highlighted in red), and SNpc (highlighted in blue) (Bottom panels).

5.3.3 EX-4 viral vector delivered stereotaxically does not prevent dopaminergic loss in the SNpc

Figure 5.4A shows representative images of the TH-positive staining in the SNpc of the different experimental groups. Specifically, the top panels depict the control hemisphere, while the bottom panels represent the injected hemisphere. Unbiased stereology (Figure 5.4B) revealed that the density of dopaminergic cells in the SNpc of the injected side of the model was significantly reduced compared with controls ($P_{\text{model vs sham + i.v. sal}} < 0.01$; $P_{\text{model vs sham}} < 0.01$). Similarly, the density of TH-positive cells in the SNpc of animals that received both 6-OHDA and the virus stereotaxically (model + stereo virus) was significantly decreased in the injected hemisphere compared with that in the control groups ($P_{\text{model + stereo virus vs sham + i.v. sal}} < 0.01$; $P_{\text{model + stereo virus vs sham}} < 0.01$), and in both ipsi and contralateral sides of the animals that received saline and the virus stereotaxically (sham + stereo virus-$P<0.01$).

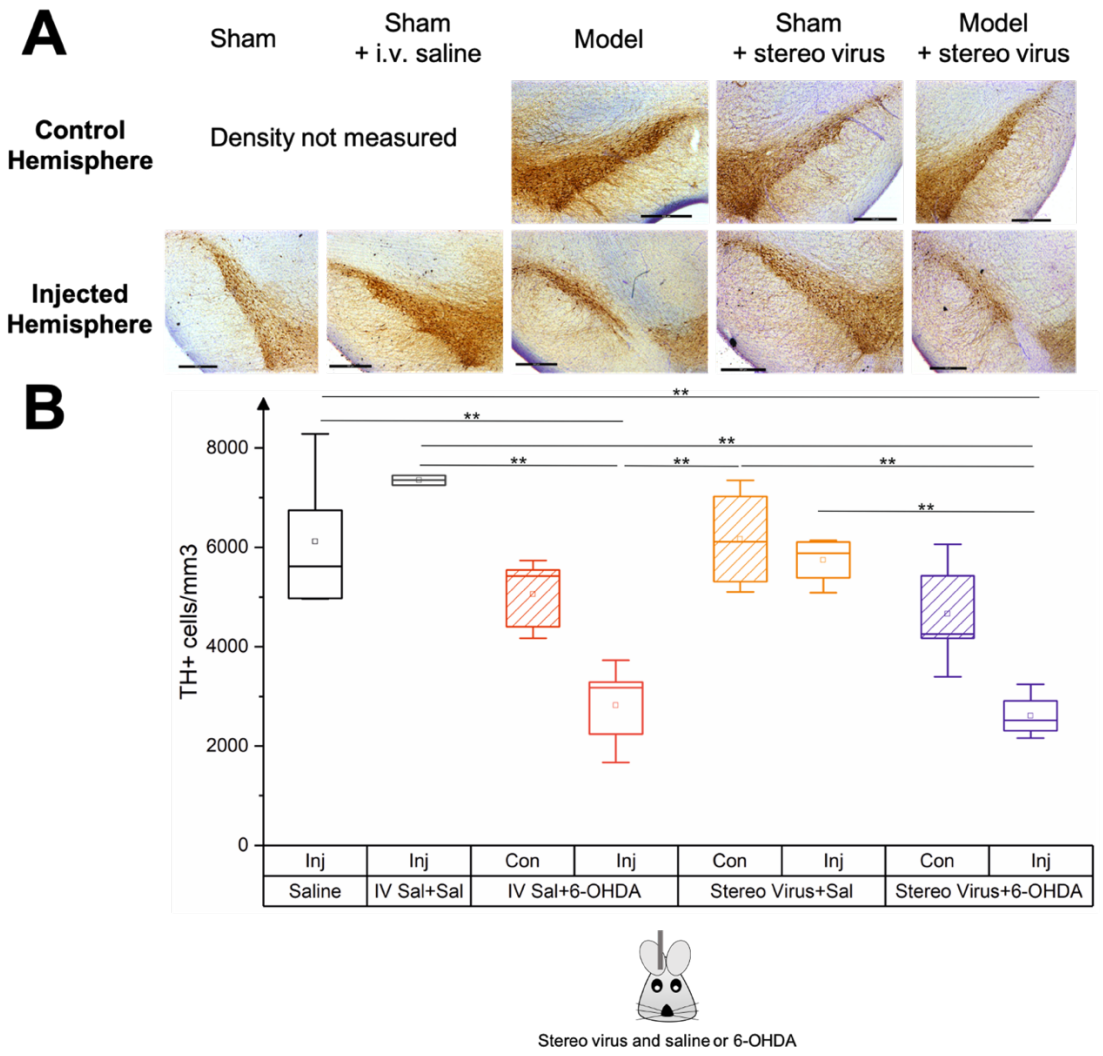

Figure 5.4 – EX-4 viral vector delivered stereotaxically does not prevent dopaminergic loss in the SNpc. A Representative images of TH staining in the SNpc of both the control hemisphere (top panels) and the injected hemisphere (bottom panels). Scale bars represent 500 μm. **B** Number of TH-positive cells in the SNpc expressed as cell density per mm^3 (n= 3 or 5 animals per experimental group). All data are presented as mean ± S.E.M. A decrease in the number of TH-positive cells was observed in the injected side of the model compared with controls (One-way ANOVA, $^{**}P_{\text{model vs sham + i.v. sal}} < 0.01$; $^{**}P_{\text{model vs sham}} < 0.01$). Similarly, the density of TH+ cells in the SNpc of the injected side of the model + stereo virus was significantly decreased compared with controls (One-way ANOVA, $^{*}P_{\text{model + stereo virus vs sham + i.v. sal}} < 0.05$; $^{**}P_{\text{model + stereo virus vs sham}} < 0.01$) and the injected side of the sham + stereo virus (One-way ANOVA, $^{**}P<0.01$), suggesting that the virus injected stereotaxically did not prevent neuronal loss.

5.3.4 EX-4 viral vector delivered intravenously prevents dopaminergic loss in the SNpc

Representative images of TH-immunopositive cells in the SNpc of the different experimental groups are shown in Figure 5.5A, with the top panels representing the control hemisphere and the bottom panels representing the injected hemisphere. The density of TH-immunopositive cells in the SNpc of the injected hemisphere of the model + i.v. virus was similar to that in the control groups ($P_{\text{model + i.v. virus vs sham + i.v. sal}} > 0.05$; $P_{\text{model + i.v. virus vs sham}} > 0.05$), whilst showing a significant *increase* compared with the injected side of the model ($P_{\text{model + i.v. virus vs model}} < 0.01$) (Figure 5.5B). Lastly, an unexpected significant *increase* in the density of TH-immunopositive cells in the SNpc was also observed in the injected side of the sham + i.v. virus compared with both hemispheres of the model ($P < 0.01$) but not with controls (Figure 5.5B).

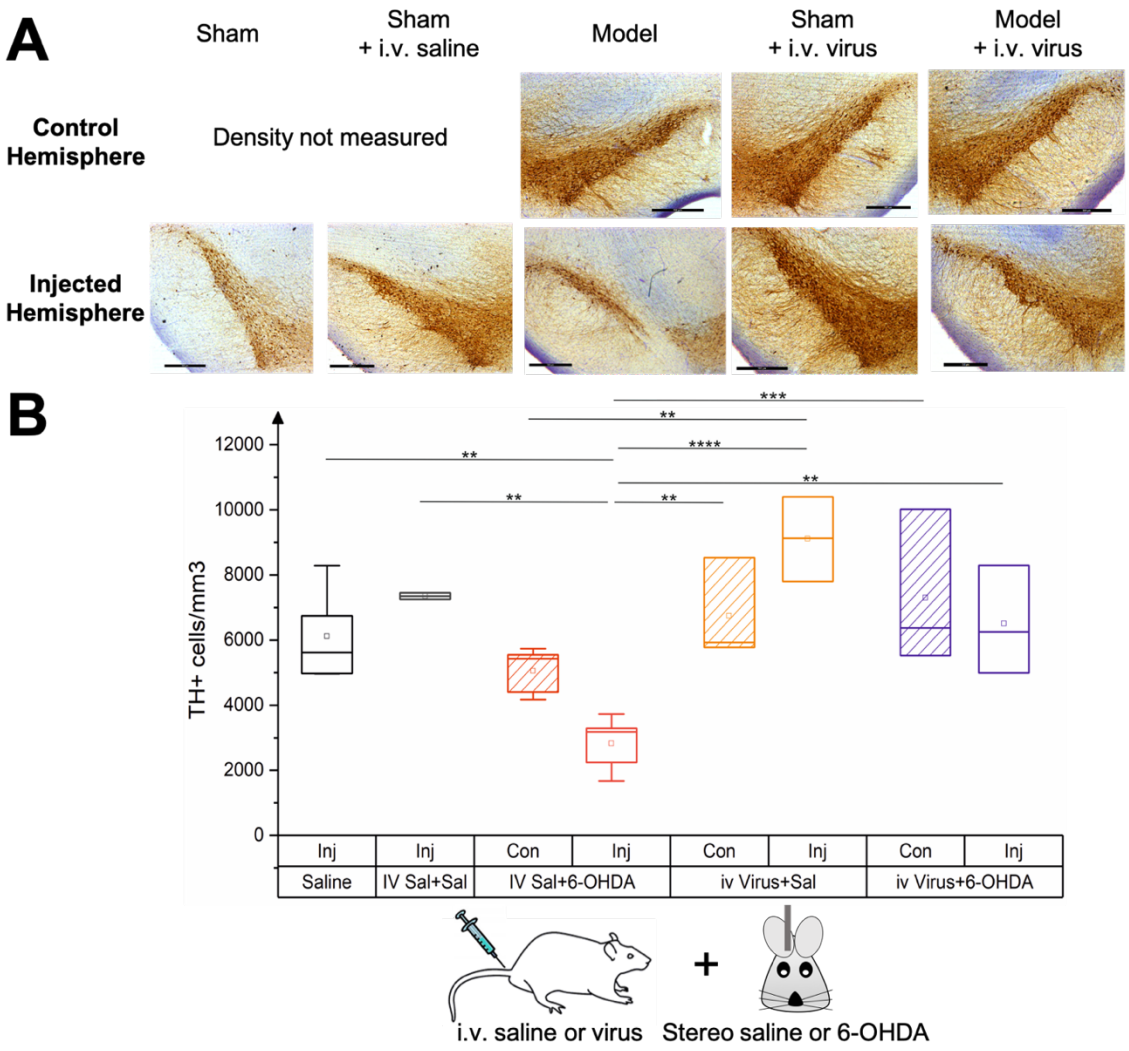

Figure 5.5 - EX-4 viral vector delivered intravenously *does* prevent dopaminergic loss in the SNpc. A Representative images of TH staining in the SNpc of both the control hemisphere (top panels) and the injected hemisphere (bottom panels). Scale bars represent 500 μm. **B** Number of TH-positive cells in the SNpc expressed as cell density per mm^3 (n= 3 or 5 animals per experimental group). All data are presented as mean ± S.E.M. Unlike stereotaxic treatment, intravenous treatment with the loaded virus prevented the dopaminergic loss in the SNpc of the model (One-way ANOVA, $P_{\text{model + i.v. virus vs sham + i.v. sal}} > 0.05$; $P_{\text{model + i.v. virus vs sham}} > 0.05$). Also, the SNpc of the injected hemisphere of animals that received the virus i.v. and stereotaxic saline showed a significant increase in the density of TH-positive cells compared with the model (One-way ANOVA, $P < 0.01$). **P<0.01, ***P<0.001, ****P<0.0001.

5.4 Discussion

The present chapter evaluated the effectiveness and tolerability of a novel AAV vector carrying the gene for EX-4, on an animal model of PD for the first time. Two different routes of AAV vector administration, intravenously and stereotaxically, were tested and compared to determine different therapeutics avenues. The virus was well-tolerated across animals, regardless of the route of administration. Lastly, the spread of the virus in several brain regions was determined by injection of the empty GFP-tagged viral vector. While the i.v. injection of the EX-4 virus effectively prevented dopaminergic neuronal loss in the SNpc observed in the PD model, the decrease in the number of TH+ cells was not prevented by stereotaxic injection of the loaded viral vector.

5.4.1 The virus travelled from the striatum to several other brain regions while being well-tolerated by the animals

In order to visualise in which areas the virus spread following injection, a GFP-tagged empty AAV vector was unilaterally injected into the striatum. Predictably, the area with the highest concentration of the virus was the striatum of the injected side. The virus then spread in the injected side to connected regions including the SNpc (Matsuda et al., 2009), the piriform cortex, the OB and the prefrontal cortex. The virus was also shown to be expressed in the prefrontal cortex in the non-injected side, suggesting that the vector can cross the corpus callosum. Similarly, Hutson and colleagues (2012) reported an AAV vector expression in the contralateral side after unilateral injection in the sensorimotor cortex of rats. The virus was therefore found to

be expressed, in addition to the injection site and its most prominent innervation site, mostly in regions known to be affected at the very early stage of the disease, specifically the olfactory bulb and the piriform cortex (Braak et al., 2003; Del Tredici and Braak, 2016; Engelender and Isacson, 2017; Perez-Pardo et al., 2017). Additionally, the fact that the virus was found to be expressed in the SNpc suggests that it may exert neuroprotective effects directly in this region rather than by triggering processes in other brain regions that then will result in preventing nigral cell loss although the mechanism of action remains to be further studied. The spread of the virus following intravenous injection also remains to be determined. Nevertheless, studies have shown that AAVs have the ability to cross the blood brain barrier when delivered i.v. (Gessler *et al.*, 2019).

The tolerability of the virus was assessed by comparing the animals' weight curves. This is due to the fact that GLP-1 has been shown to alter gastrointestinal function and decrease food intake (Gülpinar et al., 2000; Enç et al., 2001), while protracted treatment with EX-4 has been linked to substantial weight loss in human studies (Astrup et al., 2012). This is because EX-4 is a drug that was first developed for treating type II diabetes due to its functions in mediating glucose homeostasis through the stimulation of glucose-induced insulin secretion, insulin biosynthesis, and inhibition of glucagon secretion. Although the weight of animals injected i.v. with the viral vector was overall lower than the other experimental groups due to the 10 days gap between the start of the experiments, the steepness of the curve was comparable with controls, indicating that the animals did not suffer from weight loss following the gene therapy. Similarly, the steepness of the weight curve

of animals injected stereotaxically with the loaded virus was not significantly different from the other experimental groups. Nevertheless, this group were shown to be the heaviest at the beginning of the study and lighter than the sham animals at the end of the study, suggesting that the direct delivery of virus in the brain may exert different effects on glucose homeostasis. Lastly, also the GFP-tagged empty vector was well tolerated by the animals, which gained weight at the same pace as controls.

5.4.2 Intravenous injection of EX-4 viral vector prevented cell loss in the SNpc.

Interestingly, injecting the animals intravenously with the loaded virus resulted in a prevention of dopaminergic neuronal loss in the SNpc of the injected side. The exact mechanism of action by which the virus was able to prevent the damage induced by 6-OHDA has yet to be elucidated and was beyond the scope of this study. However, some speculations may be made. For instance, as mentioned in the previous chapters, systemic administered EX-4 was repeatedly found to restore DA imbalance, resulting in significant improvements in behaviour (Sancandi et al., 2018) and motor function in different PD animal models (Harkavyi et al., 2008; Kim, Moon and Park, 2009; Li et al., 2009). This effect has been ascribed to EX-4's ability, among many, to preserve mitochondrial function in dopaminergic neurones by increasing expression of complex I, the main target of 6-OHDA (Chen et al., 2015; Nassar et al., 2015). Additionally, EX-4 is also thought to indirectly upregulate both expression and activity of the TH enzyme (Kim et al., 1993), possibly providing an additional compensatory mechanism for the dopaminergic loss (Athauda

and Foltynie, 2018). Moreover, PD pathogenesis is thought to involve the gut-brain axis (Braak et al., 2003; Del Tredici and Braak, 2016; Engelender and Isacson, 2017; Perez-Pardo et al., 2017). Experimental evidence suggests that the enteric nervous system (ENS) is involved in PD genesis and progression towards the CNS, with different gut components playing a key role in the bidirectional gut-brain axis (Cryan and Dinan, 2012). Specifically, neurones of the ENS, together with those in the OB, are among the first ones to display Lewy bodies (LBs) (Del Tredici and Braak, 2016). Thus, it has been suggested that, after the immune system has been triggered, the detrimental process spreads via the vagal nerve and then olfactory tract, reaching the SNpc and other brain regions (Hawkes et al., 2009, 2010; Klingelhoefer and Reichmann, 2015). When the virus is injected intravenously, about 80% is taken up by the liver (personal communication by Professor Ahad Rahim), possibly resulting in EX-4 being produced by the peripheral nervous system as well as the CNS, which could then modulate insulin signalling both at the peripheral and central level. However, at the current stage of the project, it is not possible to determine if one effect exerts a stronger modulation than the other. Overall, It can therefore be speculated that both peripheral and central EX-4 might play a role in counteracting the genesis of the disease. This hypothesis could also explain the fact that stereotaxic injection of the AAV vector did not lead to the same effectiveness as intravenous injection of the virus. Indeed, the SNpc of the animals injected with 6-OHDA and virus showed a TH-positive density similar to the SNpc of animals treated with 6-OHDA only, implying that the virus did not prevent dopaminergic loss. However, this lack of effect of stereotaxic injection may also be due to other factors, such as the

volume injected or the time given to the virus to work, both of which could not have been enough, therefore, further studies are needed to confirm these results. Interestingly, i.v. injection of the loaded virus in sham animals led to a significant *increase* in TH-positive cells in the SNpc. Although the cause behind this increase remains unknown, other studies have reported similar results after injecting EX-4 systemically (Bertilsson et al., 2008; Harkavyi et al., 2008), and GLP-1R stimulation has been shown to indirectly upregulate both expression and activity of the TH enzyme (Kim et al., 1993), suggesting that treatment with EX-4 may result in an increased cellular density. Nevertheless, the investigation of the EX-4-induced increased TH expression was beyond the scope of this study.

5.4.3 Gene therapy limitations

Although gene therapies for neurodegenerative diseases are a promising tool, there are some general concerns, such as that the therapy is irreversible, and, in most cases, uncontrollable, whilst opto- and chemo-genetics, in contrast, can be applied when required and side effects may therefore be reduced (Chakraborty and Diwan, 2020). Firstly, the route of delivery represents a key factor for the success of any gene therapy clinical trials, considering the need to overcome the blood-brain barrier. One solution is to use vectors with inherent ability to cross the blood-brain barrier, such as AAV9, allowing for i.v. injections. However, this option requires high titers of more efficient vectors to achieve sufficient CNS distribution, which in turn increases the possibility of immune reaction and hepatotoxicity (Hinderer *et al.*, 2018). An alternative route of administration is intranasal, an attractive non-invasive delivery route

for vectors, oligonucleotides, and other biomolecules in a variety of neurologic disorders, including PD (Aly *et al.*, 2019). Nevertheless, intranasally delivered viral vectors demonstrated limited brain distribution, which is of concern for treating deeper brain structures typically targeted in PD (Merola *et al.*, 2020). Currently, several new approaches are being developed, such as nanoparticles and nanocarriers, to improve brain dispersal and to make gene therapy a more viable treatment option. The interplay between volume, optimal dosing and target coverage once the viral vector is efficiently and effectively delivered in the brain will then become the next obstacle to surmount. Some PD gene therapy clinical trials have highlighted the importance of delivering larger volumes to improve vector coverage of targeted brain regions (Christine *et al.*, 2019; Heiss *et al.*, 2019). Indeed, initial phase I studies infused between 40 and 450 µL, however, because the average putaminal volume is over 4000 µL, such small infusions only covered 5-25% of the target area (Merola *et al.*, 2020). Currently, gene therapy clinical trials opt for an average volume of administration of around 1800 µL per hemisphere in order to yield 50-80% putaminal coverage (Christine *et al.*, 2019).

5.4.4 Limitations of the study

The experiments presented in this study are the first step in evaluating the effectiveness of the AAV-EX-4 vector. These promising results however present several limitations. Firstly, the choice of intravenous and stereotaxic doses were based on previous studies that were carried out on mice. The dose used in stereotaxic experiments in rats may therefore not be sufficient enough to lead to neuroprotection in the SNpc. Secondly, the virus is expected to be

fully operative after around two weeks (personal communication by Prof Ahad Rahim, UCL - SoP) and this may explain the lack of efficacy from the stereotaxic injections as the virus was injected at the same time as the toxin and the positive results that were observed in animals that received i.v. injections. Additionally, as previously explained, the reason behind the neuroprotective effects observed with the i.v. delivery may be the result of the virus acting in the central as well as in the peripheral nervous system. Lastly, it should be noticed that these are preliminary experiments aimed at understanding if this new AAV-EX4 viral vector has the potential to help in PD progression. Therefore, the initial number of animals used is very low, with the i.v. group comprising just three animals. Hence, a larger sample size may lead to different results, especially regarding the virus injected stereotaxically.

5.4.5 Summary and future directions

The present study revealed for the first time, that i.v. injection of an AAV vector carrying the gene for EX-4 two weeks prior to 6-OHDA insult resulted in neuroprotection of dopaminergic cells in the SNpc of rats. However, this effect was not observed when the virus was given stereotaxically. This is of a particular interest, since treatment with the AAV vector may potentially allow PD patients to avoid the distressing injections required during conventional treatment with EX-4. In addition, it is also important to note that the easier i.v. administration route, clinically speaking, was also the most effective and well tolerated in these experiments.

The tolerability and shown effectiveness of the viral vector represent a promising outcome, however, further studies are required to further assess the

safety and the mechanism of action of this treatment. Indeed, although animals did not suffer from weight loss, further studies on the presence of neuroinflammation following AAV treatment are necessary. Additional experiments will also be required to study the effect of the stereotaxic delivery further. It would be of interest to determine whether a higher AAV dose may prevent neuronal loss, or whether any neuroprotective effects may be observed by extending the study and allowing the virus to have an effect for a longer period of time prior to culling the animals. Furthermore, the actual amount of EX-4 produced in the brain and the type of cells expressing the virus are currently unknown and this information will be required to determine the long-term prognosis and safety. Lastly, investigation of the spread of the virus following i.v. injection and the effect of this treatment on the liver will also be needed.

Chapter 6 - From the gut to the brain: Effects of Probiotics on early stage PD

6.1 Introduction

For several decades since its discovery, Parkinson's disease (PD) was thought to be a brain disorder mainly characterised by loss of dopaminergic (DA) neurones in the Substantia Nigra pars compacta (SNpc) (Borghammer and Van Den Berge, 2019). Recently, however, new experimental evidence emerged suggesting that PD is a multifactorial disease, involving not only the central nervous system (CNS) but also the peripheral nervous system (PNS) (Parashar and Udayabanu, 2017; Tan, Hor, *et al.*, 2020). Additionally, interest in the role of non-neurological factors in PD pathogenesis has recently increased as only around 10% of PD cases are due to genetic causes (Dutta *et al.*, 2019). Epidemiological evidence observed that Lewy bodies (LBs), which are pathological α-synuclein aggregates, were found in the enteric nervous system (ENS) of PD patients up to 20 years before the diagnosis (Svensson *et al.*, 2015; Liu *et al.*, 2017). Moreover, animal studies have shown that LBs can efficiently travel through the vagus nerve (Ulusoy *et al.*, 2013, 2017; Holmqvist *et al.*, 2014). Therefore, an increasing number of studies in recent years have taken interest in the role that the gut may play in PD pathogenesis, suggesting that the maintenance of a healthy gut may lead to potential treatments of the disease (Kim *et al.*, 2017; Parashar and Udayabanu, 2017; Dutta *et al.*, 2019; Gazerani, 2019; Castelli *et al.*, 2021).

6.1.1 The gut-brain axis

The close proximity between the gastrointestinal (GI) tract, which is the largest body surface area exposed to antigens coming from the external environment

and harbours the greatest microbial density in the human body, and enteric nerve terminal endings may explain why LBs can be found both in the gut and in the CNS in PD patients (Tan, Hor, *et al.*, 2020). Indeed, α-synuclein fibril injections performed intra-peritoneally, intra-muscularly, or intravenously were shown to lead to the formation of LBs in the brain of transgenic mice with A53T gene mutations (Breid *et al.*, 2016; Ayers *et al.*, 2017; Lohmann *et al.*, 2019). Additionally, it was recently demonstrated that α-synuclein fibrils injected into the duodenum of a transgenic PD rat model led to α-synuclein pathology in the locus coeruleus and SNpc within 4 months from the insult (Van Den Berge *et al.*, 2019). Interestingly, it was also shown that the same injection in wild-type animals led to progressive dopaminergic loss and the appearance of motor dysfunctions (Kim *et al.*, 2019), both hallmarks of PD.

LBs enter the CNS travelling through to the gut-brain axis, which links the GI tract to the CNS through biochemical signalling (Gómez-Pinilla, 2008). Disruption of the gut epithelium, which serves as a barrier for the GI, is known to lead to intestinal inflammation with elevated levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha, interferon gamma, interleukin-6, and interleukin-1 beta, and glial markers such as glial fibrillary acidic protein (GFAP), Sox-10, and S100-beta (Tan, Hor, *et al.*, 2020). Clinical and preclinical studies have demonstrated both increased intestinal permeability and the presence of inflammation in the gut of PD patients (Devos *et al.*, 2013; Houser *et al.*, 2018; Lubomski *et al.*, 2020), which are then thought to lead to the release of neuroactive peptides that modulate the CNS and ENS (Gareau, Silva and Perdue, 2008; Maes, 2008). GI symptoms such as constipation and bloating are among the first non-motor symptoms (NMSs) to arise in PD

patients, 5 to 10 years before the onset of motor dysfunction of PD (Perez-Pardo *et al.*, 2017; Radhakrishnan and Goyal, 2018; Seppi *et al.*, 2019) and an increased intestinal permeability in PD strongly correlates with the presence of intestinal α-synuclein (Forsyth *et al.*, 2011). The combination of gut inflammation and deposition of α-synuclein fibrils in the ENS may therefore initiate the process that leads to a retrograde spread via the vagal nerve to neuronal tissue in the CNS and potential role in in the pathogenesis of PD (Rietdijk *et al.*, 2017; Gazerani, 2019; Lubomski *et al.*, 2020; Tan, Hor, *et al.*, 2020; Castelli *et al.*, 2021). Although a causal relationship between GI inflammation and PD pathogenesis has yet to be confirmed, the net result remains the neuronal loss as consequence of inflammatory cascades and oxidative stress being produced (Perez-Pardo *et al.*, 2017). The gut-brain axis has therefore recently been added to the list of new promising targets for the treatment of PD (Kim *et al.*, 2018; Gazerani, 2019; Srivastav *et al.*, 2019).

6.1.2 The gut-brain axis and probiotics

The human GI tract houses different populations of bacterial species, collectively called human gut microbiota, where a symbiosis exists between the host and bacteria (Gazerani, 2019). The health of microbiota is directly associated with gut barrier integrity, immunity, function, metabolism and the correct functioning of the gut-brain axis (Delzenne and Cani, 2011; Diaz Heijtz *et al.*, 2011; Jangi *et al.*, 2016; Shi *et al.*, 2017). All of the above have also been suggested to play a potential role in several CNS-associated disorders, such as multiple sclerosis (Jangi *et al.*, 2016) and PD (Unger *et al.*, 2016; Hill-Burns *et al.*, 2017; Lubomski *et al.*, 2020). Recently, it was proposed that

alterations in gut microbiota, such as number and composition of gut microbiota and microbial metabolites, may be employed as valuable indicators for early diagnosis of several neurodegenerative disorders (Spielman, Gibson and Klegeris, 2018; Castelli *et al.*, 2021).

Probiotics are live microorganisms, comprising primarily of bacteria that occur naturally in the human gut and can be delivered in the form of drug, food, or supplements, and, if administered in sufficient amounts, exert health benefits to the host (Dutta *et al.*, 2019; Tan, Hor, *et al.*, 2020). Probiotics are indeed able to boost a wide range of host immune functions, such as phagocytosis and antibody secretion, which then translates into improved defences against pathogens (Klaenhammer *et al.*, 2012). Similarly, probiotics have also been demonstrated to upregulate various anti-inflammatory factors while downregulating pro-inflammatory cytokines and reducing intestinal inflammation (Sanders *et al.*, 2019). Additionally, probiotics have been shown to improve GI barrier function, partially through reducing GI inflammation (Dutta *et al.*, 2019). All these beneficial effects are achieved by producing or boosting in the body, the production of a wide range of bio- and neuroactive molecules, such as oxytocin, γ-aminobutyric acid (GABA), serotonin, tryptophan, tryptamine, noradrenaline, dopamine, and acetylcholine, that affect the host and its microbiota both locally and peripherally (Kim *et al.*, 2018). In recent years, therefore, several cellular, preclinical, and clinical studies have been conducted to evaluate the likely neuroprotective effects of probiotics and their potential as a treatment for PD (Dutta *et al.*, 2019; Srivastav *et al.*, 2019; Castelli *et al.*, 2020; Tan, Lim, *et al.*, 2020).

6.1.2.1 Probiotics and PD

Probiotic supplementation in PD patients has been shown to exert benefits in the treatment of constipation, a NMS with a reported prevalence of up to 70% (Knudsen *et al.*, 2017). The first clinical trial was conducted 10 years ago and demonstrated that supplementation with probiotics for 5 weeks improved stool consistency and reduced bloating and abdominal pain in PD patients (Cassani *et al.*, 2011). However, this pilot study was conducted on a small group of patients and did not include a placebo group. Treatment with probiotics was found to significantly increase the number of complete bowel movements per week, as well as improvements in daily bowel frequency and stool consistency in two double-blind placebo-controlled randomised clinical trials (Barichella *et al.*, 2016; Tan *et al.*, 2020). Additionally, a 12-week probiotics treatment was observed to improve the MDS-UPDRS (Movement Disorders Society-Unified Parkinson's Disease Rating Scale) and insulin metabolism by reducing insulin levels and insulin resistance whilst enhancing insulin sensitivity compared with placebo (Tamtaji *et al.*, 2019). Although the precise mechanisms underlying the effects of probiotics in PD are yet to be clarified, the improvement of gastrointestinal symptoms is thought to be a result of altering the gut environment or inhibiting harmful gut bacteria (Fang, 2019). Indeed, it has been reported that PD patients who are infected by *Helicobacter pylori* have lower absorption of L-DOPA (Pierantozzi *et al.*, 2001), suggesting that the eradication of *this bacterium* by treatment with probiotics may be a promising strategy in the treatment of PD (Gazerani, 2019). Lastly, given the role of oxidative stress in PD neurodegeneration (Niranjan, 2014), probiotics may be

beneficial for PD patients due to their effect in promoting production of antioxidant agents (LeBlanc *et al.*, 2013; Parashar and Udayabanu, 2017). Clinical trials assessing the effects of probiotics on PD patients have mainly focussed on the gastrointestinal symptoms as the primary end-point to evaluate the effectiveness of the treatment (Gazerani, 2019). However, PD is multifactorial disorder that includes several other symptoms, on which probiotics effects are largely unknown. For example, although probiotics have been shown help with depression associated with irritable bowel syndrome (Pinto-Sanchez *et al.*, 2017), nothing is known in the PD context. Similarly, it remains to be investigated whether and how probiotics can influence dementia and cognitive impairments in PD (Gazerani, 2019), hence, future clinical trials should increase the range of end-points evaluated in the studies to assess the effectiveness of probiotics on multiple aspects of PD.

6.1.2.2 Symprove

Among the several commercially available brands of probiotics, Symprove® has recently grown in popularity thanks to its many benefits. Symprove® is a liquid probiotic formulation containing four bacterial strains: *Lactobacillus casei*, acidophilus and plantarum as well as *Enterococcus faecium* (Bjarnason, Sisson and Ayis, 2012). A randomised double-blind placebo-controlled trial found that supplementation with Symprove led to a significant decrease in the frequency of constipation, diarrhoea, mucorrhoea and back pain in patients with symptomatic uncomplicated diverticular disease (Kvasnovsky *et al.*, 2017). Similarly, another recent randomised double-blind placebo-controlled trial observed decreased intestinal inflammation in patients with ulcerative

colitis following a 4-week treatment with Symprove (Bjarnason, Sission and Hayee, 2019). These effects were then shown to be due to changes in the bacterial composition in the microbiota. Specifically, production of short chain fatty acids (SCFAs) and lactate as well as levels of anti-inflammatory cytokines increased, whilst levels of pro-inflammatory cytokines and chemokines decreased (Ghyselinck *et al.*, 2020).

Lastly, a new clinical trial, which is based in London at King's College London, has recently concluded the recruitment phase and aims to test whether Symprove can be used in the treatment of both motor and non-motor symptoms (Parkinson's UK). Nevertheless, and despite the clinical observations, Symprove currently lacks pre-clinical evidence showing that it exerts neuroprotective effects.

6.1.3 Aims of this chapter

PD is a progressive and debilitating neurological disease with no present cure, and, over the past years, several compounds have been suggested as potential new treatments, such as probiotics. We hypothesised, hence, that supplementation with probiotics would lead to beneficial and neuroprotective effects in the PD animal model validated in Chapter 3. The aim of this Chapter was therefore to evaluate the effectiveness of the treatment using the multi-strain probiotic brand Symprove on counteracting the neurodegeneration characteristic of PD in the early-stage model. Since this was a pilot study, the latter was chosen to test whether Symprove supplementation exerted any neuroprotective effects on a mild PD model first. Currently, the scientific evidence supporting probiotics as a potential treatment for PD is still very

limited. Therefore, the pre-clinical study involving this specific multi-strain probiotic presented in this chapter mainly focussed on whether oral administration of Symprove exerted neuroprotective effects on the dopaminergic neurones in the SNpc of the rat early-stage PD model described in Chapter 3. The effect of Symprove on neuroinflammation in the striatum was also examined.

6.2 Materials and Methods

6.2.1 Experimental Groups

Rats were randomly divided into four groups: sham + placebo (n = 8), DSP-4 + 6-OHDA + placebo (n = 8), sham + Symprove (n = 5), and DSP-4 + 6-OHDA + Symprove (n = 5)[5]. The protocol used is summarised in Figure 6.1. Briefly, either placebo or Symprove® was administered orally daily from day 0. On the same day, animals were injected intraperitoneally either with saline (sham) or DSP-4 (early-stage model). Three days later, the rats underwent stereotaxic surgeries in which either vehicle (sham) or 6-OHDA (early-stage model) were delivered directly into the striatum (bilaterally). Finally, three weeks after the surgeries, animals were culled and the brains were collected. The weight of the animals was monitored daily throughout the duration of the experiments.

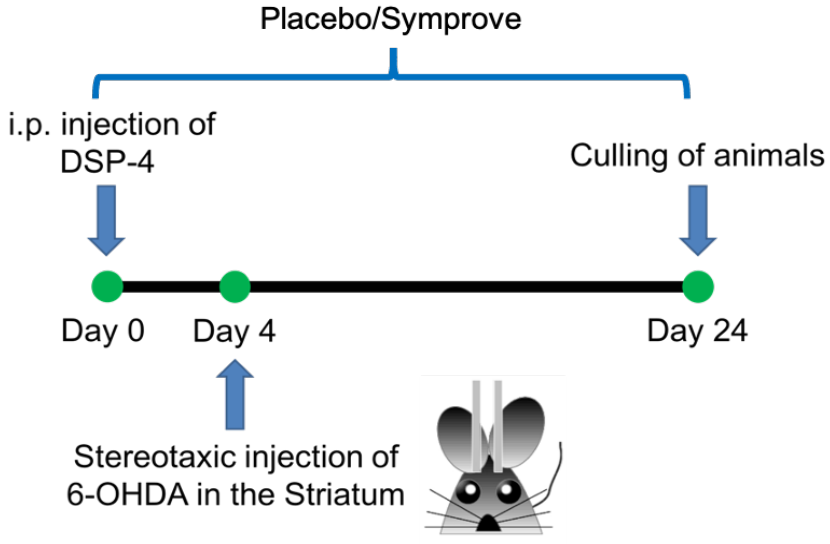

Figure 6.8 – Experimental timeline. *On day 0, animals received either saline (sham) or DSP-4 (model). Three days later, they underwent stereotaxic surgeries in which they received either saline (sham) or 6-OHDA (model), and they were sacrificed three weeks after. 0.5ml of placebo or Symprove® were given orally daily from day 0.*

[5] Due to the COVID19 pandemic, a first batch of animals had to be culled prior to the end of the 3 weeks post-surgery and, therefore, could not be included in the final analyses.

6.2.2 Drugs

N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4), a selective noradrenaline neurotoxin, and 6-hydroxydopamine (6-OHDA), a neurotoxin used to deplete both noradrenergic and dopaminergic neurones, were purchased from Sigma (Sigma Aldrich, Gillingham, UK). DSP-4 was delivered at a concentration of 25 mg/kg in saline solution and injected intraperitoneally (i.p.) 4 days prior to the 6-OHDA injection, while 6-OHDA was dissolved at a concentration of 5 mg/ml in saline solution containing 0.9% ascorbic acid and delivered bilaterally in the striatum. The optimum doses for DSP-4 and 6-OHDA were chosen to induce *partial* reduction of NA and DA levels based on previous studies (Jonsson et al., 1981; Prezedborski et al., 1995), mimicking the early stage of the disease. Both Symprove® (original flavour) and Symprove® placebo were kindly supplied by Symprove Ltd (Farnham, UK). The placebo's flavour was matched with that of Symprove®, and 0.5 ml was delivered orally using a 1 ml plastic syringe every day for 24 days. Additionally, to minimise any risk of contamination, the placebo and Symprove® experiments were carried out separately.

6.2.3 Immunohistochemistry

TH and neuroinflammation immunostainings were carried out on coronal sections containing either the SNpc or the striatum according to the immunoperoxidase protocol described in Chapter 2. Briefly, 50 µm coronal sections containing either the SNpc or the striatum were cut using a vibratome. One in every 8 sections containing the SNpc and one in every 12 sections containing the striatum were collected and 4-5 slices were used per animal

and per staining. Sections were incubated first in 1% H_2O_2 for 30 minutes and then in 1% sodium borohydride ($NaBH_4$) for 30 minutes to decrease background staining and then in 10% normal goat serum for another 60 min to block nonspecific antibody binding. Sections were incubated overnight at 4°C in a mixture of primary antibodies and triton X-100 (Sigma Aldrich) [0.1% Triton for all antibodies] made up in phosphate buffer solution (Primary antibodies are listed in Chapter 2 – Table 1). Following incubation in primary antibodies, sections were incubated overnight in secondary antibodies, biotinylated goat anti-mouse or anti-rabbit antibody (1:500, Vector Laboratories) made up in PBS. Next, sections were incubated in ABC (Vector Laboratories) overnight, washed in TRIS buffer, incubated in 3, 3' diaminobenzidine (DAB, Sigma Aldrich) for 20 minutes, and the staining was finally revealed using H_2O_2. Sections from the different experimental groups were processed together using the same immunoreagents and the DAB reaction was stopped adding TRIS buffer at the same time to allow comparison between groups. Sections were then placed onto Superfrost slides, dehydrated, cleared with Histoclear and mounted using DPX (Sigma Aldrich).

6.2.3.1 TH immunostaining quantification

The optical fractionator probe was used to determine the number of TH-immunopositive neurones in the SNpc (StereoInvestigator, MicroBrightField) using a Nikon microscope coupled to a computer-controlled x-y-z motorized stage and an MBF video camera system. Unbiased stereology was carried out on 5 slices per animal (*n* = 5 or 8 per experimental group) with the following parameters: counting frame 60 μm x 60 μm; grid size 200 μm x 200 μm;

section thickness 50 μm, dissector height 12 μm. Only neurones with visible nuclei and dendrites were counted.

6.2.3.2 Neuroinflammation

Levels of GFAP and Iba1 immunohistochemical staining were measured by quantitative thresholding image analysis as previously described (Rahim et al., 2012). Two non-overlapping images of the striatum in each section were captured using a DMR microscope and Leica Application Suite V4 (Leica Microsystems) at X5 magnification with constant light intensity, microscope calibration and video camera settings. Image-Pro Premier (Media Cybernetics, Cambridge, UK) was used to analyse the images and measure immunoreactivity using a constant threshold that was applied to all images for each respective antigen. Data are presented as the mean percentage area of immunoreactivity ± SEM.

6.2.4 Statistical analysis

All data are presented as mean ± SEM. Results were analysed using One-way ANOVA and the Tukey post-hoc test was applied. Significance was set at $P<0.05$.

6.3 Results

6.3.1 Tolerability of the probiotics

To assess the tolerability of the probiotics, the weight of the animals were monitored and recorded daily. Animals treated with the probiotics showed a weight gain comparable to the other groups (Figure 6.2). Animals that received the probiotics ended at the highest weights, with a steepness of the weight curve that was comparable with the other experimental groups. Similarly, the steepness of the weight curve of the sham + Symprove® group was not significantly different from the other experimental groups. Interestingly, the model + Symprove® group did not display the characteristic drop in weight the day following surgeries (Figure 6.2).

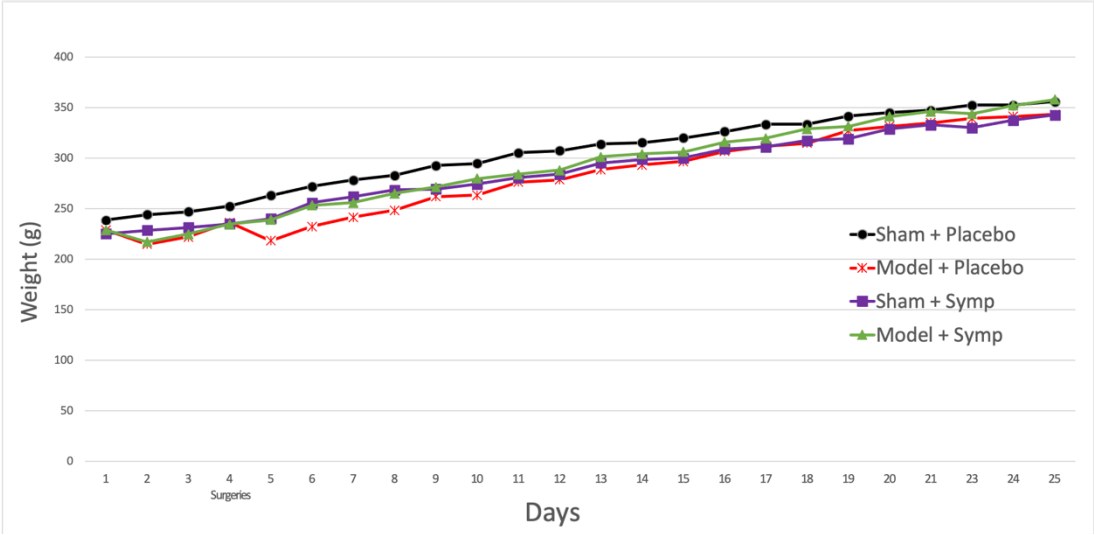

***Figure 6.2 - Animal weight curves**. Animals treated with the probiotics did not show any significant difference in weight gain overtime compared with the other experimental groups.*

6.3.2 Symprove® partially prevented dopaminergic loss in the SNpc

Figure 6.3A shows representative photomicrographs of TH-immunopositive cells in the SNpc of each experimental group: sham + placebo, model + placebo, sham + Symprove®, and model + Symprove®. Unbiased stereology revealed that the dopaminergic and noradrenergic lesions significantly decreased TH immunoreactivity in the SNpc of the early-stage model compared with that observed in both sham + placebo ($P < 0.05$) and sham + Symprove® ($P<0.01$) animals (Figure 6.3B). Treatment with Symprove® partially prevented the neuronal loss in this region in the early-stage PD model (Figure 6.3B).

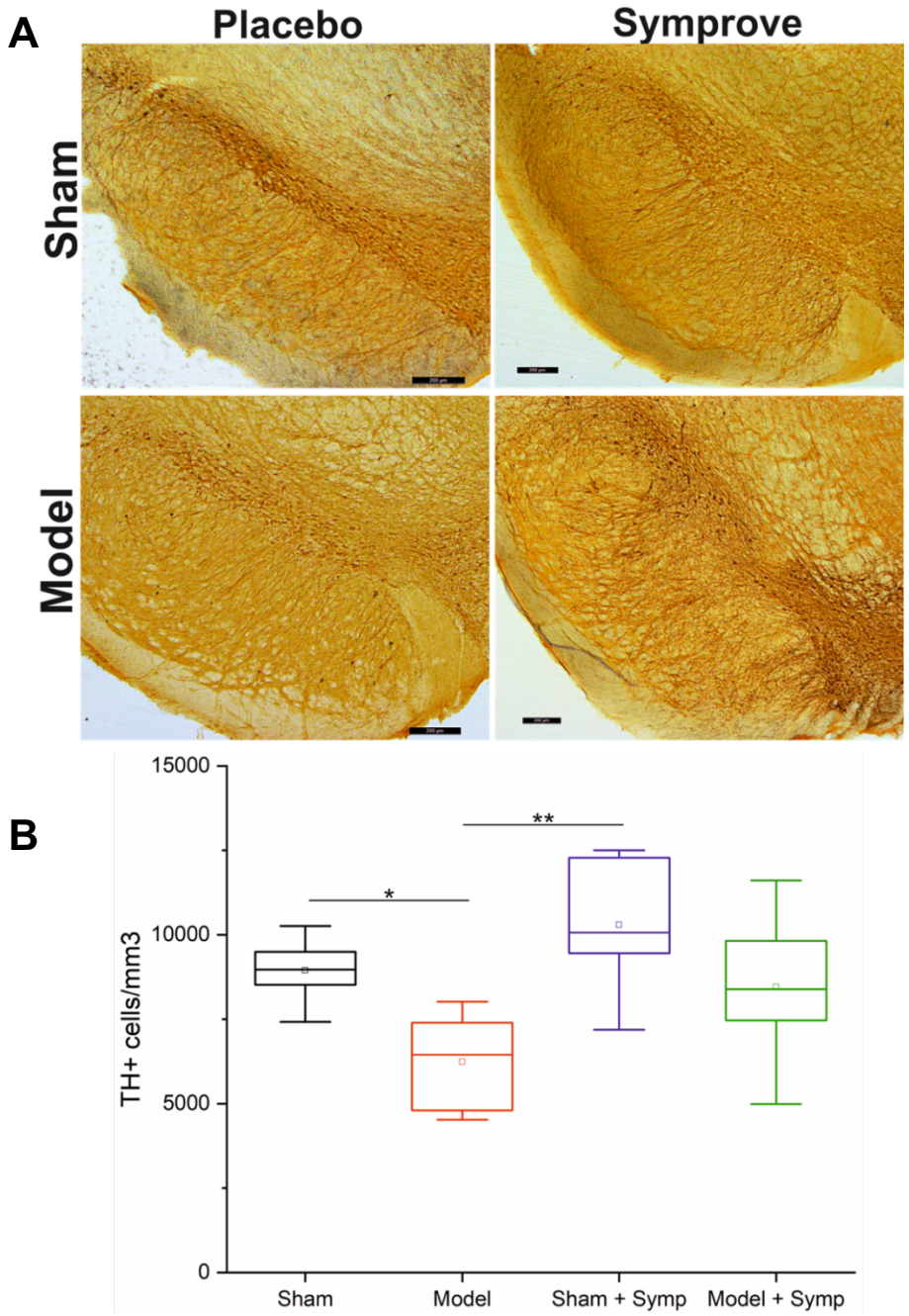

Figure 6.3 - Reduction of dopaminergic levels in a rat model of early-stage PD was partially prevented by treatment with *Symprove®.* ***A*** *Representative images of TH-staining in the Substantia Nigra pars compacta (SNpc) in the sham + placebo animals (top left panel), early-stage PD model (bottom left panel), sham + Symprove® (top right panel), and model + Symprove® (bottom right panel). Scale bars represent 200 μm.* ***B*** *Number of TH-positive cells in the SNpc expressed as cell density per mm^3 (n= 5-8 animals per experimental group). A decrease in the number of TH-positive cells was observed in the early-stage PD model compared with the sham animals treated with placebo and Symprove® (One-way ANOVA). This decrease was partially prevented when the early-stage model was treated with Symprove® *P<0.05, **P<0.01.*

6.3.3 Symprove did not prevent astrocyte activation in the striatum

To check for the presence of neuroinflammation in the striatum, slices from each experimental group were firstly stained for the glial fibrillary acidic protein (GFAP) to detect activated astrocytes (Figures 6.4A). A stronger staining was observed for GFAP-immunopositive astrocytes in the striatum of the early-stage model treated with both placebo and Symprove as well as of sham + Symprove compared with that in the sham animals treated with placebo (One-way ANOVA $P<0.001$, Figure 6.4B). The inserts in Figure 6.4A show that swollen cell bodies, extensive branching processes and overlapping domains were observed in the early-stage PD model and both groups treated with Symprove.

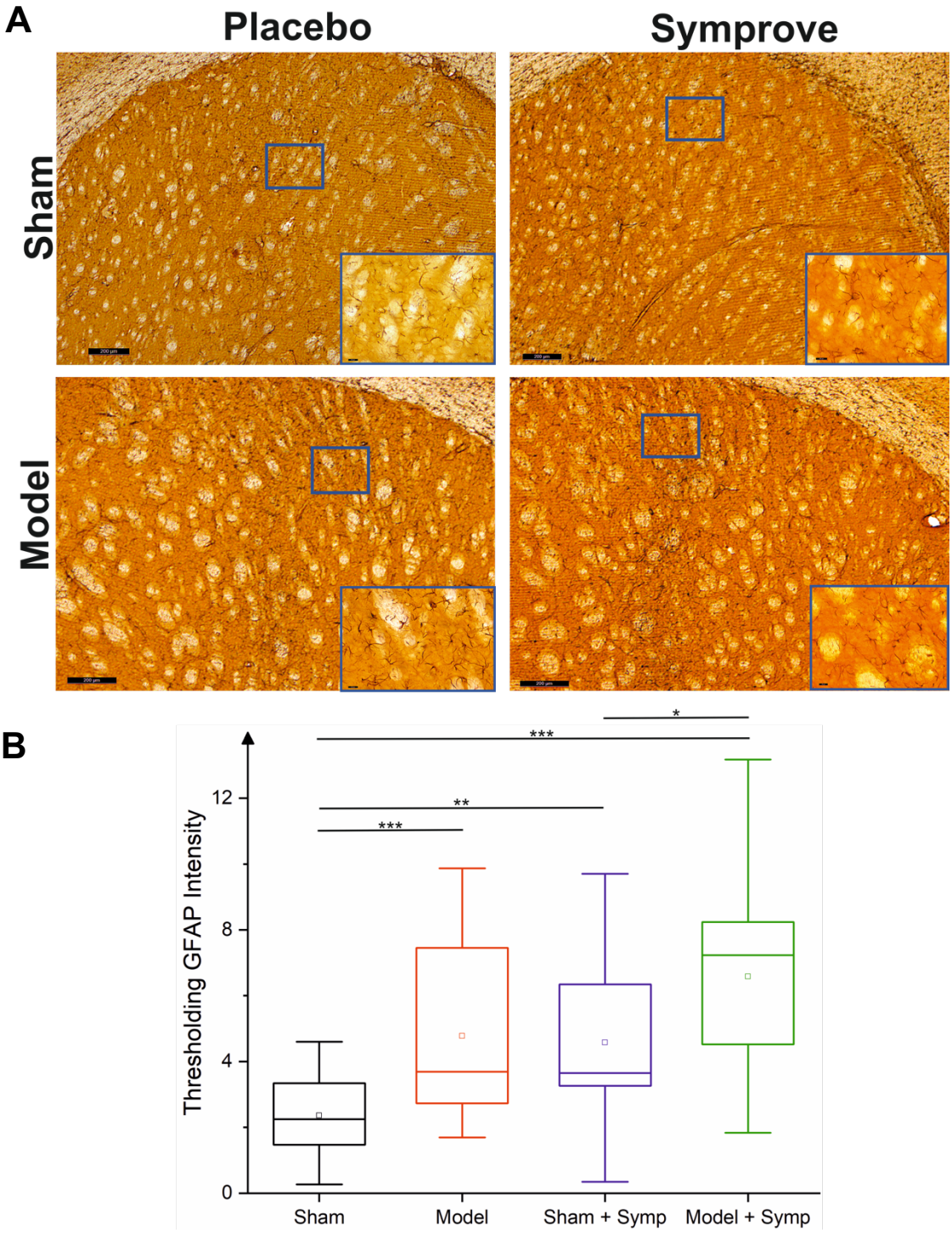

Figure 6.4 – Activation of astrocytes in the striatum of the early-stage PD model, sham + Symprove®, and model + Symprove® ***A*** *Representative immunohistochemical staining of GFAP in each experimental group. Scale bars for overview and for inserts represent 200 μm and 50 μm respectively.* ***B*** *GFAP staining intensity was stronger in all experimental groups compared with that in sham animals, suggesting that Symprove® did not prevent activation of astrocytes. Additionally, the intensity of the staining in the striatum of the model + Symprove® was also significantly stronger compared with sham + Symprove® *P<0.05, **P<0.01, ***P<0.001.*

6.3.4 Effect of Symprove® on striatal microglial activation

The activation state of microglial cells was also assessed in this study using the ionised calcium binding adaptor molecule 1 (Iba1) staining. Figure 6.5A shows the staining in the striatum of all experimental groups, with the inserts displaying more clearly their anatomical features. The intensity of the staining was not significantly different across groups (Figure 6.5B). However, a trend towards activation in the striatum of the early-stage PD model was observed. Interestingly, the intensity in both groups treated with Symprove® was comparable to that in the sham animals (One-way ANOVA $P > 0.05$).

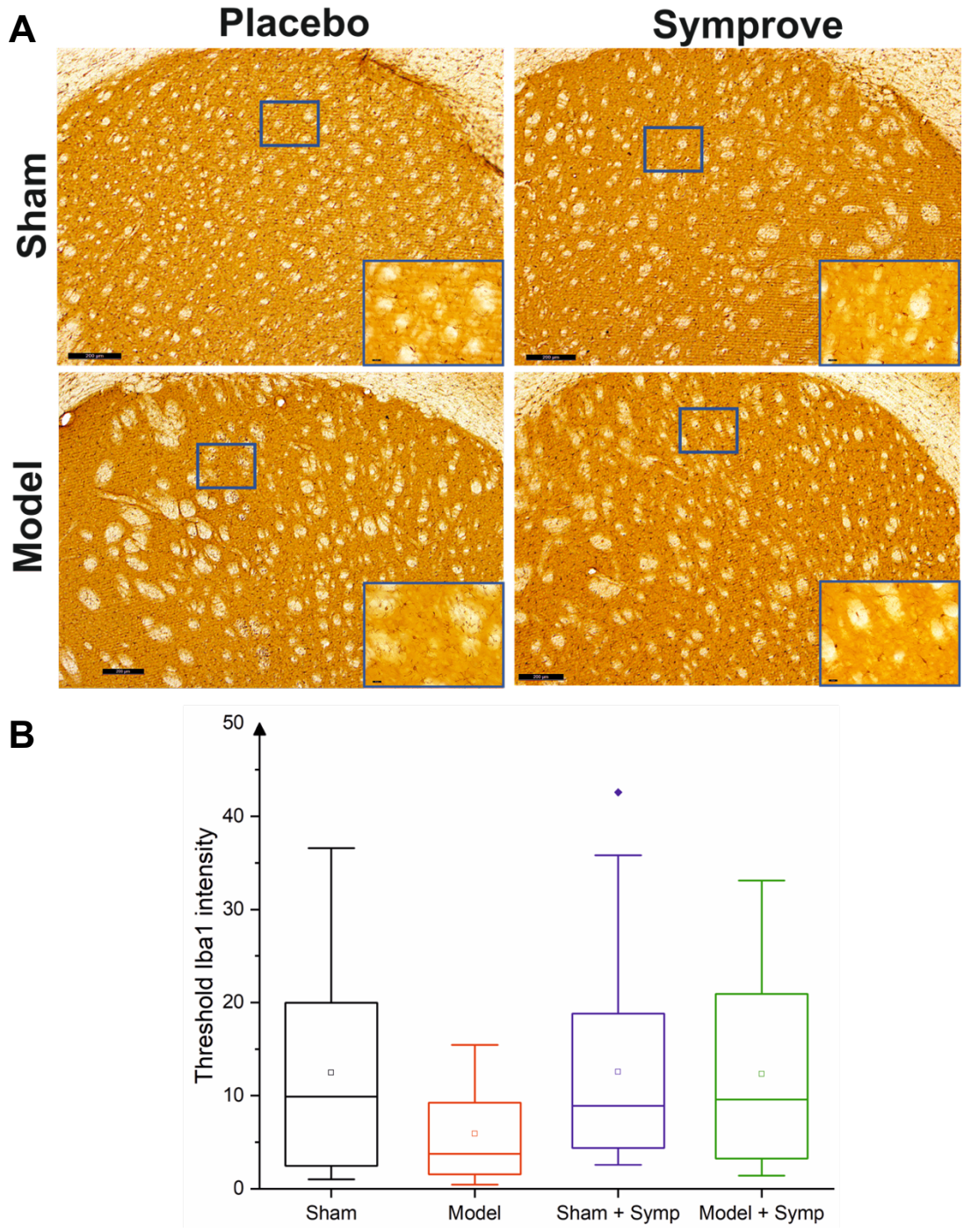

Figure 6.5 – Microglial cells activation in the striatum of the sham + Symprove® and model + Symprove® is similar to that in sham animals treated with placebo and Symprove®. A *Representative examples of Iba1 staining in the striatum of all experimental groups. Inserts show high magnification images of Iba1-positive cells. Scale bars for overview and for inserts represent 200 μm and 20 μm respectively.* ***B*** *Intensity of Iba1 staining in the striatum of the early-stage PD model was partially decreased, although not significantly, compared with that in the Sham animals. In contrast, both groups that received the probiotics showed a staining intensity very similar to sham animals, suggesting that treatment with Symprove® prevented the little activation observed in the model.*

6.4 Discussion

Presently, all current PD therapies aim at mitigating the impact of the symptoms on everyday activities, providing transient relief from the severe deficits (Maiti, Manna and Dunbar, 2017). The main PD treatments are focused on both replacing and restoring DA neurotransmission through the administration of L-DOPA (Whitton, 2007) mitigating the MSs, however they are unable to prevent degeneration of the remaining dopaminergic neurones and disease progression (Foster and Hoffer, 2004). Additionally, L-DOPA is associated with mild side effects, such as nausea, vomiting, and low blood pressure and more severe side effects, such as behavioural impediments, generation of toxic metabolites and paranoia (Paul and Borah, 2016). Similarly, as other dopamine-centred medications lead to unwanted side effects, efforts have focussed on using combinations of drugs in order to reduce the required doses while dampening associated side effects (Baker *et al.*, 2009; Radhakrishnan and Goyal, 2018; Moretti, 2019). Although the frequency and strength of side effects may be reduced with lower doses, patients will still experience them (Foster and Hoffer, 2004). The search for new potential therapies therefore focuses on finding new targets and drugs that will provide a slowing down of disease progression and low side effects.

Given the prominence of dysbiosis and colonic dysfunction in PD patients, recent evidence is suggesting that gut-derived inflammation plays an important role in neurodegenerative diseases (Houser *et al.*, 2018; Fang, 2019; Lubomski *et al.*, 2020; Tan, Hor, *et al.*, 2020). Previous studies on probiotics have shown that probiotic formulations improve CNS activity through the modulation of both inflammation and gut microbiota (Wang et al.,

2016). Additionally, probiotics do not require a prescription, are generally cheaper than medicines and, most importantly, are associated with less side effects (Felix, Karpa and Lewis, 2015). Thus, finding a supplement that exerts neuroprotective effects, and may therefore replace one drug in the mixture, could translate into a significant reduction of side effects experienced by PD patients without compromising the efficacy of the treatment. Probiotics differ in their bacteriological composition, and Symprove was shown to contain live bacteria that survive a simulated gastrointestinal environment (Fredua-Agyeman and Gaisford, 2015). Moreover, clinical trials using Symprove showed a significant improvement in the symptoms severity of irritable bowel syndrome, due to Symprove's anti-inflammatory effects in the gut (Kvasnovsky *et al.*, 2017; Bjarnason, Sission and Hayee, 2019).

To date, the beneficial effects and the underlying mechanisms of probiotic treatment for PD are yet to be determined, and studies on PD animal models are needed to clarify the benefits and underlying mechanisms of probiotic treatment for PD (Castelli *et al.*, 2021). The present study aimed, therefore, at evaluating the effectiveness of supplementation with the multi-strain probiotic Symprove on neurodegeneration in a early-stage animal model of PD. Remarkably, a partial prevention of neuronal loss in the SNpc of the early-stage model was observed following a 3-week treatment with Symprove. Nevertheless, TH+ cells in the model treated with Symprove did not significantly differ from the model treated with placebo, suggesting that the probiotics are only partially effective in preventing neurodegeneration. Thus, despite the observed beneficial effects, further research and a larger sample

size are required to study the neuroprotective effects of Symprove in greater detail.

6.4.1 Symprove partially prevented dopaminergic loss in the SNpc of an early-stage PD model

The loss of dopaminergic cells in the SNpc is considered to be the hallmark of PD, and its prevention is the first step in evaluating the effectiveness of any new treatment developed for PD (Armstrong and Okun, 2020). In this study, treatment with a multi-strain probiotic Symprove showed partial neuroprotective effects on dopaminergic neurones in the midbrain of an early-stage PD model for the first time.

Neuroprotective effects were recently reported in other animal models of more advance stages of PD (Srivastav *et al.*, 2019; Castelli *et al.*, 2020; Hsieh *et al.*, 2020). For example, treatment with probiotics on the genetic MitoPark PD model resulted in better motor performances and reduced degeneration of nigral dopaminergic neurones, indicating a neuroprotective effect of the probiotic mixture (Hsieh *et al.*, 2020). Similarly, Srivastav *et al.* (2019) reported the rescue of dopaminergic neurones following probiotic administration in MPTP- and rotenone-induced PD mouse models. Additionally, an enhanced production of butyrate, brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor, were also observed (Srivastav *et al.*, 2019).

To date, only one other study investigating the effects of probiotics on 6-OHDA models has been conducted. Castelli and colleagues (2020) have recently demonstrated that treatment with the probiotic formulation SLAB51 was able to counteract 6-OHDA-induced neurodegeneration both *in vitro* and *in vivo*.

Indeed, SLAB51 exerted anti-inflammatory effects, restored pro-survival and neuroprotective pathways such as BDNF, partially prevented dopaminergic loss, and improved behavioural impairments (Castelli *et al.*, 2020). Although the precise mechanisms by which probiotics exert neuroprotective effects are yet to be uncovered, it has been suggested that they may be due to their abilities to both enhance peripheral tyrosine decarboxylase production (van Kessel *et al.*, 2019) and modulate neuroinflammation (Castelli *et al.*, 2020; Hsieh *et al.*, 2020). In the present study, the mechanisms by which probiotics partially prevent neuronal loss are yet to be identified. However, based on previous evidence, it can be speculated that the efficacy of probiotics in PD relates to the restoration of PD-associated microbiota composition, improvement of GI function, reduction of harmful gut bacteria, and the release of proinflammatory cytokines (Barichella *et al.*, 2016; Parashar and Udayabanu, 2017; Magistrelli *et al.*, 2019; Lubomski *et al.*, 2020; Castelli *et al.*, 2021).

6.4.2 Symprove prevented the activation of microglial cells

Neuroinflammation is a major player in PD pathophysiology and progression, and it is also thought to be a key element in the aetiology of the disease (Cicchetti *et al.*, 2002; Doty, 2012a; Wang, Liu and Zhou, 2015b; Flood, Arbabzada and Sharma, 2016; O'Neill and Harkin, 2018b). Additionally, modulation of inflammation by probiotics has been shown in both CNS and ENS (Lescheid, 2014; Lubomski *et al.*, 2020). The presence of neuroinflammation in the striatum was investigated via immunohistochemical analysis of microglial cells and astrocytes, and the technique used to quantify

the neuroinflammation has been extensively described in the dedicated Discussion paragraph in Chapter 3.

In this study, probiotic supplementation prevented activation of microglia cells in the striatum, resulting in a partial prevention of neuroinflammation. The anti-inflammatory properties of probiotics are generally regarded as the mechanism by which they exert most of their beneficial effects (Klaenhammer *et al.*, 2012). For example, a study in aged rats showed that treatment with the probiotic formulation VSL#3 boosted production of BDNF and the protein synapsin, which, by reducing neuroinflammation, ultimately resulted in enhanced brain performances (Distrutti *et al.*, 2013). Similarly, it has been reported that treatment with probiotic formulation SLAB51 exerted beneficial effects on cognitive performances of transgenic 3xTg Alzheimer's disease mouse model by counteracting inflammation, in addition to reducing accumulation of amyloid plaques (Bonfili *et al.*, 2017).

Although treatment with Symprove prevented microglia activation, the opposite effects was observed with astrocytes, which were found to be activated even further when compared with the placebo groups. The mechanisms behind this activation are yet to be identified, however, this results may be partly ascribed to the analyses performed. Indeed, neuroinflammation is usually investigated by quantification of inflammatory markers such as cytokines, reactive oxygen species (ROS), and oxidative stress through biochemical techniques (Distrutti *et al.*, 2013; Wang, Liu and Zhou, 2015b; Bonfili *et al.*, 2017; D. S. Kim *et al.*, 2017b). Thus, Symprove may have impacted the release of these pro-inflammatory agents rather than astrocyte activation as shown in previous studies (Kvasnovsky *et al.*, 2017;

Bjarnason, Sission and Hayee, 2019). Additionally, it should be noticed that some probiotic strains were shown to stimulate necrosis factor-κB (Lescheid, 2014) and induce ROS production in murine macrophages (Marcinkiewicz *et al.*, 2007; Rocha-Ramírez *et al.*, 2017), suggesting they have also pro-inflammatory effects. The latter, however, is thought to be beneficial in priming the immune system responses, thereby promoting host defence against pathogens (Lescheid, 2014).

6.4.3 Limitations and future directions

These promising results, although in a preliminary stage, highlight the role that probiotics may have in the treatment of PD in the future. Further studies will be required to determine the mechanism by which Symprove exerts its effects. Similarly, the sample size will be dictated by power calculations in order to reach significance between all experimental groups. In addition, the effects of the probiotics on neuroinflammation were only assessed in one region, the striatum, which is also the region where the stereotaxic injection was performed. Thus, the study of the effects exerted by Symprove on neuroinflammation requires further investigation to untangle the contradictory results observed in this study. Lastly, additional experiments will also be needed to determine whether Symprove supplementation has an effect on the onset of non-motor symptoms observed in the model (Sancandi et al., 2018) or whether Symprove can prevent or delay the onset of the more debilitating motor dysfunctions. These preliminary experiments will lay the groundwork for more extensive investigations, including the effects of supplementation with Symprove on the gut microbiota of the early-stage model, analyses of stool

samples to detect potential changes in its composition, and the possible presence of inflammation in other internal organs related to the ENS, such as liver and intestine. Although the presence of constipation in the early-stage PD model is yet to be properly investigated, preliminary observations suggest that probiotics supplementation may have a beneficial effect on stool frequency. Blood plasma may also be extracted and inspected for inflammatory markers.

6.4.4 Summary and conclusions

In this study, a multi-strain probiotic, Symprove, currently being tested in a clinical trial funded by Parkinson's UK, was shown, for the first time, to exert beneficial effects in an animal model of PD, partially preventing neuronal loss. Albeit limited, this pilot study provides an additional piece of evidence of the potential neuroprotective effects of probiotics in PD-related neurodegeneration. Further pre-clinical research is still needed to clarify the underlying mechanisms, leading to improved therapeutic protocols of probiotics in PD or other neurodegenerative diseases. Indeed, combining probiotics and conventional PD medications appears to be a valuable option to further enhance the beneficial effects observed following probiotics supplementation, although more studies are needed to fully understand the mechanisms of action of probiotics as well as potential synergistic activities.

Chapter 7 - Discussion

Parkinson's disease is the second most common neurodegenerative disorder with no present cure and is believed to currently affect more than 10 million people worldwide (Parkinson's Foundation, 2020). Although the hallmarks of PD are the loss of DA with the subsequent development of the MSs, this disease comprises also NMSs, such as the loss of smell, cognitive deficits, depression, anxiety, and pain, most of which have been shown to precede the appearance of MSs in the early-stage (Doty, 2017; Konnova and Swanberg, 2018; Radhakrishnan and Goyal, 2018; Balestrino and Schapira, 2020; Duty *et al.*, 2020). Additionally, not all NMS can be improved using dopaminergic treatments, such as hyposmia and cognitive dysfunctions, suggesting the existence of additional underlying mechanisms (Delaville, Deurwaerdère and Benazzouz, 2011a; Barichella *et al.*, 2016; Emamzadeh and Surguchov, 2018; Seppi *et al.*, 2019). Unfortunately, the early stage of PD has not received as much attention as the late stage, with PD research mainly focussing on the dopaminergic deficit and MSs, disregarding both other symptoms and other neurotransmitters that may be involved in the aetiology of the disease (Konnova and Swanberg, 2018; Marin *et al.*, 2018; Saiz-Sanchez *et al.*, 2020). Therefore, there is currently a lack of PD animal models representing the early stage of the disease, making it difficult to investigate NMS aetiology and pathogenesis in PD (Beal, 2010; Taylor, Greene and Miller, 2010; Jagmag *et al.*, 2016). The present project was developed to address this lack, providing further insights into the deficits and treatment of the early-stage stage of the disease, with a specific focus on hyposmia. To this end, through depletion of the monoaminergic system, a dual neurotoxin animal model of early stage PD, in which hyposmia is observed in the absence of MSs, was developed and

validated as representative of the early-stage stage (Sancandi et al., 2018). The deficits displayed in the above dual neurotoxin model that underlie the loss of sense of smell were then investigated and new potential treatments, such as EX-4 and probiotics, were tested to determine whether they could prevent the observed deficits. To our knowledge, this is the first study providing evidence that hyposmia exhibited by an early-stage PD model was associated with a decrease in interneuronal CBP expression, an increase in NeuN-positive cells, and presence of neuroinflammation within the PC (Sancandi *et al.*, 2018). Additionally, a similar pattern of deficits was also observed in the PFC of the early-stage PD model, whilst an increase in expression of dopamine and interneuronal CBPs was found in the OB. Additionally, the majority of these effects were partially prevented by EX-4 treatment (at the dosage of 0.5 µg/kg used), suggesting that the observed changes may be initiated by a common denominator or that EX-4 acts on multiple sites/levels. Moreover, a new gene therapy involving a viral vector carrying the gene for EX-4 was tested for the first time in a rat 6-OHDA PD model, showing promising neuroprotective effects. Lastly, this is also the first report of potential neuroprotective effects exerted by treatment with the multi-strain probiotic Symprove on dopaminergic cells in the SNpc of an early-stage PD animal model.

7.1 A novel early-stage PD animal model

In the present project, a novel dual toxin based early-stage PD model, in which a noradrenergic deficit preceded the dopaminergic deficit, was designed as a tool to investigate the aetiology of hyposmia as well as neuroprotective agents,

such as EX-4. Chapter 3 described the validation of this novel early-stage PD model, confirming the loss of DA and NA after toxin administration and the presence of hyposmia in the absence of MSs. Additionally, the induction of the PD model was tested using either DSP-4 or 6-OHDA alone and in both cases, the animals did not show hyposmia, suggesting that the combined loss of NA and DA underlies the olfactory deficit observed in the early-stage PD model. Olfactory dysfunctions have been described in other toxin-induced PD animal models, however, these often occurred in concomitance with MSs (Tadaiesky *et al.*, 2008a; Santiago *et al.*, 2010; Carvalho *et al.*, 2013; Faggiani, Delaville and Benazzouz, 2015; Ledreux *et al.*, 2016; Nezhadi *et al.*, 2016). As hyposmia has been shown to both precede MSs in PD patients (Doty, 2017) and be a reliable indicator of disease severity (Roos *et al.*, 2019), this novel early-stage PD model is therefore thought to be a suitable model to study the pathophysiology and treatment of early stages of PD. Nevertheless, this model has some limitations as it does not fully represent the PD pathology. Indeed, PD is caused by a complex interplay between genetic and environmental factors of which the exact mechanism remains to be determined (Balestrino and Schapira, 2020). Pathological hallmarks include alterations of neurotransmitters other than just DA and NA, such as acetylcholine, serotonin, glutamate, and GABA (Müller and Bohnen, 2013; Poewe *et al.*, 2017; Doty, 2017; Konnova and Swanberg, 2018; Radhakrishnan and Goyal, 2018), aggregations of α-synuclein into LBs (Braak *et al.*, 2003; Del Tredici and Braak, 2016; Engelender and Isacson, 2017; Chakraborty and Diwan, 2020), and mitochondrial dysfunction leading to deficits in energy supply and generation of oxidative stress (Dauer and Przedborski, 2003; Nikoletopoulou

and Tavernarakis, 2012; Celardo, Martins and Gandhi, 2014; Puspita, Chung and Shim, 2017) with consequent neuroinflammation (Whitton, 2007; Dutta, Zhang and Liu, 2008; Lema Tomé *et al.*, 2013; Gelders, Baekelandt and Van der Perren, 2018a; Sancandi *et al.*, 2018). The novel early-stage PD model was developed by depleting both the noradrenergic and dopaminergic systems to mimic a parkinsonism phenotype. While potential alterations in GABAergic/glutamatergic balance have been observed in this model in response to NA and DA loss, α-synuclein depositions and alterations in other neurotransmitter systems are yet to be investigated. Therefore, to obtain a comprehensive animal model of PD, future studies may combine this novel early-stage model with other existing PD models displaying other pathological hallmarks.

7.2 Hyposmia is sustained by cellular and structural changes in the olfactory pathway of the early-stage PD model

Several studies have shown DA and NA involvement in the modulation of PC activity, with both neurotransmitters contributing to odour learning and perception through both suppression of excitatory synapses (DA: Malenka and Nicoll, 1986; NA: Hasselmo *et al.*, 1997) and enhancement of the activity of inhibitory interneurones by increasing their spontaneous inhibitory potentials (DA: Gellman and Aghajanian, 1993; NA: Kawaguchi and Shindou, 1998). Additionally, although PD pathogenesis is currently unknown, increasing evidence points at the presence of neuroinflammation as one of the key players (Gao *et al.*, 2008; Niranjan, 2014; Ortiz *et al.*, 2017). The observed neuroinflammatory response in the primary olfactory cortex of the early-stage

PD model was therefore not surprising in response to the insult with toxins. Additionally, the observation that inflammation was detected as early as 8 days after surgery and preceded the down-regulation of CBPs in the PC (Sancandi *et al.*, 2018) highlights its relevance in the aetiology of hyposmia. As suggested in previous studies, the role of inflammation in the progression of the disease may therefore lead to toxicity and damage of the surrounding cellular environment (Refolo and Stefanova, 2019). This possible role and the fact that GABAergic cells were found to be affected in hyposmic PD patients (Doty, 2017; Marin *et al.*, 2018) led to the investigation of the interneuronal population expressing CBPs as well as of PNNs in the PC of the early-stage PD model. Interestingly, PNN degradation was observed as well as CBP expression was found to be significantly reduced. The functional significance of this result is yet to be determined, however, it may be speculated that these structural changes are a direct consequence of the neuroinflammation. Indeed, the loss of PNNs could either reflect a defect in the ability of glial cells to excrete PNN components, such as chondroitin sulfate proteoglycans (CSPGs) (Berretta *et al.*, 2015a), or be triggered by the release of enzymes, such as matrix metallopeptidase-9 (MMP-9) and a disintegrin and metalloproteinase with thrombospondin motifs, from reactive astrocytes (Pollock *et al.*, 2014).

Due to a high presence of reciprocal connections, the OB were the next area investigated for any structural and cellular changes in the early-stage model, including the effects of the toxins on the separate dopaminergic population of interneurones (Halász *et al.*, 1981) (Gall *et al.*, 1987). In accordance with the literature, TH expression was found to be significantly increased in the early-

stage model compared with controls. Similarly, the expression of interneuronal CBPs was also observed to be upregulated in the PD model. An increase in CBPs was recently observed to be correlated with α-synuclein co-localisation in different areas of the anterior olfactory nucleus in PD patients, suggesting it may be a possible neuroprotective mechanism (Ubeda-Bañon *et al.*, 2017). Similarly, the increase in dopaminergic expression is thought play a key role in the aetiology of hyposmia (Huisman, Uylings and Hoogland, 2004, 2008; Belzunegui *et al.*, 2007; Pifl *et al.*, 2017; Ilkiw *et al.*, 2018). These interneurones modulate the activity of mitral cells, the main output neurones of the OB, which express both D1 and D2 dopaminergic receptors (Escanilla *et al.*, 2009). Depletion of either of these receptors was shown to impair odour discrimination and perception in rodents (Tillerson *et al.*, 2006), whilst optogenetic activation of the dopaminergic neurones in the OB resulted in long-range suppression of both the spontaneous and odour-evoked firing of the mitral cells (Banerjee *et al.*, 2015), indicating an inhibitory effect of dopaminergic modulation on mitral cell activity. Additionally, it was demonstrated that dopamine receptor activation in the OB led to a significant depression of synaptic transmission *in vitro* (Hsia, Vincent and Lledo, 1999). Therefore, a locally enhanced bulbar dopaminergic inhibition in PD may result in a more generalised inhibition of the whole pathway, with less information reaching the cortex. In support of this theory, Ilkiw and colleagues (2018) showed that injecting 6-OHDA directly in the OB of a rat PD model restored the sense of smell in the animals. The increase in the number of DA cells in the OB may thus represent a compensatory mechanism in response to neurodegeneration in the midbrain, as shown by an enhanced mobilisation of

progenitor cells in the SVZ of a genetic model of PD displaying hyposmia (Paß *et al.*, 2020).

Cognitive impairments are one of most experienced NMS by PD patients (Poewe, 2008; Narayanan, Rodnitzky and Uc, 2013; Radhakrishnan and Goyal, 2018; Seppi et al., 2019). Additionally, cognitive dysfunctions were shown to correlate with hyposmia, with the latter being a good predictor of the former (Roberts et al., 2016; Kotecha et al., 2018; Yahiaoui-Doktor et al., 2019). The early-stage PD model used in this study displayed hyposmia and memory impairments (Sancandi et al., 2018) suggesting a possible involvement of both the PFC and hippocampus. Interneuronal CBP expression was found to be significantly decreased in the PFC of the early-stage PD model compared with controls, in agreement with the findings in the PC. The CA1 and CA3 regions of the hippocampus appeared not to be affected in the early-stage model. In contrast, the CA2 region displayed a significant loss of GABAergic interneurones, which is in agreement with the evidence that this region is the first hippocampal area to be structurally affected in PD (Györfi et al., 2017). As none of the CBPs analysed here were significantly downregulated, other GABAergic cells are thought to be affected, such as somatostatin-positive interneurones, whose mRNA was recently found to be significantly decreased in GABAergic interneurones derived from iPSCs of PD patients (Iwasawa et al., 2019).

Although the precise mechanisms by which hyposmia is induced in the early-stage PD model are currently unknown, this study suggests that toxin injections have an effect on several regions associated with the olfactory pathways, including the PC, OB, PFC and hippocampus. Overall, this study

shows the importance of neuroinflammation in the early stage of the disease, which then leads to a cascade of events including PNN degradation and down- or up-regulation of CBPs, enabling possible excitation/inhibition imbalances and disrupted circuitry.

7.3 Potential new treatments for early-stage PD

Currently, no definite cure for PD has been established (Radhakrishnan and Goyal, 2018). The rationale behind the first line treatment is to counteract the dopaminergic loss in the midbrain, with little effect on the several NMSs experienced by PD patients (Schaeffer and Berg, 2017). Additionally, although the available treatments generally lead to a significant improvement of MSs, they are not able to modify the disease progression (Balestrino and Schapira, 2020). One of the aims of this project was therefore to develop and validate an early-stage PD animal model not only to study the pathogenesis of the disease, but also to test the effectiveness of new potential treatments at an early stage of the disease.

7.3.1 Exendin-4: from type 2 diabetes mellitus (T2DM) to PD

Several epidemiological studies have reported a link between diabetes and PD (Hu *et al.*, 2007; Xu *et al.*, 2011; Santiago and Potashkin, 2013), with diabetic patients often displaying parkinsonian symptoms (Arvanitakis *et al.*, 2007). More than 60% of the PD patients have impaired insulin signalling (Bosco *et al.*, 2012), and the onset of diabetes before the onset of PD appears to increase symptom severity in PD patients (Cereda *et al.*, 2012). Additionally, mitochondrial dysfunction and inflammation play a role in the aetiology and progression of both diseases (Santiago and Potashkin, 2013). Over time,

clinicians started noticing that drugs used in the treatment of diabetes had beneficial effects also on PD symptoms, as in the case of EX-4. The advantage of this drug repurposing is that clinical trials on PD patients can run concomitantly with pre-clinical studies because the drug has already been approved and declared safe (Athauda *et al.*, 2018; Sancandi *et al.*, 2018).

EX-4 has been shown to have both neuroprotective and anti-inflammatory effects, however, none of the studies tested its efficacy during the early-stage or tested its effects on disease progression (Harkavyi *et al.*, 2008; Li *et al.*, 2009; D. S. Kim *et al.*, 2017b; Athauda and Foltynie, 2018). The early-stage PD model was treated with EX-4 twice daily for 7 days prior to culling and the data revealed that treatment with EX-4 partially prevented both dopaminergic and noradrenergic loss, counteracting the loss of sense of smell. Additionally, EX-4 partially prevented all the deficits observed in the model, from the activation of the immune response to altered CBP expression. Although in the present study EX-4's mechanisms of action were not directly investigated, previous studies have shown that its neuroprotective effects may be ascribed to its ability to preserve mitochondrial function in dopaminergic neurones by increasing expression of complex I and anti-apoptotic proteins (Chen *et al.*, 2015; Nassar *et al.*, 2015) as well as EX-4's powerful anti-inflammatory properties (Li *et al.*, 2015; D. S. Kim *et al.*, 2017a; Ventorp *et al.*, 2017; Athauda and Foltynie, 2018; Reiner *et al.*, 2018). However, despite the fact that treatment with the GLP1 analogue slowed down PD progression in the early-stage animal model, the effects it exerts on later stages, when more extensive damage occurred, are currently unknown.

EX-4 has the potential to improve the long-term prognosis for millions of PD patients world-wide, however, it needs to be injected either twice daily or once weekly unless the patients have a pump, making its delivery a challenge. This led us to evaluate the effectiveness of a new and simplified method of administration: a gene therapy with a viral vector expressing EX-4. Preliminary experiments showed promising results. It was indeed observed for the first time, that i.v. injection of the viral vector not only reached the brain, but it exerted some neuroprotective effects on dopaminergic neurones in a 6-OHDA PD model, with the potential to slow down the progression of the disease. The main advantage of this therapy is that it requires only one i.v. injection. We feel that this study has laid the groundwork for new research to evaluate the use of gene therapy for PD involving EX-4, and further studies on the efficacy of this new therapy are now urgently required.

7.3.2 Probiotics: another new hope for PD?

In recent years, probiotics have grown in popularity among clinicians as an additional aid in the treatment of neurodegenerative diseases, such as PD and Alzheimer's disease, due to their beneficial effects upon stimulation of the immune system (Maldonado Galdeano *et al.*, 2019). A limited number of studies have begun reporting beneficial effects experienced by PD patients following treatment with probiotics (Parashar and Udayabanu, 2017; Fang, 2019; Hsieh *et al.*, 2020). In this study, treatment with the multi-strain probiotic Symprove for 4 weeks resulted in reduction of microglial activation and a partial prevention of dopaminergic loss in the midbrain of the early-stage PD model following toxin injections. Although more experiments are needed to

fully evaluate the effects exerted by this treatment, our results are in agreement with previous studies that showed neuroprotective effects of probiotics on dopaminergic neurones in PD animal models displaying motor dysfunctions (Srivastav *et al.*, 2019; Castelli *et al.*, 2020; Hsieh *et al.*, 2020). A growing body of evidence indicates that the positive influence on CNS diseases exerted by probiotics administration is achieved by mediating different pathways, such as neural, hormonal, immune, inflammatory, and antioxidant signalling (Wang et al., 2016; Boon Wong et al., 2018; Gazerani, 2019). Notably, a healthy intestinal microbiota was associated with a reduced risk of developing neurological and neurodegenerative disorders, including PD (Castelli *et al.*, 2021).

7.4 Methodological considerations

Despite the present project providing very interesting and encouraging results, some methodological considerations should also be highlighted. Although the early-stage PD model is representative of the early stage of the disease, it does not present the whole spectrum of deficits and symptoms proper of this stage. For example, it is unknown whether the early-stage PD model displays the characteristic LB inclusions, and the neurodegeneration in the midbrain was forcefully induced by injecting neurotoxins rather than occurring naturally. Nevertheless, it is interesting to notice that the progression of the neurodegeneration in this model follows Braak's staging scheme, with the olfactory pathway being affected before the midbrain with the consequent MSs (Hawkes, Del Tredici and Braak, 2010). Indeed, there is no current animal model of PD in which every aspect of the human disease is truthfully

replicated, indicating that each model has its own weaknesses and strengths (Beal, 2010; Taylor, Greene and Miller, 2010; Campos *et al.*, 2013). This animal model has the advantage of allowing for a better study of the deficits associated with the aetiology of hyposmia in early-stage PD by unravelling them from the more predominant MSs, the reason for which it was initially developed.

Structural and cellular changes in olfactory regions of the early-stage model were studied using immunohistochemistry and unbiased stereology. The specificity of all antibodies was previously characterised and optimum dilutions were used. The optical fractionator method was used to count cells in all brain regions studied. The parameters that were used were set using previous studies, revealing accurate estimations of the cell numbers in a particular region using the slice thickness and sample blinding was also employed.

Lastly, the studies involving gene therapy and probiotics should be regarded as promising preliminary experiments, with the result of laying the groundwork for further investigations. Indeed, despite the small sample sizes, the results provide encouraging evidence that use of gene therapy or probiotic supplementation could be part of an alternative treatment strategy, ultimately leading to a prognosis improvement for millions of PD patients worldwide.

7.5 Future directions

During the course of the studies presented here, novel and potentially important observations have been made concerning the early stage of PD and new potential therapies. To extend this project further, the use of animal PD models combined with behavioural, electrophysiological, and

immunohistochemical techniques will be used to answer a number of questions arising from these findings.

1) *Does the early-stage PD model display other NMSs?*

This thesis was centred around the investigation of olfaction, however, this stage of the disease is characterised also by other NMSs, such as sleep disorders and autonomic dysfunction, which are yet to be investigated in this PD model. Additional behavioural experiments aimed at a deeper characterisation of this model will investigate the potential presence of these other NMSs. For example, the elevated plus maze and open field test will be performed to assess anxiety. Similarly, the novel object recognition test and the Morris Water Maze could be employed to assesses cognition, specifically spatial memory and discrimination.

2) *Does the down-regulation of CBPs in the PC lead to a decreased local inhibition with subsequent cell hyperexcitability?*

As CBPs are proteins whose specific role is to bind freely circulating Ca^{2+}, their downregulation may therefore impact on the amount of Ca^{2+} which is then likely to affect the electrophysiological properties of the surrounding cells. To investigate this hypothesis, electrophysiological recordings of both pyramidal cells and interneurones in the PC may be made to study any change in the circuitry, including properties of pyramidal cells, interneurones, and LOT neurotransmission following the decrease in dopaminergic and noradrenergic inputs.

3) *Is neuroinflammation a trigger or a consequence of the disease? And is it present in other areas of the olfactory pathway?*

Neuroinflammation is known to be a key component of PD, however, its exact role in the pathogenesis of disease has yet to be fully understood. In order to answer the question whether neuroinflammation is a trigger or a consequence of PD, LPS may be employed to assess whether the PD model can be fully replicated only by eliciting an immune response. If the injection of LPS alone can fully replicate the signs of early-stage PD, it would be a first indication of the role that neuroinflammation plays in PD pathogenesis. Additionally, neuroinflammation is currently only reported in the PC of the early-stage PD model, hence, the OB, PFC, and hippocampus will be examined to assess the extent of neuroinflammation in the early-stage model. Lastly, to better understand the link between neuroinflammation and PD progression, the former will be investigated in further studies at different time points.

4) *Does the increased TH synthesis in the OB of the PD model correlate with a disrupted olfactory circuitry?*

As reported above, DA transmission within the OB exerts an inhibitory effect. Therefore, the observed increase of TH expression in the OB may lead to a suppression of neuronal activity in the very first region where the odour information is processed, ultimately leading to the appearance of hyposmia. To test this hypothesis, as well as the mechanisms underlying it, further studies involving immunohistochemical and electrophysiological techniques will be required. For example, a dopaminergic D2 agonist may be administered to untreated animals to assess whether DA receptors stimulation exerts similar effects. Similarly, a DA receptor antagonist may be employed to test whether hyposmia can be reversed.

5) *What is the exact mechanism by which EX-4 exerts its neuroprotective effects in the* early-stage *PD model, and is there a time window in which EX-4 works optimally?*

Further experiments on the cellular mechanisms of action of EX-4 as well as how GLP1-R activation leads to the prevention of the observed deficits are required to fully understand its neuroprotective effects. For example, both cAMP and cytokine levels may be quantified in specific brain regions, such as the SNpc and PC. Additionally, although the main focus of the project was the characterisation of the early-stage phase, it would be of interest to explore whether treatment with EX-4 is able to delay the appearance of MSs if the model is maintained for a longer period of time, and determine whether there is a specific stage in which EX-4 works optimally.

6) *What is the exact mechanism by which probiotics exert their neuroprotective effects in the* early-stage *model? Is gene therapy a good treatment for PD?*

Currently, no definitive cure for PD has been found and several new treatments and targets are discovered every year. In this thesis, two pilot studies were conducted to test two new potential treatments. Following promising initial results, further experiments will explore and evaluate the therapeutic potential of both treatments. For example, the effects of both drugs on both NMSs and MSs and on neuroinflammation will be investigated using behavioural tests and immunohistochemistry. Additionally, the viral vector will be analysed to assess its biodistribution when injected i.v. as well as the amount of EX-4 produced compared with injection of the exogenous EX-4. Lastly, its tolerability will be investigated for a protracted period of time to ensure its safety.

7.6 Conclusions

The lack of PD animal models representing the early stage of the disease is limiting our current understanding of its aetiology. This Ph.D. project therefore focussed on developing, validating, and characterising an early-stage PD animal model that also served as a tool to test new potential treatments. Hyposmia displayed by this model, was shown to be sustained by the presence of neuroinflammation in the primary olfactory cortex together with several structural changes in the olfactory pathway, such as an altered expression of CBPs in PC, OB, and PFC and an increased synthesis of dopamine in the OB. These results are the first report of some of the deficits/changes occurring within the olfactory circuit during the early stage of the disease. Additionally, treatment with EX-4 was shown to prevent both neuroinflammation and structural changes in the olfactory pathway, suggesting that it may be effective in slowing down the progression of the disease. Similarly, preliminary experiments assessing the effectiveness of a new viral vector expressing EX-4 and probiotic supplementation showed promising results, providing encouraging evidence in the search for a potential new and life-changing treatment for early stage PD.

Bibliography

A Berardelli *et al.* (2001) 'Pathophysiology of Bradykinesia in Parkinson's Disease', *Brain*, pp. 2131–2146. doi: 10.1155/2020/4260501.

Abbott, R. J., Pye, I. F. and Nahorski, S. R. (1982) 'CSF and plasma GABA levels in Parkinson's disease.', *Journal of neurology, neurosurgery, and psychiatry*, 45(3), pp. 253–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7086446 (Accessed: 7 August 2017).

Adipietro, K. A. *et al.* (2012) 'Functional Evolution of Mammalian Odorant Receptors', *PLoS Genetics*. Edited by J. Zhang. Academic Press, 8(7), p. e1002821. doi: 10.1371/journal.pgen.1002821.

Agrawal, N. *et al.* (2003) 'RNA interference: biology, mechanism, and applications.', *Microbiology and molecular biology reviews : MMBR*, 67(4), pp. 657–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14665679 (Accessed: 5 December 2018).

Alpár, A. *et al.* (2006) 'Distribution of pyramidal cells associated with perineuronal nets in the neocortex of rat', *Brain Research*, 1120(1), pp. 13–22. doi: 10.1016/j.brainres.2006.08.069.

Altinayar, S. *et al.* (2014) 'Olfactory disfunction and its relation olfactory bulb volume in Parkinson's disease.', *European review for medical and pharmacological sciences*, 18(23), pp. 3659–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25535137 (Accessed: 6 August 2017).

Aly, A. E.-E. *et al.* (2019) 'Intranasal Delivery of pGDNF DNA Nanoparticles Provides Neuroprotection in the Rat 6-Hydroxydopamine Model of Parkinson's Disease', *Molecular Neurobiology*, 56(1), pp. 688–701. doi: 10.1007/s12035-018-1109-6.

Anderson, A. K. *et al.* (2003) 'Dissociated neural representations of intensity and valence in human olfaction', *Nature Neuroscience*. Nat Neurosci, 6(2), pp. 196–202. doi: 10.1038/nn1001.

Andrews, C. J., Burke, D. and Lance, J. W. (1972) *THE RESPONSE TO MUSCLE STRETCH AND SHORTENING IN PARKINSONIAN RIGIDITY*, *Brain*. Available at: https://academic.oup.com/brain/article-abstract/95/4/795/350726 (Accessed: 22 May 2020).

Appel, S. H. (2012) 'Inflammation in Parkinson's disease: Cause or consequence?', *Movement Disorders*. John Wiley & Sons, Ltd, 27(9), pp. 1075–1077. doi: https://doi.org/10.1002/mds.25111.

Archibald, N. K. *et al.* (2013) 'Visual Exploration in Parkinson's Disease and Parkinson's Disease Dementia', *Brain*, pp. 739–750. Available at: https://pubmed.ncbi.nlm.nih.gov/23436502/ (Accessed: 23 May 2020).

Armstrong, M. J. and Okun, M. S. (2020) 'Diagnosis and Treatment of Parkinson Disease: A Review', *JAMA - Journal of the American Medical Association*, 323(6), pp. 548–560. doi: 10.1001/jama.2019.22360.

Aroniadou-Anderjaska, V. *et al.* (2000) 'Tonic and Synaptically Evoked Presynaptic Inhibition of Sensory Input to the Rat Olfactory Bulb Via GABA $_B$ Heteroreceptors', *Journal of Neurophysiology*, 84(3), pp. 1194–1203. doi: 10.1152/jn.2000.84.3.1194.

Arshad, A. R. *et al.* (2017) 'MicroRNAs and Target Genes As Biomarkers for the Diagnosis of Early Onset of Parkinson Disease.', *Frontiers in molecular neuroscience*, 10, p. 352. doi: 10.3389/fnmol.2017.00352.

Arvanitakis, Z. *et al.* (2007) 'Diabetes and Parkinsonian Signs in Older Persons', *Alzheimer Disease & Associated Disorders*, 21(2). Available at: https://journals.lww.com/alzheimerjournal/Fulltext/2007/04000/Diabetes_and_Parkinsonian_Signs_in_Older_Persons.13.aspx.

Asokan, A., Schaffer, D. V and Jude Samulski, R. (2012) 'The AAV Vector Toolkit: Poised at the Clinical Crossroads', *Molecular Therapy*, 20(4), pp. 699–708. doi: 10.1038/mt.2011.287.

Astrup, A. *et al.* (2012) 'Safety, tolerability and sustained weight loss over 2 years with the once-daily human GLP-1 analog, liraglutide', *International Journal of Obesity*. Nature Publishing Group, 36(6), pp. 843–854. doi: 10.1038/ijo.2011.158.

Athauda, D. *et al.* (2018) 'What effects might exenatide have on non-motor symptoms in Parkinson's disease: A post Hoc analysis', *Journal of Parkinson's Disease*. IOS Press, 8(2), pp. 247–258. doi: 10.3233/JPD-181329.

Athauda, D. *et al.* (2019) 'Utility of Neuronal-Derived Exosomes to Examine Molecular Mechanisms That Affect Motor Function in Patients with Parkinson Disease: A Secondary Analysis of the Exenatide-PD Trial', *JAMA Neurology*. American Medical Association, 76(4), pp. 420–429. doi: 10.1001/jamaneurol.2018.4304.

Athauda, D. and Foltynie, T. (2016) 'The glucagon-like peptide 1 (GLP) receptor as a therapeutic target in Parkinson's disease: mechanisms of action', *Drug Discovery Today*. Elsevier Current Trends, 21(5), pp. 802–818. doi: 10.1016/J.DRUDIS.2016.01.013.

Athauda, D. and Foltynie, T. (2018) 'Protective effects of the GLP-1 mimetic exendin-4 in Parkinson's disease', *Neuropharmacology*. Pergamon, 136, pp. 260–270. doi: 10.1016/J.NEUROPHARM.2017.09.023.

Aviles-Olmos, I. *et al.* (2013) 'Exenatide and the treatment of patients with Parkinson's disease.', *The Journal of clinical investigation*, 123(6), pp. 2730–2736. doi: 10.1172/JCI68295.

Aviles-Olmos, I. *et al.* (2014) 'Motor and cognitive advantages persist 12 months after exenatide exposure in Parkinson's disease.', *Journal of Parkinson's disease*. Netherlands, 4(3), pp. 337–344. doi: 10.3233/JPD-140364.

Axelsen, T. M. and Woldbye, D. P. D. (2018) 'Gene therapy for Parkinson's disease, an update', *Journal of Parkinson's Disease*, 8(2), pp. 195–215. doi: 10.3233/JPD-181331.

Ayers, J. I. *et al.* (2017) 'Robust Central Nervous System Pathology in Transgenic Mice following Peripheral Injection of α-Synuclein Fibrils', *Journal of virology*. American Society for Microbiology, 91(2), pp. e02095-16. doi: 10.1128/JVI.02095-16.

Baig, S., Wilcock, G. K. and Love, S. (2005a) 'Loss of perineuronal net N-acetylgalactosamine in Alzheimer's disease', *Acta Neuropathologica*, 110(4), pp. 393–401. doi: 10.1007/s00401-005-1060-2.

Baig, S., Wilcock, G. K. and Love, S. (2005b) 'Loss of perineuronal net N-acetylgalactosamine in Alzheimer's disease', *Acta Neuropathologica*, 110(4), pp. 393–401. doi: 10.1007/s00401-005-1060-2.

Baker, W. L. *et al.* (2009) 'Dopamine agonists in the treatment of early Parkinson's disease: A meta-analysis', *Parkinsonism & Related Disorders*, 15(4), pp. 287–294. doi: 10.1016/j.parkreldis.2008.07.004.

Balestrino, R. and Schapira, A. H. V. (2020) 'Parkinson disease', *European Journal of Neurology*. Blackwell Publishing Ltd, 27(1), pp. 27–42. doi: 10.1111/ene.14108.

Balmer, T. S. (2016) 'Perineuronal Nets Enhance the Excitability of Fast-Spiking Neurons.', *eNeuro*. Society for Neuroscience, 3(4). doi: 10.1523/ENEURO.0112-16.2016.

Banerjee, A. *et al.* (2015) 'An Interglomerular Circuit Gates Glomerular Output and Implements Gain Control in the Mouse Olfactory Bulb.', *Neuron*, 87(1), pp. 193–207. doi: 10.1016/j.neuron.2015.06.019.

Barichella, M. *et al.* (2016) 'Probiotics and prebiotic fiber for constipation associated with Parkinson disease: An RCT.', *Neurology*. United States, 87(12), pp. 1274–1280. doi: 10.1212/WNL.0000000000003127.

Batista-Brito, R. *et al.* (2008) 'The Distinct Temporal Origins of Olfactory Bulb Interneuron Subtypes', *Journal of Neuroscience*, 28(15), pp. 3966–3975. doi: 10.1523/JNEUROSCI.5625-07.2008.

Beach, T. G. *et al.* (2020) 'Severe hyposmia distinguishes neuropathologically confirmed dementia with Lewy bodies from Alzheimer's disease dementia', *PLoS ONE*. Public Library of Science, 15(4). doi: 10.1371/journal.pone.0231720.

Beal, F. (2010) 'Parkinson's disease: a model dilemma', *Nature*, (Outlooks), pp. S8-10. doi: 10.1038/466S8a.

Beasley, C. L. *et al.* (2002) 'Selective deficits in prefrontal cortical GABAergic neurons in schizophrenia defined by the presence of calcium-binding proteins', *Biological Psychiatry*, 52(7), pp. 708–715. doi: https://doi.org/10.1016/S0006-3223(02)01360-4.

Becker, B. *et al.* (2017) 'Effect of Intrastriatal 6-OHDA Lesions on Extrastriatal Brain Structures in the Mouse'. doi: 10.1007/s12035-017-0637-9.

Bédard, A. and Parent, A. (2004) 'Evidence of newly generated neurons in the human olfactory bulb', *Developmental Brain Research*, 151(1–2), pp. 159–168. doi: 10.1016/j.devbrainres.2004.03.021.

Beitz, J. M. (2014a) 'Parkinson's disease: a review.', *Frontiers in bioscience (Scholar edition)*, 6, pp. 65–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24389262 (Accessed: 9 June 2017).

Beitz, J. M. (2014b) 'Parkinson's disease: a review.', *Frontiers in bioscience (Scholar edition)*, 6, pp. 65–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24389262 (Accessed: 29 November 2018).

Bekkers, J. M. and Suzuki, N. (2013) 'Neurons and circuits for odor processing in the piriform cortex', *Trends in Neurosciences*, 36(7), pp. 429–438. doi: 10.1016/j.tins.2013.04.005.

Belzunegui, S. *et al.* (2007) 'The number of dopaminergic cells is increased in the olfactory bulb of monkeys chronically exposed to MPTP', *Synapse*, 61(12), pp. 1006–1012. doi: 10.1002/syn.20451.

Berendse, H. W. *et al.* (2011) 'Motor and non-motor correlates of olfactory dysfunction in Parkinson's disease', *Journal of the Neurological Sciences*, 310(1–2), pp. 21–24. doi: 10.1016/j.jns.2011.06.020.

Van Den Berge, N. *et al.* (2019) 'Evidence for bidirectional and trans-synaptic parasympathetic and sympathetic propagation of alpha-synuclein in rats', *Acta neuropathologica*. 2019/06/26. Springer Berlin Heidelberg, 138(4), pp. 535–550. doi: 10.1007/s00401-019-02040-w.

Berkowicz, D. A. and Trombley, P. Q. (2000) 'Dopaminergic modulation at the olfactory nerve synapse.', *Brain research*, 855(1), pp. 90–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10650134 (Accessed: 2 December 2018).

Berretta, S. (2012) 'Extracellular matrix abnormalities in schizophrenia', *Neuropharmacology*, 62(3), pp. 1584–1597. doi: 10.1016/j.neuropharm.2011.08.010.

Berretta, S. *et al.* (2015a) 'Losing the sugar coating: Potential impact of perineuronal net abnormalities on interneurons in schizophrenia', *Schizophrenia Research*. Elsevier, 167(1–3), pp. 18–27. doi: 10.1016/J.SCHRES.2014.12.040.

Berretta, S. *et al.* (2015b) 'Losing the sugar coating: Potential impact of perineuronal net abnormalities on interneurons in schizophrenia', *Schizophrenia Research*, 167(1–3), pp. 18–27. doi: 10.1016/j.schres.2014.12.040.

Bertilsson, G. *et al.* (2008) 'Peptide hormone exendin-4 stimulates subventricular zone neurogenesis in the adult rodent brain and induces recovery in an animal model of parkinson's disease', *Journal of Neuroscience Research*. Wiley-Blackwell, 86(2), pp. 326–338. doi: 10.1002/jnr.21483.

Betarbet, R. *et al.* (2000) 'Chronic systemic pesticide exposure reproduces features of Parkinson's disease', *Nature Neuroscience*, 3(12), pp. 1301–1306. doi: 10.1038/81834.

Bezdicek, O. *et al.* (2019) 'Memory impairment in Parkinson's disease: The retrieval versus associative deficit hypothesis revisited and reconciled', *Neuropsychology*. American Psychological Association Inc., 33(3), pp. 391–405. doi: 10.1037/neu0000503.

Bischop, D. P. *et al.* (2012) 'Control of Neuronal Excitability by Calcium Binding Proteins: A New Mathematical Model for Striatal Fast-Spiking Interneurons', *Frontiers in Molecular Neuroscience*. Frontiers, 5, p. 78. doi: 10.3389/fnmol.2012.00078.

Biundo, R., Weis, L. and Antonini, A. (2016) 'Cognitive decline in Parkinson's disease: the complex picture', *npj Parkinson's Disease*. Nature Research, pp. 1–7. doi: 10.1038/npjparkd.2016.18.

Bjarnason, I., Sission, G. and Hayee, B. (2019) 'A randomised, double-blind, placebo-controlled trial of a multi-strain probiotic in patients with asymptomatic ulcerative colitis and Crohn's disease.', *Inflammopharmacology*, 27(3), pp. 465–473. doi: 10.1007/s10787-019-00595-4.

Bjarnason, I., Sisson, G. and Ayis, S. (2012) 'PMO-076 Assessment of the probiotic symprove in patients with IBS: a randomised double blind placebo controlled trial', *Gut*, 61(Suppl 2), p. A104 LP-A104. doi: 10.1136/gutjnl-2012-302514b.76.

Björklund, A. and Dunnett, S. B. (2007) 'Dopamine neuron systems in the brain: an update', *Trends in Neurosciences*, 30(5), pp. 194–202. doi: 10.1016/j.tins.2007.03.006.

Błaszczyk, Janusz W (2016) 'Parkinson's Disease and Neurodegeneration: GABA-Collapse Hypothesis.', *Frontiers in neuroscience*. Frontiers Media SA, 10, p. 269. doi: 10.3389/fnins.2016.00269.

Błaszczyk, Janusz W. (2016) 'Parkinson's Disease and Neurodegeneration: GABA-Collapse Hypothesis', *Frontiers in Neuroscience*, 10, p. 269. doi: 10.3389/fnins.2016.00269.

Blesa, J. *et al.* (2012a) 'Classic and New Animal Models of Parkinson's Disease', *Journal of Biomedicine and Biotechnology*. Hindawi, 2012, pp. 1–10. doi: 10.1155/2012/845618.

Blesa, J. *et al.* (2012b) 'Classic and New Animal Models of Parkinson's Disease', *Journal of Biomedicine and Biotechnology*. Hindawi Publishing Corporation, 2012, pp. 1–10. doi: 10.1155/2012/845618.

Bohnen, Nicolaas I *et al.* (2008) 'Selective hyposmia in Parkinson disease: association with hippocampal dopamine activity.', *Neuroscience letters*. NIH Public Access, 447(1), pp. 12–6. doi: 10.1016/j.neulet.2008.09.070.

Bohnen, Nicolaas I. *et al.* (2008) 'Selective hyposmia in Parkinson disease: Association with hippocampal dopamine activity', *Neuroscience Letters*. Elsevier, 447(1), pp. 12–16. doi:

10.1016/j.neulet.2008.09.070.

Bonfili, L. *et al.* (2017) 'Microbiota modulation counteracts Alzheimer's disease progression influencing neuronal proteolysis and gut hormones plasma levels', *Scientific Reports*, 7(1), p. 2426. doi: 10.1038/s41598-017-02587-2.

Bonito-Oliva, A., Masini, D. and Fisone, G. (2014) 'A mouse model of non-motor symptoms in Parkinson's disease: focus on pharmacological interventions targeting affective dysfunctions', *Frontiers in Behavioral Neuroscience*, 8, p. 290. doi: 10.3389/fnbeh.2014.00290.

Borghammer, P. and Van Den Berge, N. (2019) 'Brain-First versus Gut-First Parkinson's Disease: A Hypothesis', *Journal of Parkinson's Disease*, 9(s2), pp. S281–S295. doi: 10.3233/JPD-191721.

Borisovska, M. *et al.* (2013) 'Distinct modes of dopamine and GABA release in a dual transmitter neuron.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*. NIH Public Access, 33(5), pp. 1790–6. doi: 10.1523/JNEUROSCI.4342-12.2013.

Borovac, J. A. (2016) 'Side effects of a dopamine agonist therapy for Parkinson's disease: a mini-review of clinical pharmacology.', *The Yale journal of biology and medicine*. Yale Journal of Biology and Medicine, 89(1), pp. 37–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27505015 (Accessed: 30 November 2018).

Van Den Bosch, L. *et al.* (2002) 'Protective Effect of Parvalbumin on Excitotoxic Motor Neuron Death', *Experimental Neurology*, 174(2), pp. 150–161. doi: 10.1006/exnr.2001.7858.

Bosco, D. *et al.* (2012) 'Dementia is associated with Insulin Resistance in patients with Parkinson's Disease', *Journal of the Neurological Sciences*, 315(1), pp. 39–43. doi: https://doi.org/10.1016/j.jns.2011.12.008.

Bose, A. and Beal, M. F. (2016) 'Mitochondrial dysfunction in Parkinson's disease', *Journal of Neurochemistry*, 139, pp. 216–231. doi: 10.1111/jnc.13731.

Bouret, S. and Sara, S. J. (2002) 'Locus coeruleus activation modulates firing rate and temporal organization of odour-induced single-cell responses in rat piriform cortex.', *The European journal of neuroscience*, 16(12), pp. 2371–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12492432 (Accessed: 6 August 2017).

Braak, H. *et al.* (2003) 'Staging of brain pathology related to sporadic Parkinson's disease', *Neurobiology of Aging*. Elsevier, 24(2), pp. 197–211. doi: 10.1016/S0197-4580(02)00065-9.

Branch, S. Y. *et al.* (2016) 'Dopaminergic neurons exhibit an age-dependent decline in electrophysiological parameters in the MitoPark mouse model of Parkinson's disease', *Journal of Neuroscience*. Society for Neuroscience, 36(14), pp. 4026–4037. doi: 10.1523/JNEUROSCI.1395-15.2016.

Breid, S. *et al.* (2016) 'Neuroinvasion of α-Synuclein Prionoids after Intraperitoneal and Intraglossal Inoculation', *Journal of virology*. American Society for Microbiology, 90(20), pp. 9182–9193. doi: 10.1128/JVI.01399-16.

Brodoehl, S. *et al.* (2012) 'Decreased olfactory bulb volume in idiopathic Parkinson's disease detected by 3.0-Tesla magnetic resonance imaging', *Movement Disorders*, 27(8), pp. 1019–1025. doi: 10.1002/mds.25087.

Brück, A. *et al.* (2004) 'Hippocampal and prefrontal atrophy in patients with early non-demented Parkinson's disease is related to cognitive impairment', *Journal of Neurology, Neurosurgery and Psychiatry*. BMJ Publishing Group Ltd, 75(10), pp. 1467–1469. doi: 10.1136/jnnp.2003.031237.

Buddhala, C. *et al.* (2015) 'Dopaminergic, serotonergic, and noradrenergic deficits in Parkinson disease.', *Annals of clinical and translational neurology*, 2(10), pp. 949–959. doi: 10.1002/acn3.246.

Bullitt, E. (1990) 'Expression ofC-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat', *The Journal of Comparative Neurology*, 296(4), pp. 517–530. doi: 10.1002/cne.902960402.

Burke, R. E., Dauer, W. T. and Vonsattel, J. P. G. (2008) 'A critical evaluation of the Braak staging scheme for Parkinson's disease', *Annals of Neurology*. Ann Neurol, pp. 485–491. doi: 10.1002/ana.21541.

Cabezas, R. *et al.* (2014) 'Astrocytic modulation of blood brain barrier: perspectives on Parkinsonâ€™s disease', *Frontiers in Cellular Neuroscience*. Frontiers, 8, p. 211. doi: 10.3389/fncel.2014.00211.

Cabezas, R. *et al.* (2016) 'Growth Factors and Astrocytes Metabolism: Possible Roles for Platelet Derived Growth Factor.', *Medicinal chemistry (Shariqah (United Arab Emirates))*, 12(3), pp. 204–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26477707 (Accessed: 5 December 2018).

Cabezas, R. *et al.* (2018) 'Growth Factors and Neuroglobin in Astrocyte Protection Against Neurodegeneration and Oxidative Stress', *Molecular Neurobiology*. Springer US, pp. 1–13. doi: 10.1007/s12035-018-1203-9.

Cabungcal, J.-H. *et al.* (2013) 'Perineuronal nets protect fast-spiking interneurons against oxidative stress', *Proceedings of the National Academy of Sciences*, 110(22), pp. 9130–9135. doi: 10.1073/pnas.1300454110.

del Campo, N. *et al.* (2011) 'The Roles of Dopamine and Noradrenaline in the Pathophysiology and Treatment of Attention-Deficit/Hyperactivity Disorder', *Biological Psychiatry*, 69(12), pp. e145–e157. doi: 10.1016/j.biopsych.2011.02.036.

Campos, F. L. *et al.* (2013) 'Rodent models of Parkinson's disease: beyond the motor symptomatology', *Frontiers in behavioral neuroscience*. Frontiers Media S.A., 7, p. 175. doi: 10.3389/fnbeh.2013.00175.

Carulli, D. *et al.* (2006) 'Composition of perineuronal nets in the adult rat cerebellum and the cellular origin of their components', *The Journal of Comparative Neurology*, 494(4), pp. 559–577. doi: 10.1002/cne.20822.

Carvalho, M. M. *et al.* (2013) 'Behavioral characterization of the 6-hydroxidopamine model of Parkinson's disease and pharmacological rescuing of non-motor deficits', *Molecular Neurodegeneration*, 8(1), p. 14. doi: 10.1186/1750-1326-8-14.

Cassani, E. *et al.* (2011) 'Use of probiotics for the treatment of constipation in Parkinson's disease patients.', *Minerva gastroenterologica e dietologica*. Italy, 57(2), pp. 117–121.

Castelli, V. *et al.* (2020) 'Effects of the probiotic formulation SLAB51 in in vitro and in vivo Parkinson's disease models', *Aging*. 2020/03/09. Impact Journals, 12(5), pp. 4641–4659. doi: 10.18632/aging.102927.

Castelli, V. *et al.* (2021) 'The emerging role of probiotics in neurodegenerative diseases: New hope for Parkinson's disease?', *Neural Regeneration Research*, 16(4), pp. 628–634. doi: 10.4103/1673-5374.295270.

Castrioto, A. *et al.* (2014) 'The pathogenesis of Pisa syndrome in Parkinson's disease', *Movement Disorders*. John Wiley and Sons Inc., pp. 1100–1107. doi: 10.1002/mds.25925.

Celardo, I., Martins, L. M. and Gandhi, S. (2014) 'Unravelling mitochondrial pathways to

Parkinson's disease', *British Journal of Pharmacology*. John Wiley and Sons Inc., 171(8), pp. 1943–1957. doi: 10.1111/bph.12433.

Cereda, E. *et al.* (2012) 'Clinical features of Parkinson disease when onset of diabetes came first', *Neurology*, 78(19), pp. 1507 LP – 1511. doi: 10.1212/WNL.0b013e3182553cc9.

Chakraborty, A. and Diwan, A. (2020) 'The Neuroscience Chronicles Parkinson's disease: cell therapy vs. gene therapy', *Neurosci Chron*, 1(1), pp. 6–10.

Chaudhury, D., Escanilla, O. and Linster, C. (2009) 'Bulbar acetylcholine enhances neural and perceptual odor discrimination', *Journal of Neuroscience*. Society for Neuroscience, 29(1), pp. 52–60. doi: 10.1523/JNEUROSCI.4036-08.2009.

Chen, A. Y. *et al.* (2014) 'Olfactory Deficits in an Alpha-Synuclein Fly Model of Parkinson's Disease', *PLoS ONE*. Edited by M. B. Feany. Public Library of Science, 9(5), p. e97758. doi: 10.1371/journal.pone.0097758.

Chen, L. *et al.* (2014) 'SK channel blockade reverses cognitive and motor deficits induced by nigrostriatal dopamine lesions in rats.', *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)*, 17(8), pp. 1295–306. doi: 10.1017/S1461145714000236.

Chen, Y. *et al.* (2015) 'Neuroprotective effects of geniposide in the MPTP mouse model of Parkinson's disease', *European Journal of Pharmacology*, 768, pp. 21–27. doi: 10.1016/j.ejphar.2015.09.029.

Chen, Y., Lü, Y.-H. and Li, Z.-F. (2014) '[Advances in the application of gene therapy for Parkinson's disease with adeno-associated virus].', *Yao xue xue bao = Acta pharmaceutica Sinica*, 49(5), pp. 576–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25151724 (Accessed: 4 December 2018).

Chiaravalloti, N. D. *et al.* (2014) 'The source of the memory impairment in Parkinson's disease: Acquisition versus retrieval', *Movement Disorders*. John Wiley and Sons Inc., 29(6), pp. 765–771. doi: 10.1002/mds.25842.

Chiu, W.-H. *et al.* (2014) 'Selegiline normalizes, while l-DOPA sustains the increased number of dopamine neurons in the olfactory bulb in a 6-OHDA mouse model of Parkinson's disease', *Neuropharmacology*, 79, pp. 212–221. doi: 10.1016/j.neuropharm.2013.11.014.

Chou, K. L. *et al.* (2018) 'The spectrum of "off" in Parkinson's disease: What have we learned over 40 years?', *Parkinsonism and Related Disorders*. Elsevier Ltd, pp. 9–16. doi: 10.1016/j.parkreldis.2018.02.001.

Chowen, J. A. *et al.* (1999) 'Increased glucagon-like peptide-1 receptor expression in glia after mechanical lesion of the rat brain', *Neuropeptides*, 33(3), pp. 212–215. doi: 10.1054/npep.1999.0757.

Christine, C. W. *et al.* (2019) 'Magnetic resonance imaging-guided phase 1 trial of putaminal AADC gene therapy for Parkinson's disease', *Annals of neurology*. 2019/03/26. John Wiley & Sons, Inc., 85(5), pp. 704–714. doi: 10.1002/ana.25450.

Christopher, L. *et al.* (2014) 'Combined insular and striatal dopamine dysfunction are associated with executive deficits in Parkinson's disease with mild cognitive impairment', *Brain*. Oxford University Press, 137(2), pp. 565–575. doi: 10.1093/brain/awt337.

Cicchetti, F. *et al.* (2002) 'Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging.', *The European journal of neuroscience*, 15(6), pp. 991–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11918659 (Accessed: 13 August 2017).

Ciranna, L. (2006) 'Serotonin as a modulator of glutamate- and GABA-mediated neurotransmission: implications in physiological functions and in pathology.', *Current neuropharmacology*. Bentham Science Publishers, 4(2), pp. 101–14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18615128 (Accessed: 7 August 2017).

Cools, R. *et al.* (2001) 'Enhanced or impaired cognitive function in Parkinson's disease as a function of dopaminergic medication and task demands', *Cerebral Cortex*. Oxford University Press, 11(12), pp. 1136–1143. doi: 10.1093/cercor/11.12.1136.

Cools, R. *et al.* (2002) 'Dopaminergic modulation of high-level cognition in Parkinson's disease: The role of the prefrontal cortex revealed by PET', *Brain*. Oxford University Press, 125(3), pp. 584–594. doi: 10.1093/brain/awf052.

Cork, S. C. *et al.* (2015) 'Distribution and characterisation of Glucagon-like peptide-1 receptor expressing cells in the mouse brain', *Molecular Metabolism*. Elsevier, 4(10), pp. 718–731. doi: 10.1016/J.MOLMET.2015.07.008.

Coune, P. G., Schneider, B. L. and Aebischer, P. (2012) 'Parkinson's disease: gene therapies.', *Cold Spring Harbor perspectives in medicine*. Cold Spring Harbor Laboratory Press, 2(4), p. a009431. doi: 10.1101/cshperspect.a009431.

Crapser, J. D. *et al.* (2020) 'Microglia facilitate loss of perineuronal nets in the Alzheimer's disease brain', *EBioMedicine*. Elsevier, 58. doi: 10.1016/j.ebiom.2020.102919.

Cryan, J. F. and Dinan, T. G. (2012) 'Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour', *Nature Reviews Neuroscience*. Nature Publishing Group, 13(10), pp. 701–712. doi: 10.1038/nrn3346.

D'Amelio, M., Puglisi-Allegra, S. and Mercuri, N. (2018) 'The role of dopaminergic midbrain in Alzheimer's disease: Translating basic science into clinical practice', *Pharmacological Research*. Academic Press, 130, pp. 414–419. doi: 10.1016/J.PHRS.2018.01.016.

Daniels, D. and Mietlicki-Baase, E. G. (2019) 'Glucagon-Like Peptide 1 in the Brain: Where Is It Coming From, Where Is It Going?', *Diabetes*, pp. 15–17. doi: 10.2337/dbi18-0045.

Datiche, F. and Cattarelli, M. (1996) 'Catecholamine innervation of the piriform cortex: a tracing and immunohistochemical study in the rat', *Brain Research*. Elsevier, 710(1–2), pp. 69–78. doi: 10.1016/0006-8993(95)01279-6.

Dauer, W. and Przedborski, S. (2003) 'Parkinson's disease: mechanisms and models.', *Neuron*, 39(6), pp. 889–909. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12971891 (Accessed: 20 August 2018).

Davalos, D. *et al.* (2005) 'ATP mediates rapid microglial response to local brain injury in vivo', *Nature Neuroscience*, 8(6), pp. 752–758. doi: 10.1038/nn1472.

Dave, K. D. *et al.* (2014) 'Phenotypic characterization of recessive gene knockout rat models of Parkinson's disease', *Neurobiology of Disease*. Academic Press, 70, pp. 190–203. doi: 10.1016/J.NBD.2014.06.009.

Delaville, C., Deurwaerdère, P. De and Benazzouz, A. (2011a) 'Noradrenaline and Parkinson's disease.', *Frontiers in systems neuroscience*. Frontiers Media SA, 5, p. 31. doi: 10.3389/fnsys.2011.00031.

Delaville, C., Deurwaerdère, P. De and Benazzouz, A. (2011b) 'Noradrenaline and Parkinson's Disease', *Frontiers in Systems Neuroscience*. Frontiers, 5, p. 31. doi: 10.3389/fnsys.2011.00031.

Delzenne, N. M. and Cani, P. D. (2011) 'Interaction between obesity and the gut microbiota: relevance in nutrition.', *Annual review of nutrition*. United States, 31, pp. 15–31. doi:

10.1146/annurev-nutr-072610-145146.

Desplats, P. *et al.* (2012) 'α-Synuclein induces alterations in adult neurogenesis in Parkinson disease models via p53-mediated repression of notch', *Journal of Biological Chemistry*. JBC Papers in Press, 287(38), pp. 31691–31702. doi: 10.1074/jbc.M112.354522.

Deverman, B. E. *et al.* (2018) 'Gene therapy for neurological disorders: progress and prospects', *Nature Reviews Drug Discovery*. Nature Publishing Group, 17(9), pp. 641–659. doi: 10.1038/nrd.2018.110.

Devi, L. *et al.* (2008) 'Mitochondrial Import and Accumulation of α-Synuclein Impair Complex I in Human Dopaminergic Neuronal Cultures and Parkinson Disease Brain', *Journal of Biological Chemistry*, 283(14), pp. 9089–9100. doi: 10.1074/jbc.M710012200.

Devore, S., Manella, L. C. and Linster, C. (2012) 'Blocking muscarinic receptors in the olfactory bulb impairs performance on an olfactory short-term memory task', *Frontiers in Behavioral Neuroscience*. Frontiers, 6(AUGUST), p. 59. doi: 10.3389/fnbeh.2012.00059.

Devos, D. *et al.* (2013) 'Colonic inflammation in Parkinson's disease.', *Neurobiology of disease*. United States, 50, pp. 42–48. doi: 10.1016/j.nbd.2012.09.007.

Diaz Heijtz, R. *et al.* (2011) 'Normal gut microbiota modulates brain development and behavior.', *Proceedings of the National Academy of Sciences of the United States of America*, 108(7), pp. 3047–3052. doi: 10.1073/pnas.1010529108.

Dirnberger, G., Frith, C. D. and Jahanshahi, M. (2005) 'Executive dysfunction in Parkinson's disease is associated with altered pallidal-frontal processing', *NeuroImage*. Academic Press Inc., 25(2), pp. 588–599. doi: 10.1016/j.neuroimage.2004.11.023.

Distrutti, E. *et al.* (2013) 'Probiotics VSL#3 protect against development of visceral pain in murine model of irritable bowel syndrome', *PloS one*. Public Library of Science, 8(5), pp. e63893–e63893. doi: 10.1371/journal.pone.0063893.

Doder, M. *et al.* (2003) 'Tremor in Parkinson's disease and serotonergic dysfunction: An 11C-WAY 100635 PET study', *Neurology*. Lippincott Williams and Wilkins, 60(4), pp. 601–605. doi: 10.1212/01.WNL.0000031424.51127.2B.

Le Dorze, C. *et al.* (2018) 'Behavioral and Noradrenergic Sensitizations in Vulnerable Traumatized Rats Suggest Common Bases with Substance Use Disorders', *Molecular Neurobiology*. Springer US, pp. 1–10. doi: 10.1007/s12035-018-1053-5.

Doty, R. L. *et al.* (1992) 'Bilateral olfactory dysfunction in early stage treated and untreated idiopathic Parkinson's disease.', *Journal of neurology, neurosurgery, and psychiatry*, 55(2), pp. 138–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1538221 (Accessed: 4 August 2017).

Doty, R. L. (2012a) 'Olfaction in Parkinson's disease and related disorders.', *Neurobiol Dis.*, 46(3), pp. 527–552. doi: 10.1016/j.nbd.2011.10.026.Olfaction.

Doty, R. L. (2012b) 'Olfaction in Parkinson's disease and related disorders', *Neurobiology of Disease*, 46(3), pp. 527–552. doi: 10.1016/j.nbd.2011.10.026.

Doty, R. L. (2017) 'Olfactory dysfunction in neurodegenerative diseases: is there a common pathological substrate?', *The Lancet Neurology*, 16(6), pp. 478–488. doi: 10.1016/S1474-4422(17)30123-0.

Drouin-Ouellet, J. and Cicchetti, F. (2012) 'Inflammation and neurodegeneration: the story "retolled"', *Trends in Pharmacological Sciences*, 33(10), pp. 542–551. doi: 10.1016/j.tips.2012.07.002.

Dunbar, M.D, C. E. (1996) 'GENE TRANSFER TO HEMATOPOIETIC STEM CELLS: Implications for Gene Therapy of Human Disease', *Annual Review of Medicine*, 47(1), pp. 11–20. doi: 10.1146/annurev.med.47.1.11.

Dunbar, C. E. *et al.* (2018) 'Gene therapy comes of age.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 359(6372), p. eaan4672. doi: 10.1126/science.aan4672.

Dutta, G., Zhang, P. and Liu, B. (2008) 'The lipopolysaccharide Parkinson's disease animal model: mechanistic studies and drug discovery', *Fundamental & clinical pharmacology*, 22(5), pp. 453–464. doi: 10.1111/j.1472-8206.2008.00616.x.The.

Dutta, S. K. *et al.* (2019) 'Parkinson's disease: The emerging role of gut dysbiosis, antibiotics, probiotics, and fecal microbiota transplantation', *Journal of Neurogastroenterology and Motility*, 25(3), pp. 363–376. doi: 10.5056/jnm19044.

Duty, S. *et al.* (2020) 'Potential of animal models for advancing the understanding and treatment of pain in Parkinson's disease', *Nature Parkinson's Journal*, 6(1), pp. 1–7. doi: 10.1038/s41531-019-0104-6.

Dwir, D. *et al.* (2020) 'MMP9/RAGE pathway overactivation mediates redox dysregulation and neuroinflammation, leading to inhibitory/excitatory imbalance: a reverse translation study in schizophrenia patients', *Molecular Psychiatry*, 25(11), pp. 2889–2904. doi: 10.1038/s41380-019-0393-5.

Dzyubenko, E. *et al.* (2018) 'Topological remodeling of cortical perineuronal nets in focal cerebral ischemia and mild hypoperfusion', *Matrix Biology*. Elsevier, 74, pp. 121–132. doi: 10.1016/J.MATBIO.2018.08.001.

Eberling, J. L. *et al.* (2009) 'Functional Effects of AAV2-GDNF on the Dopaminergic Nigrostriatal Pathway in Parkinsonian Rhesus Monkeys', *Human Gene Therapy*, 20(5), pp. 511–518. doi: 10.1089/hum.2008.201.

Van Den Eeden, S. K. *et al.* (2003) 'Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity.', *American journal of epidemiology*, 157(11), pp. 1015–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12777365 (Accessed: 28 November 2018).

Eisinger, R. S. *et al.* (2017) 'Motor subtype changes in early Parkinson's disease.', *Parkinsonism & related disorders*. NIH Public Access, 43, pp. 67–72. doi: 10.1016/j.parkreldis.2017.07.018.

Ekstrand, J. J. *et al.* (2001) 'Immunocytochemical analysis of basket cells in rat piriform cortex', *The Journal of Comparative Neurology*. John Wiley & Sons, Inc., 434(3), pp. 308–328. doi: 10.1002/cne.1179.

Ekstrand, M. I. and Galter, D. (2009) 'The MitoPark Mouse - An animal model of Parkinson's disease with impaired respiratory chain function in dopamine neurons', *Parkinsonism and Related Disorders*. Parkinsonism Relat Disord, 15(SUPPL. 3). doi: 10.1016/S1353-8020(09)70811-9.

Emamzadeh, F. N. and Surguchov, A. (2018) 'Parkinson's Disease: Biomarkers, Treatment, and Risk Factors', *Frontiers in neuroscience*. Frontiers Media S.A., 12, p. 612. doi: 10.3389/fnins.2018.00612.

Emborg, M. E. (2007) 'Nonhuman primate models of Parkinson's disease', *ILAR Journal*. Oxford University Press, pp. 339–355. doi: 10.1093/ilar.48.4.339.

Enç, F. Y. *et al.* (2001) 'Inhibition of gastric emptying by acarbose is correlated with GLP-1 response and accompanied by CCK release', *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 281(3), pp. G752–G763. doi:

10.1152/ajpgi.2001.281.3.G752.

Engelender, S. and Isacson, O. (2017) 'The Threshold Theory for Parkinson's Disease', *Trends in Neurosciences*. Elsevier Current Trends, 40(1), pp. 4–14. doi: 10.1016/J.TINS.2016.10.008.

Engert, V. and Pruessner, J. C. (2008) 'Dopaminergic and noradrenergic contributions to functionality in ADHD: the role of methylphenidate.', *Current neuropharmacology*. Bentham Science Publishers, 6(4), pp. 322–8. doi: 10.2174/157015908787386069.

Escanilla, O. *et al.* (2009) 'Dopaminergic modulation of olfactory bulb processing affects odor discrimination learning in rats.', *Behavioral neuroscience*, 123(4), pp. 828–833. doi: 10.1037/a0015855.

Espay, A. J., LeWitt, P. A. and Kaufmann, H. (2014) 'Norepinephrine deficiency in Parkinson's disease: The case for noradrenergic enhancement', *Movement Disorders*. John Wiley & Sons, Ltd, 29(14), pp. 1710–1719. doi: https://doi.org/10.1002/mds.26048.

F. Rainer Von Coelln, Erik Barr, Ann Gruber-Baldini, Stephen Reich, Melissa Armstrong, L. S. *et al.* (2015) 'Motor Subtypes of Parkinson Disease are Unstable Over Time', *Neurology*. Advanstar Communications, 84(14 Supplement), p. S48.002. Available at: http://n.neurology.org/content/84/14_Supplement/S48.002 (Accessed: 29 November 2018).

Faggiani, E., Delaville, C. and Benazzouz, A. (2015) 'The combined depletion of monoamines alters the effectiveness of subthalamic deep brain stimulation.', *Neurobiology of disease*. United States, 82, pp. 342–348. doi: 10.1016/j.nbd.2015.07.010.

Fallon, J. H., Riley, J. N. and Moore, R. Y. (1978) 'Substantia nigra dopamine neurons: separate populations project to neostriatum and allocortex.', *Neuroscience letters*, 7(2–3), pp. 157–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19605105 (Accessed: 6 August 2017).

Fama, R. *et al.* (2000) 'Extent, pattern, and correlates of remote memory impairment in Alzheimer's disease and Parkinson's disease.', *Neuropsychology*. American Psychological Association (APA), 14(2), pp. 265–276. doi: 10.1037//0894-4105.14.2.265.

Fang, C. *et al.* (2020) 'Cognition Deficits in Parkinson's Disease: Mechanisms and Treatment', *Hindawi*. doi: 10.1155/2020/2076942.

Fang, X. (2019) 'Microbial treatment: the potential application for Parkinson's disease.', *Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology*. Italy, 40(1), pp. 51–58. doi: 10.1007/s10072-018-3641-6.

Felix, T. M., Karpa, K. D. and Lewis, P. R. (2015) 'Adverse Effects of Common Drugs: Dietary Supplements.', *FP essentials*. United States, 436, pp. 31–40.

Ferreira, M. and Massano, J. (2017) 'An updated review of Parkinson's disease genetics and clinicopathological correlations', *Acta Neurologica Scandinavica*. Wiley/Blackwell (10.1111), 135(3), pp. 273–284. doi: 10.1111/ane.12616.

Fiorelli, R. *et al.* (2015) 'Adding a spatial dimension to postnatal ventricular-subventricular zone neurogenesis', *Development*, 142(12), pp. 2109–2120. doi: 10.1242/dev.119966.

Flood, P., Arbabzada, N. and Sharma, M. (2016) 'Inflammation: Role in Parkinson's Disease and Target for Therapy', in *Challenges in Parkinson's Disease*. InTech. doi: 10.5772/63164.

Foo, H. *et al.* (2016) 'Associations of hippocampal subfields in the progression of cognitive decline related to Parkinson's disease', *NeuroImage: Clinical*. Elsevier Inc., 14, pp. 37–42. doi: 10.1016/j.nicl.2016.12.008.

Forsyth, C. B. *et al.* (2011) 'Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease.', *PloS one*, 6(12), p. e28032. doi: 10.1371/journal.pone.0028032.

Foster, H. D. and Hoffer, A. (2004) 'The two faces of L-DOPA: benefits and adverse side effects in the treatment of Encephalitis lethargica, Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis', *Medical Hypotheses*, 62(2), pp. 177–181. doi: 10.1016/S0306-9877(03)00318-9.

Fredua-Agyeman, M. and Gaisford, S. (2015) 'Comparative survival of commercial probiotic formulations: tests in biorelevant gastric fluids and real-time measurements using microcalorimetry.', *Beneficial microbes*. Netherlands, 6(1), pp. 141–151. doi: 10.3920/BM2014.0051.

Frielingsdorf, H. *et al.* (2004) 'No evidence for new dopaminergic neurons in the adult mammalian substantia nigra', *Proceedings of the National Academy of Sciences*, 101(27), pp. 10177–10182. doi: 10.1073/pnas.0401229101.

Furini, C. R. G. *et al.* (2017) 'Extinction memory is facilitated by methylphenidate and regulated by dopamine and noradrenaline receptors', *Behavioural Brain Research*. Elsevier, 326, pp. 303–306. doi: 10.1016/J.BBR.2017.03.027.

Gall, C. M. *et al.* (1987) 'Evidence for coexistence of GABA and dopamine in neurons of the rat olfactory bulb.', *The Journal of comparative neurology*, 266(3), pp. 307–18. doi: 10.1002/cne.902660302.

Gao, H.-M. *et al.* (2008) 'Neuroinflammation and Oxidation/Nitration of -Synuclein Linked to Dopaminergic Neurodegeneration', *Journal of Neuroscience*, 28(30), pp. 7687–7698. doi: 10.1523/JNEUROSCI.0143-07.2008.

Garbayo, E. *et al.* (2009) 'Effective GDNF brain delivery using microspheres-A promising strategy for Parkinson's disease', *Journal of Controlled Release*. J Control Release, 135(2), pp. 119–126. doi: 10.1016/j.jconrel.2008.12.010.

Garbayo, E. *et al.* (2011) 'Long-term neuroprotection and neurorestoration by glial cell-derived neurotrophic factor microspheres for the treatment of Parkinson's disease', *Movement Disorders*. John Wiley & Sons, Ltd, 26(10), pp. 1943–1947. doi: 10.1002/mds.23793.

Garbayo, E. *et al.* (2016) 'Brain delivery of microencapsulated GDNF induces functional and structural recovery in parkinsonian monkeys', *Biomaterials*. Elsevier Ltd, 110, pp. 11–23. doi: 10.1016/j.biomaterials.2016.09.015.

Garcia-Ruiz, P. J. *et al.* (2014) 'Impulse control disorder in patients with Parkinson's disease under dopamine agonist therapy: A multicentre study', *Journal of Neurology, Neurosurgery and Psychiatry*. BMJ Publishing Group, 85(8), pp. 841–845. doi: 10.1136/jnnp-2013-306787.

Garcia, R. A. *et al.* (2014) 'P2Y6 receptor potentiates pro-inflammatory responses in macrophages and exhibits differential roles in atherosclerotic lesion development.', *PloS one*. Public Library of Science, 9(10), p. e111385. doi: 10.1371/journal.pone.0111385.

Gareau, M. G., Silva, M. A. and Perdue, M. H. (2008) 'Pathophysiological mechanisms of stress-induced intestinal damage.', *Current molecular medicine*. Netherlands, 8(4), pp. 274–281. doi: 10.2174/156652408784533760.

Gazerani, P. (2019) 'Probiotics for Parkinson Disease Marked.Pdf'.

Gelders, G., Baekelandt, V. and Van der Perren, A. (2018a) 'Linking Neuroinflammation and Neurodegeneration in Parkinson's Disease', *Journal of Immunology Research*. Hindawi, 2018, pp. 1–12. doi: 10.1155/2018/4784268.

Gelders, G., Baekelandt, V. and Van der Perren, A. (2018b) 'Linking Neuroinflammation and Neurodegeneration in Parkinson's Disease', *Journal of Immunology Research*. Hindawi, 2018, pp. 1–12. doi: 10.1155/2018/4784268.

Gellman, R. L. and Aghajanian, G. K. (1993) 'Pyramidal cells in piriform cortex receive a convergence of inputs from monoamine activated GABAergic interneurons.', *Brain research*, 600(1), pp. 63–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8422591 (Accessed: 6 August 2017).

Gessler, D. J. *et al.* (2019) 'Intravenous Infusion of AAV for Widespread Gene Delivery to the Nervous System', *Methods in molecular biology (Clifton, N.J.)*, 1950, pp. 143–163. doi: 10.1007/978-1-4939-9139-6_8.

Ghyselinck, J. *et al.* (2020) 'A 4-strain probiotic supplement influences gut microbiota composition and gut wall function in patients with ulcerative colitis.', *International journal of pharmaceutics*. Netherlands, 587, p. 119648. doi: 10.1016/j.ijpharm.2020.119648.

Goldman, J. G. *et al.* (2018) 'Cognitive impairment in Parkinson's disease: a report from a multidisciplinary symposium on unmet needs and future directions to maintain cognitive health', *npj Parkinson's Disease*. Springer US, 4(1), pp. 1–11. doi: 10.1038/s41531-018-0055-3.

Gonçalves, G. A. R. and Paiva, R. de M. A. (2017) 'Gene therapy: advances, challenges and perspectives.', *Einstein (Sao Paulo, Brazil)*. Instituto de Ensino e Pesquisa Albert Einstein, 15(3), pp. 369–375. doi: 10.1590/S1679-45082017RB4024.

Grabenhorst, F. *et al.* (2007) 'How pleasant and unpleasant stimuli combine in different brain regions: Odor mixtures', *Journal of Neuroscience*. Society for Neuroscience, 27(49), pp. 13532–13540. doi: 10.1523/JNEUROSCI.3337-07.2007.

Grieco, M. *et al.* (2019) 'Glucagon-Like Peptide-1: A Focus on Neurodegenerative Diseases', *Frontiers in Neuroscience*. Frontiers Media S.A., 13, p. 1112. doi: 10.3389/fnins.2019.01112.

Grow, D. A., McCarrey, J. R. and Navara, C. S. (2016) 'Advantages of nonhuman primates as preclinical models for evaluating stem cell-based therapies for Parkinson's disease', *Stem Cell Research*. Elsevier B.V., pp. 352–366. doi: 10.1016/j.scr.2016.08.013.

Gülpinar, M. A. *et al.* (2000) 'Glucagon-like peptide (GLP-1) is involved in the central modulation of fecal output in rats', *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 278(6), pp. G924–G929. doi: 10.1152/ajpgi.2000.278.6.G924.

Györfi, O. *et al.* (2017) 'Reduced CA2-CA3 Hippocampal Subfield Volume Is Related to Depression and Normalized by l-DOPA in Newly Diagnosed Parkinson's Disease.', *Frontiers in neurology*, 8, p. 84. doi: 10.3389/fneur.2017.00084.

Hagiwara, A. *et al.* (2012) 'Optophysiological analysis of associational circuits in the olfactory cortex', *Frontiers in Neural Circuits*, 6, p. 18. doi: 10.3389/fncir.2012.00018.

Halász, N. *et al.* (1981) 'Immunohistochemical identification of two types of dopamine neuron in the rat olfactory bulb as seen by serial sectioning', *Journal of Neurocytology*, 10(2), pp. 251–259. doi: 10.1007/BF01257970.

Hall, S. D. *et al.* (2010) 'GABA(A) alpha-1 subunit mediated desynchronization of elevated low frequency oscillations alleviates specific dysfunction in stroke – A case report', *Clinical Neurophysiology*, 121(4), pp. 549–555. doi: https://doi.org/10.1016/j.clinph.2009.11.084.

Hall, S. D. *et al.* (2014) 'GABA-mediated changes in inter-hemispheric beta frequency activity in early-stage Parkinson's disease', *Neuroscience*, 281, pp. 68–76. doi: https://doi.org/10.1016/j.neuroscience.2014.09.037.

Hallett, M. (2012) 'Parkinson's disease tremor: Pathophysiology', *Parkinsonism and Related Disorders*. Elsevier Ltd. doi: 10.1016/s1353-8020(11)70027-x.

Harkavyi, A. *et al.* (2008) 'Glucagon-like peptide 1 receptor stimulation by exendin-4 reverses key deficits in distinct rodent models of Parkinson's disease', *Journal of Neuroinflammation*, 5(1), p. 19. doi: 10.1186/1742-2094-5-19.

Harrington, D. L. *et al.* (2011) 'Neurobehavioral mechanisms of temporal processing deficits in Parkinson's disease', *PLoS ONE*. PLoS One, 6(2). doi: 10.1371/journal.pone.0017461.

Hassanzadeh, K. and Rahimmi, A. (2019) 'Oxidative stress and neuroinflammation in the story of Parkinson's disease: Could targeting these pathways write a good ending?', *Journal of Cellular Physiology*. John Wiley & Sons, Ltd, 234(1), pp. 23–32. doi: 10.1002/jcp.26865.

Hasselmo, M. E. *et al.* (no date) 'Noradrenergic Suppression of Synaptic Transmission May Influence Cortical Signal-to-Noise Ratio'. Available at: http://jn.physiology.org/content/jn/77/6/3326.full.pdf (Accessed: 6 August 2017).

Hawkes, C. H. and Shephard, B. C. (1993) 'Selective anosmia in Parkinson's disease?', *Lancet*. Elsevier, p. 436. doi: 10.1016/0140-6736(93)93027-X.

Hawkes, C. H., Del Tredici, K. and Braak, H. (2009) 'Parkinson's Disease', *Annals of the New York Academy of Sciences*. Wiley/Blackwell (10.1111), 1170(1), pp. 615–622. doi: 10.1111/j.1749-6632.2009.04365.x.

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

He, X. J. and Nakayama, H. (2009) 'Neurogenesis in Neurotoxin-induced Animal Models for Parkinson's Disease-A Review of the Current Status.', *Journal of toxicologic pathology*. The Japanese Society of Toxicologic Pathology, 22(2), pp. 101–8. doi: 10.1293/tox.22.101.

Healy, D. G. *et al.* (2008) 'Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study', *The Lancet Neurology*. Elsevier, 7(7), pp. 583–590. doi: 10.1016/S1474-4422(08)70117-0.

Heiss, J. D. *et al.* (2019) 'Trial of magnetic resonance-guided putaminal gene therapy for advanced Parkinson's disease', *Movement disorders : official journal of the Movement Disorder Society*. 2019/05/30, 34(7), pp. 1073–1078. doi: 10.1002/mds.27724.

Helmich, R. C. *et al.* (2012) 'Cerebral causes and consequences of parkinsonian resting tremor: a tale of two circuits?', *Brain : a journal of neurology*. 2012/03/01. Oxford University Press, 135(Pt 11), pp. 3206–3226. doi: 10.1093/brain/aws023.

Hely, M. A. *et al.* (2008) 'The Sydney multicenter study of Parkinson's disease: The inevitability of dementia at 20 years', *Movement Disorders*, 23(6), pp. 837–844. doi: 10.1002/mds.21956.

Henderson, J. L. *et al.* (2015) 'Discovery and preclinical profiling of 3-[4-(morpholin-4-yl)-7H-pyrrolo[2,3-d]pyrimidin-5-yl]benzonitrile (PF-06447475), a highly potent, selective, brain penetrant, and in vivo active LRRK2 kinase inhibitor', *Journal of Medicinal Chemistry*. American Chemical Society, 58(1), pp. 419–432. doi: 10.1021/jm5014055.

Hernán, M. A., Logroscino, G. and García Rodríguez, L. A. (2006) 'Nonsteroidal anti-inflammatory drugs and the incidence of Parkinson disease.', *Neurology*. Wolters Kluwer Health, Inc. on behalf of the American Academy of Neurology, 66(7), pp. 1097–9. doi: 10.1212/01.wnl.0000204446.82823.28.

Hernandez-Baltazar, D., Zavala-Flores, L. M. and Villanueva-Olivo, A. (2017) 'The 6-hydroxydopamine model and parkinsonian pathophysiology: Novel findings in an older model',

Neurología (English Edition). Elsevier Doyma, 32(8), pp. 533–539. doi: 10.1016/J.NRLENG.2015.06.019.

Hernandez, N. S. *et al.* (2017) 'Activation of glucagon-like peptide-1 receptors in the nucleus accumbens attenuates cocaine seeking in rats', *Addiction Biology*. doi: 10.1111/adb.12583.

Hernandez, N. S. *et al.* (2018) 'Glucagon-like peptide-1 receptor activation in the ventral tegmental area attenuates cocaine seeking in rats', *Neuropsychopharmacology*, 43(10), pp. 2000–2008. doi: 10.1038/s41386-018-0010-3.

Hill-Burns, E. M. *et al.* (2017) 'Parkinson's disease and Parkinson's disease medications have distinct signatures of the gut microbiome.', *Movement disorders : official journal of the Movement Disorder Society*, 32(5), pp. 739–749. doi: 10.1002/mds.26942.

Hinderer, C. *et al.* (2018) 'Evaluation of Intrathecal Routes of Administration for Adeno-Associated Viral Vectors in Large Animals', *Human gene therapy*. 2017/10/03. Mary Ann Liebert, Inc., 29(1), pp. 15–24. doi: 10.1089/hum.2017.026.

Hirsch, E. C. and Hunot, S. (2009) 'Neuroinflammation in Parkinson's disease: a target for neuroprotection?', *The Lancet Neurology*, 8(4), pp. 382–397. doi: 10.1016/S1474-4422(09)70062-6.

Holmqvist, S. *et al.* (2014) 'Direct evidence of Parkinson pathology spread from the gastrointestinal tract to the brain in rats', *Acta Neuropathologica*, 128(6), pp. 805–820. doi: 10.1007/s00401-014-1343-6.

Holst, J. J., Burcelin, R. and Nathanson, E. (2011) 'Neuroprotective properties of GLP-1: theoretical and practical applications', *Current Medical Research and Opinion*, 27(3), pp. 547–558. doi: 10.1185/03007995.2010.549466.

Hoogland, J. *et al.* (2017) 'Mild cognitive impairment as a risk factor for Parkinson's disease dementia', *Movement Disorders*. John Wiley and Sons Inc., 32(7), pp. 1056–1065. doi: 10.1002/mds.27002.

Hornung, J.-P. (2003) 'The human raphe nuclei and the serotonergic system.', *Journal of chemical neuroanatomy*, 26(4), pp. 331–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14729135 (Accessed: 30 November 2018).

Hou, J.-G. G. and Lai, E. C. (2007) 'Non-motor Symptoms of Parkinson's Disease', *International Journal of Gerontology*, 1(2), pp. 53–64. doi: 10.1016/S1873-9598(08)70024-3.

Houser, M. C. *et al.* (2018) 'Stool Immune Profiles Evince Gastrointestinal Inflammation in Parkinson's Disease', *Movement disorders : official journal of the Movement Disorder Society*. 2018/03/23, 33(5), pp. 793–804. doi: 10.1002/mds.27326.

Howell, M. J. and Schenck, C. H. (2015) 'Rapid eyemovement sleep behavior disorder and neurodegenerative disease', *JAMA Neurology*. American Medical Association, pp. 707–712. doi: 10.1001/jamaneurol.2014.4563.

Hsia, a Y., Vincent, J. D. and Lledo, P. M. (1999) 'Dopamine depresses synaptic inputs into the olfactory bulb.', *Journal of neurophysiology*, 82(2), pp. 1082–1085.

Hsieh, T.-H. *et al.* (2020) 'Probiotics Alleviate the Progressive Deterioration of Motor Functions in a Mouse Model of Parkinson's Disease', *Brain sciences*. MDPI, 10(4), p. 206. doi: 10.3390/brainsci10040206.

Hu, G. *et al.* (2007) 'Type 2 Diabetes and the Risk of Parkinson's Disease', *Diabetes Care*, 30(4), pp. 842 LP – 847. doi: 10.2337/dc06-2011.

Huang, C. *et al.* (2007) 'Metabolic brain networks associated with cognitive function in

Parkinson's disease', *NeuroImage*. Neuroimage, 34(2), pp. 714–723. doi: 10.1016/j.neuroimage.2006.09.003.

Huang, L. *et al.* (2013) 'Reciprocal connectivity between mitral cells and external plexiform layer interneurons in the mouse olfactory bulb', *Frontiers in Neural Circuits*, 7, p. 32. doi: 10.3389/fncir.2013.00032.

Huang, Z., Thiebaud, N. and Fadool, D. A. (2017) 'Differential serotonergic modulation across the main and accessory olfactory bulbs', *Journal of Physiology*. Blackwell Publishing Ltd, 595(11), pp. 3515–3533. doi: 10.1113/JP273945.

Huisman, E., Uylings, H. B. M. and Hoogland, P. V. (2004) 'A 100% increase of dopaminergic cells in the olfactory bulb may explain hyposmia in parkinson's disease', *Movement Disorders*, 19(6), pp. 687–692. doi: 10.1002/mds.10713.

Huisman, E., Uylings, H. B. M. and Hoogland, P. V. (2008) 'Gender-related changes in increase of dopaminergic neurons in the olfactory bulb of Parkinson's disease patients', *Movement Disorders*, 23(10), pp. 1407–1413. doi: 10.1002/mds.22009.

Hurley, M. J. *et al.* (2013) 'Parkinson's disease is associated with altered expression of CaV1 channels and calcium-binding proteins', *Brain*, 136(7), pp. 2077–2097. doi: 10.1093/brain/awt134.

Hutson, T. H. *et al.* (2012) 'Corticospinal tract transduction: a comparison of seven adeno-associated viral vector serotypes and a non-integrating lentiviral vector.', *Gene therapy*. Europe PMC Funders, 19(1), pp. 49–60. doi: 10.1038/gt.2011.71.

Hwang, E. J. *et al.* (2019) 'Magnetic resonance imaging assessment of the substrate for hyposmia in patients with Parkinson's disease', *Clinical Radiology*. W.B. Saunders Ltd, 74(6), pp. 489.e9-489.e15. doi: 10.1016/j.crad.2019.02.003.

Iannilli, E. *et al.* (2017) 'Olfactory impairment in Parkinson's disease is a consequence of central nervous system decline', *Journal of Neurology*. Dr. Dietrich Steinkopff Verlag GmbH and Co. KG, 264(6), pp. 1236–1246. doi: 10.1007/s00415-017-8521-0.

Ilkiw, J. L. *et al.* (2018) 'Dopaminergic Lesion in the Olfactory Bulb Restores Olfaction and Induces Depressive-Like Behaviors in a 6-OHDA Model of Parkinson's Disease', *Molecular Neurobiology*. Molecular Neurobiology, pp. 1–14. doi: 10.1007/s12035-018-1134-5.

Ille, R. *et al.* (2015) 'Role of Disgust Proneness in Parkinson's Disease: A Voxel-Based Morphometry Study', *Journal of the International Neuropsychological Society*, 21(04), pp. 314–317. doi: 10.1017/S135561771500017X.

Imbriani, P. *et al.* (2018) 'Promising rodent models in Parkinson's disease', *Parkinsonism & Related Disorders*. Elsevier, 46, pp. S10–S14. doi: 10.1016/J.PARKRELDIS.2017.07.027.

Isacson, R. *et al.* (2011) 'The glucagon-like peptide 1 receptor agonist exendin-4 improves reference memory performance and decreases immobility in the forced swim test.', *European journal of pharmacology*. Netherlands, 650(1), pp. 249–255. doi: 10.1016/j.ejphar.2010.10.008.

Iwasawa, C. *et al.* (2019) 'Reduced expression of somatostatin in GABAergic interneurons derived from induced pluripotent stem cells of patients with parkin mutations', *Molecular Brain*, 12(1), p. 5. doi: 10.1186/s13041-019-0426-7.

Izco, M. *et al.* (2019) 'Systemic Exosomal Delivery of shRNA Minicircles Prevents Parkinsonian Pathology', *Molecular Therapy*, 27(12), pp. 2111–2122. doi: https://doi.org/10.1016/j.ymthe.2019.08.010.

Jacob, S. N. and Nienborg, H. (2018) 'Monoaminergic Neuromodulation of Sensory

Processing.', *Frontiers in neural circuits*. Frontiers Media SA, 12, p. 51. doi: 10.3389/fncir.2018.00051.

Jagmag, S. A. *et al.* (2016) 'Evaluation of Models of Parkinson's Disease', *Frontiers in Neuroscience | www.frontiersin.org*, 1, p. 503. doi: 10.3389/fnins.2015.00503.

Jahanshahi, M. *et al.* (2010) 'Dopaminergic modulation of striato-frontal connectivity during motor timing in Parkinson's disease', *Brain*. Brain, 133(3), pp. 727–745. doi: 10.1093/brain/awq012.

Jamebozorgi, K. *et al.* (2018) 'Cellular and Molecular Aspects of Parkinson Treatment: Future Therapeutic Perspectives', *Molecular Neurobiology*. Springer US, pp. 1–13. doi: 10.1007/s12035-018-1419-8.

Jangi, S. *et al.* (2016) 'Alterations of the human gut microbiome in multiple sclerosis.', *Nature communications*, 7, p. 12015. doi: 10.1038/ncomms12015.

Jankovic, J. (2008) 'Parkinson's disease: clinical features and diagnosis', *Journal of Neurology, Neurosurgery & Psychiatry*, 79(4), pp. 368–376. doi: 10.1136/jnnp.2007.131045.

Janvin, C. C. *et al.* (2006) 'Subtypes of mild cognitive impairment in Parkinson's disease: Progression to dementia', *Movement Disorders*. Mov Disord, 21(9), pp. 1343–1349. doi: 10.1002/mds.20974.

Jarraya, B. *et al.* (2009) 'Dopamine Gene Therapy for Parkinson's Disease in a Nonhuman Primate Without Associated Dyskinesia', *Science Translational Medicine*, 1(2), pp. 2ra4-2ra4. doi: 10.1126/scitranslmed.3000130.

Jawabri, K. H. and Cascella, M. (2020) *Physiology, Explicit Memory*, *StatPearls*. StatPearls Publishing. Available at: http://www.ncbi.nlm.nih.gov/pubmed/32119438 (Accessed: 25 November 2020).

Jordan, J. T. (2020) 'The rodent hippocampus as a bilateral structure: A review of hemispheric lateralization', *Hippocampus*. John Wiley and Sons Inc., 30(3), pp. 278–292. doi: 10.1002/hipo.23188.

Kadohisa, M. (2013) 'Effects of odor on emotion, with implications', *Frontiers in Systems Neuroscience*, 7(OCT). doi: 10.3389/fnsys.2013.00066.

Kato, H. K. *et al.* (2013) 'Parvalbumin-Expressing Interneurons Linearly Control Olfactory Bulb Output', *Neuron*. Oxford University Press, New York, 80(5), pp. 1218–1231. doi: 10.1016/j.neuron.2013.08.036.

Kawaguchi, Y. and Shindou, T. (no date) 'Noradrenergic Excitation and Inhibition of GABAergic Cell Types in Rat Frontal Cortex'. Available at: https://pdfs.semanticscholar.org/790a/2dfe7634983bec58aee34d6e49938179549f.pdf (Accessed: 6 August 2017).

Kay, D. M. *et al.* (2007) 'Heterozygous *parkin* point mutations are as common in control subjects as in Parkinson's patients', *Annals of Neurology*. John Wiley & Sons, Ltd, 61(1), pp. 47–54. doi: 10.1002/ana.21039.

Kesby, J. *et al.* (2018) 'Dopamine, psychosis and schizophrenia: the widening gap between basic and clinical neuroscience', *Translational Psychiatry*. Nature Publishing Group, 8(1), p. 30. doi: 10.1038/s41398-017-0071-9.

Kesner, R. P., Gilbert, P. E. and Barua, L. A. (2002) 'The role of the hippocampus in memory for the temporal order of a sequence of odors.', *Behavioral Neuroscience*. American Psychological Association (APA), 116(2), pp. 286–290. doi: 10.1037//0735-7044.116.2.286.

van Kessel, S. P. *et al.* (2019) 'Gut bacterial tyrosine decarboxylases restrict levels of levodopa in the treatment of Parkinson's disease.', *Nature communications*, 10(1), p. 310. doi: 10.1038/s41467-019-08294-y.

Khakpour-Taleghani, B. *et al.* (2009) 'Effect of reversible inactivation of locus ceruleus on spatial reference and working memory', *Neuroscience*, 158(4), pp. 1284–1291. doi: 10.1016/j.neuroscience.2008.11.001.

Killinger, B. A. and Kordower, J. H. (2019) 'Spreading of alpha-synuclein - relevant or epiphenomenon?', *Journal of neurochemistry*. England, 150(5), pp. 605–611. doi: 10.1111/jnc.14779.

Kim, D. S. *et al.* (2017a) 'A New Treatment Strategy for Parkinson's Disease through the Gut–Brain Axis', *Cell Transplantation*, 26(9), pp. 1560–1571. doi: 10.1177/0963689717721234.

Kim, D. S. *et al.* (2017b) 'A New Treatment Strategy for Parkinson's Disease through the Gut–Brain Axis', *Cell Transplantation*. SAGE PublicationsSage CA: Los Angeles, CA, 26(9), pp. 1560–1571. doi: 10.1177/0963689717721234.

Kim, J. Y., Choe, J. and Moon, C. (2020) 'Distinct developmental features of olfactory bulb interneurons', *Molecules and Cells*. Korean Society for Molecular and Cellular Biology, pp. 215–221. doi: 10.14348/molcells.2020.0033.

Kim, N. *et al.* (2018) 'Mind-altering with the gut: Modulation of the gut-brain axis with probiotics.', *Journal of microbiology (Seoul, Korea)*. Korea (South), 56(3), pp. 172–182. doi: 10.1007/s12275-018-8032-4.

Kim, S. *et al.* (2019) 'Transneuronal Propagation of Pathologic α-Synuclein from the Gut to the Brain Models Parkinson's Disease', *Neuron*. 2019/06/26, 103(4), pp. 627-641.e7. doi: 10.1016/j.neuron.2019.05.035.

Kim, S., Moon, M. and Park, S. (2009) 'Exendin-4 protects dopaminergic neurons by inhibition of microglial activation and matrix metalloproteinase-3 expression in an animal model of Parkinson's disease', *Journal of Endocrinology*, 202(3), pp. 431–439. doi: 10.1677/JOE-09-0132.

Kim, S. Y. *et al.* (2017) 'TGFβ signaling is associated with changes in inflammatory gene expression and perineuronal net degradation around inhibitory neurons following various neurological insults', *Scientific Reports*. Nature Publishing Group, 7(1), p. 7711. doi: 10.1038/s41598-017-07394-3.

Kim, Y.-K., Kim, O. Y. and Song, J. (2020) 'Alleviation of Depression by Glucagon-Like Peptide 1 Through the Regulation of Neuroinflammation, Neurotransmitters, Neurogenesis, and Synaptic Function', *Frontiers in pharmacology*. Frontiers Media S.A., 11, p. 1270. doi: 10.3389/fphar.2020.01270.

Klaenhammer, T. R. *et al.* (2012) 'The impact of probiotics and prebiotics on the immune system.', *Nature reviews. Immunology*. England, pp. 728–734. doi: 10.1038/nri3312.

Klempin, F. *et al.* (2011) 'Properties of Doublecortin-(DCX)-Expressing Cells in the Piriform Cortex Compared to the Neurogenic Dentate Gyrus of Adult Mice', *PLoS ONE*. Edited by C. V. Borlongan, 6(10), p. e25760. doi: 10.1371/journal.pone.0025760.

Klingelhoefer, L. and Reichmann, H. (2015) 'Pathogenesis of Parkinson disease—the gut–brain axis and environmental factors', *Nature Reviews Neurology*. Nature Publishing Group, 11(11), pp. 625–636. doi: 10.1038/nrneurol.2015.197.

Knudsen, K. *et al.* (2017) 'Constipation in parkinson's disease: Subjective symptoms, objective markers, and new perspectives.', *Movement disorders : official journal of the Movement Disorder Society*. United States, 32(1), pp. 94–105. doi: 10.1002/mds.26866.

Kohl, Z. *et al.* (2016) 'Severely impaired hippocampal neurogenesis associates with an early serotonergic deficit in a BAC α-synuclein transgenic rat model of Parkinson's disease', *Neurobiology of disease*. 2015/10/30, 85, pp. 206–217. doi: 10.1016/j.nbd.2015.10.021.

Kolli, N. *et al.* (2018) 'Application of the gene editing tool, CRISPR-Cas9, for treating neurodegenerative diseases', *Neurochemistry International*. Pergamon, 112, pp. 187–196. doi: 10.1016/J.NEUINT.2017.07.007.

Konnova, E. A. and Swanberg, M. (2018) *Parkinson's Disease Pathogenesis and Clinical Aspects*. Available at: http://dx.doi.org/10.15586/codonpublications.parkinsonsdisease.2018.ch5 (Accessed: 23 May 2020).

Kosaka, K. and Kosaka, T. (2005) 'Synaptic organization of the glomerulus in the main olfactory bulb: Compartments of the glomerulus and heterogeneity of the periglomerular cells', *Anatomical Science International*, 80(2), pp. 80–90. doi: 10.1111/j.1447-073x.2005.00092.x.

Kosaka, T. and Kosaka, K. (2010) 'Olfactory Bulb Anatomy', *Encyclopedia of Neuroscience*, pp. 59–69. doi: 10.1016/B978-008045046-9.01686-7.

Kosaka, T. and Kosaka, K. (2016) 'Neuronal organization of the main olfactory bulb revisited', *Anatomical Science International*. Springer Japan, 91(2), pp. 115–127. doi: 10.1007/s12565-015-0309-7.

Kotecha, A. M. *et al.* (2018) 'Olfactory Dysfunction as a Global Biomarker for Sniffing out Alzheimer's Disease: A Meta-Analysis', *Biosensors*. MDPI, 8(2), p. 41. doi: 10.3390/bios8020041.

Krause, B., Márquez-Ruiz, J. and Kadosh, R. C. (2013) 'The effect of transcranial direct current stimulation: a role for cortical excitation/inhibition balance?', *Frontiers in Human Neuroscience*, 7, p. 602. doi: 10.3389/fnhum.2013.00602.

Kumar, S. R. *et al.* (2016) 'Clinical development of gene therapy: results and lessons from recent successes.', *Molecular therapy. Methods & clinical development*. Elsevier, 3, p. 16034. doi: 10.1038/mtm.2016.34.

Kvasnovsky, C. L. *et al.* (2017) 'A randomized double-blind placebo-controlled trial of a multi-strain probiotic in treatment of symptomatic uncomplicated diverticular disease.', *Inflammopharmacology*. Switzerland. doi: 10.1007/s10787-017-0363-y.

La, C. *et al.* (2019) 'Hippocampal CA1 subfield predicts episodic memory impairment in Parkinson's disease', *NeuroImage: Clinical*. Elsevier Inc., 23, p. 101824. doi: 10.1016/j.nicl.2019.101824.

Lanoue, A. C., Blatt, G. J. and Soghomonian, J.-J. (2013) 'Decreased parvalbumin mRNA expression in dorsolateral prefrontal cortex in Parkinson's disease.', *Brain research*, 1531, pp. 37–47. doi: 10.1016/j.brainres.2013.07.025.

Larsson, M. and Willander, J. (2009) 'Autobiographical odor memory', in *Annals of the New York Academy of Sciences*. Blackwell Publishing Inc., pp. 318–323. doi: 10.1111/j.1749-6632.2009.03934.x.

LeBlanc, J. G. *et al.* (2013) 'Bacteria as vitamin suppliers to their host: a gut microbiota perspective.', *Current opinion in biotechnology*. England, 24(2), pp. 160–168. doi: 10.1016/j.copbio.2012.08.005.

Ledreux, A. *et al.* (2016) 'BDNF levels are increased by aminoindan and rasagiline in a double lesion model of Parkinson's disease.', *Brain research*. Netherlands, 1631, pp. 34–45. doi: 10.1016/j.brainres.2015.11.028.

Lee, E.-Y. *et al.* (2014) 'Olfactory-related cortical atrophy is associated with olfactory dysfunction in Parkinson's disease', *Movement Disorders*, 29(9), pp. 1205–1208. doi: 10.1002/mds.25829.

Lee, H.-J. *et al.* (2010) 'Direct Transfer of α-Synuclein from Neuron to Astroglia Causes Inflammatory Responses in Synucleinopathies', *Journal of Biological Chemistry*, 285(12), pp. 9262–9272. doi: 10.1074/jbc.M109.081125.

Lee, J. M. *et al.* (2012) 'Postural instability and cognitive dysfunction in early Parkinson's disease.', *The Canadian journal of neurological sciences. Le journal canadien des sciences neurologiques*, 39(4), pp. 473–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22728854 (Accessed: 29 November 2018).

Lema Tomé, C. M. *et al.* (2013) 'Inflammation and α-Synuclein's Prion-like Behavior in Parkinson's Disease—Is There a Link?', *Molecular Neurobiology*, 47(2), pp. 561–574. doi: 10.1007/s12035-012-8267-8.

Lendvai, D. *et al.* (2013) 'Neurochemical mapping of the human hippocampus reveals perisynaptic matrix around functional synapses in Alzheimer's disease', *Acta Neuropathologica*, 125(2), pp. 215–229. doi: 10.1007/s00401-012-1042-0.

Lensjø, K. K. *et al.* (2017) 'Removal of Perineuronal Nets Unlocks Juvenile Plasticity Through Network Mechanisms of Decreased Inhibition and Increased Gamma Activity', *The Journal of Neuroscience*, 37(5), pp. 1269–1283. doi: 10.1523/JNEUROSCI.2504-16.2016.

Lescheid, D. W. (2014) 'Probiotics as regulators of inflammation: A review', *Functional Foods in Health and Disease*, 4(7), pp. 299–311. doi: 10.31989/ffhd.v4i7.2.

Levite, M., Marino, F. and Cosentino, M. (2017) 'Dopamine, T cells and multiple sclerosis (MS)', *Journal of Neural Transmission*. Springer Vienna, 124(5), pp. 525–542. doi: 10.1007/s00702-016-1640-4.

LeWitt, P. A. *et al.* (2011) 'AAV2-GAD gene therapy for advanced Parkinson's disease: a double-blind, sham-surgery controlled, randomised trial', *The Lancet Neurology*, 10(4), pp. 309–319. doi: 10.1016/S1474-4422(11)70039-4.

Li, H. *et al.* (2015) 'Neuroprotective effects of exendin-4 in rat model of spinal cord injury via inhibiting mitochondrial apoptotic pathway.', *International journal of clinical and experimental pathology*, 8(5), pp. 4837–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26191175 (Accessed: 6 November 2018).

Li, J. *et al.* (2016) 'Changes in Olfactory Bulb Volume in Parkinson's Disease: A Systematic Review and Meta-Analysis', *PLOS ONE*. Edited by R. E. Jung. Wiley-Blackwell, 11(2), p. e0149286. doi: 10.1371/journal.pone.0149286.

Li, M. *et al.* (2019) 'Impaired D2 receptor-dependent dopaminergic transmission in prefrontal cortex of awake mouse model of Parkinson's disease', *Brain*. Oxford University Press, 142(10), pp. 3099–3115. doi: 10.1093/brain/awz243.

Li, Y. *et al.* (2009) 'GLP-1 receptor stimulation preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinsonism', *Proceedings of the National Academy of Sciences*, 106(4), pp. 1285–1290. doi: 10.1073/pnas.0806720106.

Liddelow, S. A. *et al.* (2017) 'Neurotoxic reactive astrocytes are induced by activated microglia.', *Nature*. Nature Publishing Group, 541(7638), pp. 481–487. doi: 10.1038/nature21029.

Liddle, R. A. (2018) 'Parkinson's disease from the gut.', *Brain research*, 1693(Pt B), pp. 201–206. doi: 10.1016/j.brainres.2018.01.010.

van Lier, B., Hierlemann, A. and Knoflach, F. (2018) 'Parvalbumin expression and gamma oscillation occurrence increase over time in a neurodevelopmental model of NMDA receptor dysfunction', *PeerJ*, 6, p. e5543. doi: 10.7717/peerj.5543.

Lisman, J. *et al.* (2017) 'Viewpoints: How the hippocampus contributes to memory, navigation and cognition', *Nature Neuroscience*. Nature Publishing Group, pp. 1434–1447. doi: 10.1038/nn.4661.

Liu, B. *et al.* (2002) 'Role of nitric oxide in inflammation-mediated neurodegeneration.', *Annals of the New York Academy of Sciences*, 962, pp. 318–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12076984 (Accessed: 4 August 2017).

Liu, B. *et al.* (2017) 'Vagotomy and Parkinson disease: A Swedish register-based matched-cohort study', *Neurology*. 2017/04/26. Lippincott Williams & Wilkins, 88(21), pp. 1996–2002. doi: 10.1212/WNL.0000000000003961.

Liu, B. *et al.* (2021) 'Irritable bowel syndrome and Parkinson's disease risk: register-based studies', *npj Parkinson's Disease*, 7(1), p. 5. doi: 10.1038/s41531-020-00145-8.

Liu, H. *et al.* (2013) 'Perineuronal nets increase inhibitory GABAergic currents during the critical period in rats.', *International journal of ophthalmology*, 6(2), pp. 120–5. doi: 10.3980/j.issn.2222-3959.2013.02.02.

Lledo, P.-M. and Valley, M. (2016) 'Adult Olfactory Bulb Neurogenesis', *Cold Spring Harbor Perspectives in Biology*, 8(8), p. a018945. doi: 10.1101/cshperspect.a018945.

Lodovichi, C. and Belluscio, L. (no date) 'Odorant Receptors in the Formation of the Olfactory Bulb Circuitry Organization of the Mammalian Olfactory System'. doi: 10.1152/physiol.00015.2012.

Lohmann, E. *et al.* (2003) 'How much phenotypic variation can be attributed to *parkin* genotype?', *Annals of Neurology*. John Wiley & Sons, Ltd, 54(2), pp. 176–185. doi: 10.1002/ana.10613.

Lohmann, S. *et al.* (2019) 'Oral and intravenous transmission of α-synuclein fibrils to mice', *Acta neuropathologica*. 2019/06/22. Springer Berlin Heidelberg, 138(4), pp. 515–533. doi: 10.1007/s00401-019-02037-5.

Lorenzo Bozzelli, P. *et al.* (2018) 'Proteolytic Remodeling of Perineuronal Nets: Effects on Synaptic Plasticity and Neuronal Population Dynamics.', *Neural plasticity*. Hindawi Limited, 2018, p. 5735789. doi: 10.1155/2018/5735789.

Louter, M. *et al.* (2013) 'Sleep matters in Parkinson's disease: Use of a priority list to assess the presence of sleep disturbances', *European Journal of Neurology*. Eur J Neurol, 20(2), pp. 259–265. doi: 10.1111/j.1468-1331.2012.03836.x.

Lubomski, M. *et al.* (2020) 'Parkinson's disease and the gastrointestinal microbiome', *Journal of Neurology*, 267(9), pp. 2507–2523. doi: 10.1007/s00415-019-09320-1.

Luo, D. *et al.* (2016) 'Novel multifunctional dopamine D 2 /D 3 receptors agonists with potential neuroprotection and anti-alpha synuclein protein aggregation properties', *Bioorganic & Medicinal Chemistry*, 24(21), pp. 5088–5102. doi: 10.1016/j.bmc.2016.08.021.

Maes, M. (2008) 'The cytokine hypothesis of depression: inflammation, oxidative & nitrosative stress (IO&NS) and leaky gut as new targets for adjunctive treatments in depression.', *Neuro endocrinology letters*. Sweden, 29(3), pp. 287–291.

Magistrelli, L. *et al.* (2019) 'Probiotics may have beneficial effects in Parkinson's disease: In vitro evidence', *Frontiers in Immunology*, 10(May), pp. 1–9. doi: 10.3389/fimmu.2019.00969.

Magrinelli, F. *et al.* (2016) 'Pathophysiology of Motor Dysfunction in Parkinson's Disease as the Rationale for Drug Treatment and Rehabilitation'. doi: 10.1155/2016/9832839.

Maidan, I. *et al.* (2018) 'Evidence for Differential Effects of 2 Forms of Exercise on Prefrontal Plasticity During Walking in Parkinson's Disease', *Neurorehabilitation and Neural Repair*. SAGE Publications Inc., 32(3), pp. 200–208. doi: 10.1177/1545968318763750.

Maier, J. X., Wachowiak, M. and Katz, D. B. (2012) 'Chemosensory Convergence on Primary Olfactory Cortex', *Journal of Neuroscience*, 32(48). Available at: http://www.jneurosci.org/content/32/48/17037 (Accessed: 6 August 2017).

Maiti, P., Manna, J. and Dunbar, G. L. (2017) 'Current understanding of the molecular mechanisms in Parkinson's disease: Targets for potential treatments.', *Translational neurodegeneration*. BioMed Central, 6, p. 28. doi: 10.1186/s40035-017-0099-z.

Maldonado Galdeano, C. *et al.* (2019) 'Beneficial Effects of Probiotic Consumption on the Immune System', *Annals of Nutrition and Metabolism*, 74(2), pp. 115–124. doi: 10.1159/000496426.

Malenka, R. C. and Nicoll, R. A. (1986) 'Dopamine decreases the calcium-activated afterhyperpolarization in hippocampal CA1 pyramidal cells.', *Brain research*, 379(2), pp. 210–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3017510 (Accessed: 6 August 2017).

Malvaut, S. and Saghatelyan, A. (2016) 'The Role of Adult-Born Neurons in the Constantly Changing Olfactory Bulb Network', *Neural Plasticity*, 2016, pp. 1–8. doi: 10.1155/2016/1614329.

De Marchis, S. *et al.* (2007) 'Generation of Distinct Types of Periglomerular Olfactory Bulb Interneurons during Development and in Adult Mice: Implication for Intrinsic Properties of the Subventricular Zone Progenitor Population', *Journal of Neuroscience*, 27(3), pp. 657–664. doi: 10.1523/JNEUROSCI.2870-06.2007.

Marcinkiewicz, J. *et al.* (2007) 'Differential inflammatory mediator response in vitro from murine macrophages to lactobacilli and pathogenic intestinal bacteria', *International Journal of Experimental Pathology*. John Wiley & Sons, Ltd, 88(3), pp. 155–164. doi: https://doi.org/10.1111/j.1365-2613.2007.00530.x.

Marek, G. J. and Aghajanian, G. K. (1994) 'Excitation of interneurons in piriform cortex by 5-hydroxytryptamine: blockade by MDL 100,907, a highly selective 5-HT2A receptor antagonist.', *European journal of pharmacology*, 259(2), pp. 137–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7957607 (Accessed: 7 August 2017).

Marin, C. *et al.* (2017) 'Olfactory function in an excitotoxic model for secondary neuronal degeneration: Role of dopaminergic interneurons', *Neuroscience*. Elsevier Ltd, 364, pp. 28–44. doi: 10.1016/j.neuroscience.2017.09.008.

Marin, C. *et al.* (2018) 'Olfactory Dysfunction in Neurodegenerative Diseases', *Current Allergy and Asthma Reports*. Current Medicine Group LLC 1, pp. 1–19. doi: 10.1007/s11882-018-0796-4.

Marks Jr, W. J. *et al.* (2008) 'Safety and tolerability of intraputaminal delivery of CERE-120 (adeno-associated virus serotype 2–neurturin) to patients with idiopathic Parkinson's disease: an open-label, phase I trial', *The Lancet Neurology*. Elsevier, 7(5), pp. 400–408. doi: 10.1016/S1474-4422(08)70065-6.

Marras, C. *et al.* (2016) 'Nomenclature of genetic movement disorders: Recommendations of the international Parkinson and movement disorder society task force', *Movement Disorders*, 31(4), pp. 436–457. doi: 10.1002/mds.26527.

Masurkar, A. V. and Chen, W. R. (2010) 'Olfactory Bulb Physiology', *Encyclopedia of*

Neuroscience, pp. 77–86. doi: 10.1016/B978-008045046-9.01687-9.

Matsuda, W. *et al.* (2009) 'Single Nigrostriatal Dopaminergic Neurons Form Widely Spread and Highly Dense Axonal Arborizations in the Neostriatum'. doi: 10.1523/JNEUROSCI.4029-08.2009.

Matuskova, M. and Durinikov, E. (2016) 'Retroviral Vectors in Gene Therapy', in *Advances in Molecular Retrovirology*. InTech. doi: 10.5772/61844.

McKenzie, J. *et al.* (2017) 'Neuroinflammation as a Common Mechanism Associated with the Modifiable Risk Factors for Alzheimer's and Parkinson`s Diseases', *Current Aging Science*, 10(999), pp. 1–1. doi: 10.2174/1874609810666170315113244.

Mehta, S. H. and Tanner, C. M. (2016) 'Role of Neuroinflammation in Parkinson Disease: The Enigma Continues.', *Mayo Clinic proceedings*. Elsevier, 91(10), pp. 1328–1330. doi: 10.1016/j.mayocp.2016.08.010.

Merola, A. *et al.* (2020) 'Gene therapy for Parkinson's disease: contemporary practice and emerging concepts', *Expert Review of Neurotherapeutics*. Taylor & Francis, 20(6), pp. 577–590. doi: 10.1080/14737175.2020.1763794.

Mesholam, R. I. *et al.* (1998) 'Olfaction in neurodegenerative disease: a meta-analysis of olfactory functioning in Alzheimer's and Parkinson's diseases.', *Archives of neurology*, 55(1), pp. 84–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9443714 (Accessed: 4 August 2017).

Mishra, A. *et al.* (2019) 'Dopamine D1 receptor activation improves adult hippocampal neurogenesis and exerts anxiolytic and antidepressant-like effect via activation of Wnt/β-catenin pathways in rat model of Parkinson's disease', *Neurochemistry International*. Elsevier Ltd, 122, pp. 170–186. doi: 10.1016/j.neuint.2018.11.020.

Miyamichi, K. *et al.* (2013) 'Dissecting Local Circuits: Parvalbumin Interneurons Underlie Broad Feedback Control of Olfactory Bulb Output', *Neuron*, 80(5), pp. 1232–1245. doi: 10.1016/j.neuron.2013.08.027.

Moretti, D. V. (2019) 'Available and future treatments for atypical parkinsonism. A systematic review', *CNS Neuroscience and Therapeutics*, 25(2), pp. 159–174. doi: 10.1111/cns.13068.

Mori, K. (2010) 'Olfactory Bulb Mapping', *Encyclopedia of Neuroscience*, pp. 71–75. doi: 10.1016/B978-008045046-9.01688-0.

Morishita, H. *et al.* (2015) 'Prolonged Period of Cortical Plasticity upon Redox Dysregulation in Fast-Spiking Interneurons', *Biological Psychiatry*, 78(6), pp. 396–402. doi: 10.1016/j.biopsych.2014.12.026.

Morris, N. P. and Henderson, Z. (2000) 'Perineuronal nets ensheath fast spiking, parvalbumin-immunoreactive neurons in the medial septum/diagonal band complex.', *The European journal of neuroscience*, 12(3), pp. 828–38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10762312 (Accessed: 7 August 2017).

Moustafa, A. A. *et al.* (2016) 'Motor symptoms in Parkinson's disease: A unified framework', *Neuroscience & Biobehavioral Reviews*. Pergamon, 68, pp. 727–740. doi: 10.1016/J.NEUBIOREV.2016.07.010.

Müller, M. L. T. M. and Bohnen, N. I. (2013) 'Cholinergic dysfunction in parkinson's disease', *Current Neurology and Neuroscience Reports*. Curr Neurol Neurosci Rep, 13(9). doi: 10.1007/s11910-013-0377-9.

Mundiñano, I.-C. *et al.* (2011) 'Increased dopaminergic cells and protein aggregates in the olfactory bulb of patients with neurodegenerative disorders', *Acta Neuropathologica*, 122(1),

pp. 61–74. doi: 10.1007/s00401-011-0830-2.

Mure, H. *et al.* (2011) 'Parkinson's disease tremor-related metabolic network: Characterization, progression, and treatment effects', *NeuroImage*. Academic Press, 54(2), pp. 1244–1253. doi: 10.1016/J.NEUROIMAGE.2010.09.028.

Murphy, G. J., Darcy, D. P. and Isaacson, J. S. (2005) 'Intraglomerular inhibition: signaling mechanisms of an olfactory microcircuit', *Nature Neuroscience*, 8(3), pp. 354–364. doi: 10.1038/nn1403.

Murthy, V. N. (2011) 'Olfactory Maps in the Brain', *Annual Review of Neuroscience*, 34(1), pp. 233–258. doi: 10.1146/annurev-neuro-061010-113738.

Murueta-Goyena, A. *et al.* (2019) 'Contribution of the GABAergic system to non-motor manifestations in premotor and early stages of Parkinson's disease', *Frontiers in Pharmacology*. Frontiers Media S.A., 10(OCT). doi: 10.3389/fphar.2019.01294.

Muslimović, D. *et al.* (2007) 'Motor procedural learning in Parkinson's disease', *Brain*. Oxford University Press, 130(11), pp. 2887–2897. doi: 10.1093/brain/awm211.

Muthuraman, M. *et al.* (2018) 'Deep Brain Stimulation and L-DOPA Therapy: Concepts of Action and Clinical Applications in Parkinson's Disease', *Frontiers in Neurology*. Frontiers, 9, p. 711. doi: 10.3389/fneur.2018.00711.

Nacher, J., Crespo, C. and McEwen, B. S. (2001) 'Doublecortin expression in the adult rat telencephalon.', *The European journal of neuroscience*, 14(4), pp. 629–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11556888 (Accessed: 26 November 2018).

Nadkarni, P., Chepurny, O. G. and Holz, G. G. (2014) 'Regulation of glucose homeostasis by GLP-1.', *Progress in molecular biology and translational science*. NIH Public Access, 121, pp. 23–65. doi: 10.1016/B978-0-12-800101-1.00002-8.

Nagayama, S., Homma, R. and Imamura, F. (2014) 'Neuronal organization of olfactory bulb circuits.', *Frontiers in neural circuits*, 8(September), p. 98. doi: 10.3389/fncir.2014.00098.

Nahimi, A. *et al.* (2018) 'Noradrenergic Deficits in Parkinson Disease Imaged with (11)C-MeNER.', *Journal of nuclear medicine : official publication, Society of Nuclear Medicine*. United States, 59(4), pp. 659–664. doi: 10.2967/jnumed.117.190975.

Narayanan, N. S., Rodnitzky, R. L. and Uc, E. Y. (2013) 'Prefrontal dopamine signaling and cognitive symptoms of Parkinson's disease', *Reviews in the Neurosciences*. Walter de Gruyter GmbH, 24(3), pp. 267–278. doi: 10.1515/revneuro-2013-0004.

Nassar, N. N. *et al.* (2015) 'Saxagliptin: A novel antiparkinsonian approach', *Neuropharmacology*. Pergamon, 89, pp. 308–317. doi: 10.1016/J.NEUROPHARM.2014.10.007.

Nebe, A. and Ebersbach, G. (2007) 'Selective diplopia in Parkinson's disease: A special subtype of visual hallucination?', *Movement Disorders*, 22(8), pp. 1175–1178. doi: 10.1002/mds.21298.

Nezhadi, A. *et al.* (2016) 'Neurosteroid allopregnanolone attenuates cognitive dysfunctions in 6-OHDA-induced rat model of Parkinson's disease.', *Behavioural brain research*. Netherlands, 305, pp. 258–264. doi: 10.1016/j.bbr.2016.03.019.

Nikoletopoulou, V. and Tavernarakis, N. (2012) 'Calcium homeostasis in aging neurons.', *Frontiers in genetics*. Frontiers Media SA, 3, p. 200. doi: 10.3389/fgene.2012.00200.

Niranjan, R. (2014) 'The Role of Inflammatory and Oxidative Stress Mechanisms in the Pathogenesis of Parkinson's Disease: Focus on Astrocytes', *Molecular Neurobiology*, 49(1),

pp. 28–38. doi: 10.1007/s12035-013-8483-x.

Nishioka, K. *et al.* (2006) 'Clinical heterogeneity of α-synuclein gene duplication in Parkinson's disease', *Annals of Neurology*. Wiley-Blackwell, 59(2), pp. 298–309. doi: 10.1002/ana.20753.

Novellino, F. *et al.* (2018) 'Relationship between Hippocampal Subfields and Category Cued Recall in AD and PDD: A Multimodal MRI Study', *Neuroscience*. Elsevier Ltd, 371, pp. 506–517. doi: 10.1016/j.neuroscience.2017.12.028.

Nowicka, D. *et al.* (2009) 'Parvalbumin-containing neurons, perineuronal nets and experience-dependent plasticity in murine barrel cortex', *European Journal of Neuroscience*, 30(11), pp. 2053–2063. doi: 10.1111/j.1460-9568.2009.06996.x.

O'Neill, E. and Harkin, A. (2018a) 'Targeting the noradrenergic system for anti-inflammatory and neuroprotective effects: implications for Parkinson's disease.', *Neural regeneration research*. Wolters Kluwer -- Medknow Publications, 13(8), pp. 1332–1337. doi: 10.4103/1673-5374.235219.

O'Neill, E. and Harkin, A. (2018b) 'Targeting the noradrenergic system for anti-inflammatory and neuroprotective effects: implications for Parkinson's disease.', *Neural regeneration research*. Wolters Kluwer -- Medknow Publications, 13(8), pp. 1332–1337. doi: 10.4103/1673-5374.235219.

Obeso, J. A. *et al.* (2000) 'Pathophysiology of the basal ganglia in Parkinson's disease', *Trends in Neurosciences*. Elsevier Ltd, 23(10 SUPPL.). doi: 10.1016/S1471-1931(00)00028-8.

Oertel, W. and Schulz, J. B. (2016) 'Current and experimental treatments of Parkinson disease: A guide for neuroscientists', *Journal of Neurochemistry*. Wiley/Blackwell (10.1111), 139, pp. 325–337. doi: 10.1111/jnc.13750.

Ortiz, G. G. *et al.* (2017) 'Physiology and Pathology of Neuroimmunology: Role of Inflammation in Parkinson's Disease', in *Physiology and Pathology of Immunology*. InTech. doi: 10.5772/intechopen.70377.

De Pablo-Fernandez, E. *et al.* (2017) 'Association of Autonomic Dysfunction With Disease Progression and Survival in Parkinson Disease', *JAMA Neurology*. American Medical Association, 74(8), p. 970. doi: 10.1001/jamaneurol.2017.1125.

Padoa-Schioppa, C. (2011) 'Neurobiology of economic choice: A good-based model', *Annual Review of Neuroscience*. NIH Public Access, 34, pp. 333–359. doi: 10.1146/annurev-neuro-061010-113648.

Pagonabarraga, J. *et al.* (2016) 'Minor hallucinations occur in drug-naive Parkinson's disease patients, even from the premotor phase', *Movement Disorders*. John Wiley and Sons Inc., 31(1), pp. 45–52. doi: 10.1002/mds.26432.

Pandey, S. and Srivanitchapoom, P. (2017) 'Levodopa-induced Dyskinesia: Clinical Features, Pathophysiology, and Medical Management', *Annals of Indian Academy of Neurology*. Medknow Publications & Media Pvt Ltd, 20(3), pp. 190–198. doi: 10.4103/aian.AIAN_239_17.

Pantcheva, P. *et al.* (2015) 'Treating non-motor symptoms of Parkinson's disease with transplantation of stem cells.', *Expert review of neurotherapeutics*. NIH Public Access, 15(10), pp. 1231–40. doi: 10.1586/14737175.2015.1091727.

Parashar, A. and Udayabanu, M. (2017) 'Gut microbiota: Implications in Parkinson's disease', *Parkinsonism and Related Disorders*. Elsevier Ltd, 38, pp. 1–7. doi: 10.1016/j.parkreldis.2017.02.002.

Pascual-Sedano, B. *et al.* (2008) 'Levodopa and executive performance in Parkinson's

disease: A randomized study', *Journal of the International Neuropsychological Society*. J Int Neuropsychol Soc, 14(5), pp. 832–841. doi: 10.1017/S1355617708081010.

Paß, T. *et al.* (2020) 'The Impact of Mitochondrial Dysfunction on Dopaminergic Neurons in the Olfactory Bulb and Odor Detection', *Molecular Neurobiology*. Springer, 57(9), pp. 3646–3657. doi: 10.1007/s12035-020-01947-w.

Paul, R. and Borah, A. (2016) 'L-DOPA-induced hyperhomocysteinemia in Parkinson's disease: Elephant in the room', *Biochimica et Biophysica Acta (BBA) - General Subjects*. Elsevier, 1860(9), pp. 1989–1997. doi: 10.1016/J.BBAGEN.2016.06.018.

Paxinos, George *et al.* (1985) 'Bregma, lambda and the interaural midpoint in stereotaxic surgery with rats of different sex, strain and weight', *Journal of Neuroscience Methods*, 13, pp. 139–143. Available at: http://ac.els-cdn.com/0165027085900263/1-s2.0-0165027085900263-main.pdf?_tid=55619470-7ba3-11e7-b22a-00000aacb35d&acdnat=1502132890_ed5db6d6d1ef4f274c4eefa87cd652ed (Accessed: 7 August 2017).

Pekcec, A., Löscher, W. and Potschka, H. (2006) 'Neurogenesis in the adult rat piriform cortex.', *Neuroreport*, 17(6), pp. 571–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16603913 (Accessed: 26 November 2018).

Pereira, J. B. *et al.* (2009) 'Neuroanatomical substrate of visuospatial and visuoperceptual impairment in Parkinson's disease', *Movement Disorders*. Mov Disord, 24(8), pp. 1193–1199. doi: 10.1002/mds.22560.

Perello, A. S. and Andres, P. (2020) 'Episodic Memory Impairment in Parkinson's Disease: Disentangling the Role of Encoding and Retrieval', *Article in Journal of the International Neuropsychological Society*. doi: 10.1017/S1355617720000909.

Perez-Pardo, P. *et al.* (2017) 'The gut-brain axis in Parkinson's disease: Possibilities for food-based therapies', *European Journal of Pharmacology*. Elsevier, 817, pp. 86–95. doi: 10.1016/J.EJPHAR.2017.05.042.

Peterson, A. C. and Li, C.-S. R. (2018) 'Noradrenergic Dysfunction in Alzheimer's and Parkinson's Diseases-An Overview of Imaging Studies.', *Frontiers in aging neuroscience*, 10, p. 127. doi: 10.3389/fnagi.2018.00127.

Pierantozzi, M. *et al.* (2001) 'Reduced L-dopa absorption and increased clinical fluctuations in Helicobacter pylori-infected Parkinson's disease patients.', *Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology*. Italy, 22(1), pp. 89–91. doi: 10.1007/s100720170061.

Pifl, C. *et al.* (2017) 'Early Paradoxical Increase of Dopamine: A Neurochemical Study of Olfactory Bulb in Asymptomatic and Symptomatic MPTP Treated Monkeys', *Frontiers in Neuroanatomy*. Frontiers, 11, p. 46. doi: 10.3389/fnana.2017.00046.

Pignatelli, A. and Belluzzi, O. (2017) 'Dopaminergic Neurones in the Main Olfactory Bulb: An Overview from an Electrophysiological Perspective', *Frontiers in Neuroanatomy*. Frontiers, 11, p. 7. doi: 10.3389/fnana.2017.00007.

Pinto-Sanchez, M. I. *et al.* (2017) 'Probiotic Bifidobacterium longum NCC3001 Reduces Depression Scores and Alters Brain Activity: A Pilot Study in Patients With Irritable Bowel Syndrome', *Gastroenterology*, 153(2), pp. 448-459.e8. doi: https://doi.org/10.1053/j.gastro.2017.05.003.

Pirogovsky-Turk, E. *et al.* (2015) 'Structural MRI correlates of episodic memory processes in Parkinson's disease without mild cognitive impairment', *Journal of Parkinson's Disease*. IOS Press, 5(4), pp. 971–981. doi: 10.3233/JPD-150652.

Pitkänen, A. *et al.* (2000) 'Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat', in *Annals of the New York Academy of Sciences*. New York Academy of Sciences, pp. 369–391. doi: 10.1111/j.1749-6632.2000.tb06738.x.

Pizzorusso, T. *et al.* (2002) 'Reactivation of Ocular Dominance Plasticity in the Adult Visual Cortex', *Science*, 298(5596), pp. 1248–1251. doi: 10.1126/science.1072699.

Poewe, W. (2008) 'Non-motor symptoms in Parkinson?s disease', *European Journal of Neurology*, 15(s1), pp. 14–20. doi: 10.1111/j.1468-1331.2008.02056.x.

Poewe, W. *et al.* (2017) 'Parkinson disease', *Nature Reviews Disease Primers*. Nature Publishing Group, 3, p. 17013. doi: 10.1038/nrdp.2017.13.

Pollock, E. *et al.* (2014) 'Metalloproteinase inhibition prevents inhibitory synapse reorganization and seizure genesis', *Neurobiology of Disease*, 70, pp. 21–31. doi: 10.1016/j.nbd.2014.06.003.

Pondal, M. *et al.* (2013) 'Clinical features of dopamine agonist withdrawal syndrome in a movement disorders clinic', *Journal of Neurology, Neurosurgery and Psychiatry*. BMJ Publishing Group, 84(2), pp. 130–135. doi: 10.1136/jnnp-2012-302684.

Postuma, R. B. *et al.* (2015) 'MDS clinical diagnostic criteria for Parkinson's disease', *Movement Disorders*. John Wiley and Sons Inc., pp. 1591–1601. doi: 10.1002/mds.26424.

Puspita, L., Chung, S. Y. and Shim, J. (2017) 'Oxidative stress and cellular pathologies in Parkinson's disease', *Molecular Brain*. BioMed Central, 10(1), p. 53. doi: 10.1186/s13041-017-0340-9.

Pyka, M. *et al.* (2011) 'Chondroitin sulfate proteoglycans regulate astrocyte-dependent synaptogenesis and modulate synaptic activity in primary embryonic hippocampal neurons', *European Journal of Neuroscience*, 33(12), pp. 2187–2202. doi: 10.1111/j.1460-9568.2011.07690.x.

Rabinak, C. A. and Nirenberg, M. J. (2010) 'Dopamine agonist withdrawal syndrome in parkinson disease', *Archives of Neurology*. American Medical Association, 67(1), pp. 58–63. doi: 10.1001/archneurol.2009.294.

Radhakrishnan, D. M. and Goyal, V. (2018) 'Parkinson's disease: A review'. doi: 10.4103/0028-3886.226451.

Rajkowska, G. *et al.* (2007) 'GABAergic neurons immunoreactive for calcium binding proteins are reduced in the prefrontal cortex in major depression', *Neuropsychopharmacology*, 32(2), pp. 471–482. doi: 10.1038/sj.npp.1301234.

Ransohoff, R. M. (2016) 'How neuroinflammation contributes to neurodegeneration', *Science*, 353(6301), pp. 777–783. doi: 10.1126/science.aag2590.

Rees, K. *et al.* (2011) 'Non-steroidal anti-inflammatory drugs as disease-modifying agents for Parkinson's disease: evidence from observational studies', *Cochrane Database of Systematic Reviews*. doi: 10.1002/14651858.CD008454.pub2.

Refolo, V. and Stefanova, N. (2019) 'Neuroinflammation and Glial Phenotypic Changes in Alpha-Synucleinopathies', *Frontiers in Cellular Neuroscience*, 13, p. 263. doi: 10.3389/fncel.2019.00263.

Regensburger, M., Prots, I. and Winner, B. (2014) 'Adult hippocampal neurogenesis in Parkinson's disease: Impact on neuronal survival and plasticity', *Neural Plasticity*. Hindawi Publishing Corporation. doi: 10.1155/2014/454696.

Reiner, D. J. *et al.* (2016) 'Astrocytes Regulate GLP-1 Receptor-Mediated Effects on Energy Balance.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*. Society for Neuroscience, 36(12), pp. 3531–40. doi: 10.1523/JNEUROSCI.3579-15.2016.

Reiner, D. J. *et al.* (2018) 'Glucagon-Like Peptide-1 Receptor Signaling in the Lateral Dorsal Tegmental Nucleus Regulates Energy Balance', *Neuropsychopharmacology*, 43(3), pp. 627–637. doi: 10.1038/npp.2017.225.

Rey, N. L. *et al.* (2016) 'Widespread transneuronal propagation of α-synucleinopathy triggered in olfactory bulb mimics prodromal Parkinson's disease', *Journal of Experimental Medicine*. Rockefeller University Press, 213(9), pp. 1759–1778. doi: 10.1084/jem.20160368.

Del Rey, N. L. G. *et al.* (2018) 'Advances in parkinson's disease: 200 years later', *Frontiers in Neuroanatomy*, 12(December), pp. 1–14. doi: 10.3389/fnana.2018.00113.

Rietdijk, C. D. *et al.* (2017) 'Exploring Braak's Hypothesis of Parkinson's Disease.', *Frontiers in neurology*. Frontiers Media SA, 8, p. 37. doi: 10.3389/fneur.2017.00037.

Roberts, R. O. *et al.* (2016) 'Association Between Olfactory Dysfunction and Amnestic Mild Cognitive Impairment and Alzheimer Disease Dementia', *JAMA Neurology*, 73(1), pp. 93–101. doi: 10.1001/jamaneurol.2015.2952.

Rocha-Ferreira, E. *et al.* (2018) 'Neuroprotective exendin-4 enhances hypothermia therapy in a model of hypoxic-ischaemic encephalopathy.', *Brain : a journal of neurology*. Oxford University Press, 141(10), pp. 2925–2942. doi: 10.1093/brain/awy220.

Rocha-Ramírez, L. M. *et al.* (2017) 'Probiotic *Lactobacillus* Strains Stimulate the Inflammatory Response and Activate Human Macrophages', *Journal of Immunology Research*. Hindawi, 2017, p. 4607491. doi: 10.1155/2017/4607491.

Rodriguez-Oroz, M. C. *et al.* (2009) 'Initial clinical manifestations of Parkinson's disease: features and pathophysiological mechanisms', *The Lancet Neurology*. Lancet Neurol, pp. 1128–1139. doi: 10.1016/S1474-4422(09)70293-5.

Rodriguez-Pallares, J. *et al.* (2007) 'Mechanism of 6-hydroxydopamine neurotoxicity: the role of NADPH oxidase and microglial activation in 6-hydroxydopamine-induced degeneration of dopaminergic neurons', *Journal of Neurochemistry*, 0(0), pp. 070615193023005-??? doi: 10.1111/j.1471-4159.2007.04699.x.

Rolls, E. T. (2010) 'A computational theory of episodic memory formation in the hippocampus', *Behavioural Brain Research*. Behav Brain Res, pp. 180–196. doi: 10.1016/j.bbr.2010.03.027.

Rolls, E. T. (2012) 'Taste, olfactory and food texture reward processing in the brain and the control of appetite', in *Proceedings of the Nutrition Society*. Proc Nutr Soc, pp. 488–501. doi: 10.1017/S0029665112000821.

Rolls, E. T. and Kesner, R. P. (2006) 'A computational theory of hippocampal function, and empirical tests of the theory', *Progress in Neurobiology*. Pergamon, pp. 1–48. doi: 10.1016/j.pneurobio.2006.04.005.

Romito, A. and Cobellis, G. (2016) 'Pluripotent Stem Cells: Current Understanding and Future Directions.', *Stem cells international*. Hindawi Limited, 2016, p. 9451492. doi: 10.1155/2016/9451492.

Roos, D. S. *et al.* (2019) 'Hyposmia as a marker of (non-)motor disease severity in Parkinson's disease', *Journal of Neural Transmission*, 126, pp. 1471–1478. doi: 10.1007/s00702-019-02074-0.

Ross, S. B. and Stenfors, C. (2014) 'DSP4, a Selective Neurotoxin for the Locus Coeruleus Noradrenergic System. A Review of Its Mode of Action', *Neurotoxicity Research*, pp. 15–30.

doi: 10.1007/s12640-014-9482-z.

ROTHWELL, J. C. (2011) 'THE MOTOR FUNCTIONS OF THE BASAL GANGLIA', *Journal of Integrative Neuroscience*, 10(03), pp. 303–315. doi: 10.1142/S0219635211002798.

De Rui, M. *et al.* (2020) 'Parkinson's disease and the non-motor symptoms: hyposmia, weight loss, osteosarcopenia'. doi: 10.1007/s40520-020-01470-x.

Sachs, C. and Jonsson, G. (1975) 'Mechanisms of action of 6-hydroxydopamine.', *Biochemical pharmacology*, 24(1), pp. 1–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1092302 (Accessed: 5 August 2017).

Saijo, K. *et al.* (2009) 'A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death.', *Cell*. NIH Public Access, 137(1), pp. 47–59. doi: 10.1016/j.cell.2009.01.038.

Saiz-Sanchez, D. *et al.* (2020) 'Somatostatin, Olfaction, and Neurodegeneration', *Frontiers in Neuroscience*. Frontiers Media S.A. doi: 10.3389/fnins.2020.00096.

Sakai, T. *et al.* (2008) 'Changes in density of calcium-binding-protein-immunoreactive GABAergic neurons in prefrontal cortex in schizophrenia and bipolar disorder', *Neuropathology*. John Wiley & Sons, Ltd, 28(2), pp. 143–150. doi: 10.1111/j.1440-1789.2007.00867.x.

Salimi, M. *et al.* (2019) 'Distraction of olfactory bulb-medial prefrontal cortex circuit may induce anxiety-like behavior in allergic rhinitis', *PLoS ONE*. Public Library of Science, 14(9). doi: 10.1371/journal.pone.0221978.

Sampaio, T. F. *et al.* (2018) 'MAO-B and COMT Genetic Variations Associated With Levodopa Treatment Response in Patients With Parkinson's Disease', *The Journal of Clinical Pharmacology*, 58(7), pp. 920–926. doi: 10.1002/jcph.1096.

Sampson, T. R. *et al.* (2016) 'Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease', *Cell*. Cell Press, 167(6), pp. 1469-1480.e12. doi: 10.1016/j.cell.2016.11.018.

Sancandi, M. *et al.* (2018) 'Structural changes observed in the piriform cortex in a rat model of pre-motor Parkinson's disease.', *Frontiers in Cellular Neuroscience*. Frontiers, 12, p. 479. doi: 10.3389/FNCEL.2018.00479.

Sancandi, M. *et al.* (2020) 'Protein deimination signatures in plasma and plasma-evs and protein deimination in the brain vasculature in a rat model of pre-motor parkinson's disease', *International Journal of Molecular Sciences*, 21(8). doi: 10.3390/ijms21082743.

Sander, J. D. and Joung, J. K. (2014) 'CRISPR-Cas systems for editing, regulating and targeting genomes', *Nature Biotechnology*, 32(4), pp. 347–355. doi: 10.1038/nbt.2842.

Sanders, M. E. *et al.* (2019) 'Probiotics and prebiotics in intestinal health and disease: from biology to the clinic.', *Nature reviews. Gastroenterology & hepatology*. England, 16(10), pp. 605–616. doi: 10.1038/s41575-019-0173-3.

Sanjari Moghaddam, H. *et al.* (2017) 'Neurotransmission systems in Parkinson's disease', *Reviews in the Neurosciences*. De Gruyter, 28(5), pp. 509–536. doi: 10.1515/revneuro-2016-0068.

Santana, N., Mengod, G. and Artigas, F. (2009) 'Quantitative Analysis of the Expression of Dopamine D1 and D2 Receptors in Pyramidal and GABAergic Neurons of the Rat Prefrontal Cortex', *Cerebral Cortex*. Oxford University Press, 19(4), pp. 849–860. doi: 10.1093/cercor/bhn134.

Santiago, J. A. and Potashkin, J. A. (2013) 'Shared dysregulated pathways lead to Parkinson's disease and diabetes', *Trends in Molecular Medicine*, 19(3), pp. 176–186. doi: https://doi.org/10.1016/j.molmed.2013.01.002.

Santiago, R. M. *et al.* (2010) 'Depressive-like behaviors alterations induced by intranigral MPTP, 6-OHDA, LPS and rotenone models of Parkinson's disease are predominantly associated with serotonin and dopamine', *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 34(6), pp. 1104–1114. doi: 10.1016/j.pnpbp.2010.06.004.

Dos Santos, M. C. T. *et al.* (2018) 'miRNA-based signatures in cerebrospinal fluid as potential diagnostic tools for early stage Parkinson's disease.', *Oncotarget*, 9(25), pp. 17455–17465. doi: 10.18632/oncotarget.24736.

Sara, S. J. (2009) 'The locus coeruleus and noradrenergic modulation of cognition'. doi: 10.1038/nrn2573.

Satoh, J. *et al.* (1991) 'Parvalbumin-immunoreactive neurons in the human central nervous system are decreased in Alzheimer's disease', *Acta Neuropathologica*. Springer-Verlag, 81(4), pp. 388–395. doi: 10.1007/BF00293459.

Sauer, H. and Oertel, W. H. (1994) 'Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: A combined retrograde tracing and immunocytochemical study in the rat', *Neuroscience*. Pergamon, 59(2), pp. 401–415. doi: 10.1016/0306-4522(94)90605-X.

Savica, R. *et al.* (2013) 'Incidence and Pathology of Synucleinopathies and Tauopathies Related to Parkinsonism', *JAMA Neurology*, 70(7), p. 859. doi: 10.1001/jamaneurol.2013.114.

Schaeffer, E. and Berg, D. (2017) 'Dopaminergic Therapies for Non-motor Symptoms in Parkinson's Disease.', *CNS drugs*. New Zealand, 31(7), pp. 551–570. doi: 10.1007/s40263-017-0450-z.

Schapira, A. H., Ray Chaudhuri, K. and Jenner, P. (2017) 'Non-motor features of Parkinson disease', *Nature Publishing Group*, 18. doi: 10.1038/nrn.2017.62.

Schärer, Y.-P. Z. *et al.* (2012) 'Dopaminergic modulation of synaptic transmission and neuronal activity patterns in the zebrafish homolog of olfactory cortex', *Frontiers in Neural Circuits*. Frontiers, 6, p. 76. doi: 10.3389/fncir.2012.00076.

Scheperjans, F. *et al.* (2015) 'Gut microbiota are related to Parkinson's disease and clinical phenotype', *Movement Disorders*. John Wiley and Sons Inc., 30(3), pp. 350–358. doi: 10.1002/mds.26069.

Schildknecht, S. *et al.* (2017) 'Tipping Points and Endogenous Determinants of Nigrostriatal Degeneration by MPTP', *Trends in Pharmacological Sciences*, 38(6), pp. 541–555. doi: 10.1016/j.tips.2017.03.010.

Schmidt, H. D. *et al.* (2016) 'Glucagon-Like Peptide-1 Receptor Activation in the Ventral Tegmental Area Decreases the Reinforcing Efficacy of Cocaine', *Neuropsychopharmacology*, 41(7), pp. 1917–1928. doi: 10.1038/npp.2015.362.

Schober, A. (2004) 'Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP', *Cell Tissue Res*, 318, pp. 215–224. doi: 10.1007/s00441-004-0938-y.

Schrag, A. and Schott, J. M. (2006) 'Epidemiological, clinical, and genetic characteristics of early-onset parkinsonism', *Lancet Neurology*. Lancet Neurol, pp. 355–363. doi: 10.1016/S1474-4422(06)70411-2.

Schwaller, B. (2010) 'Cytosolic Ca2+ Buffers', *Cold Spring Harbor Perspectives in Biology*, 2(11), pp. a004051–a004051. doi: 10.1101/cshperspect.a004051.

Sedelis, M., Schwarting, R. K. W. and Huston, J. P. (2001) 'Behavioral phenotyping of the MPTP mouse model of Parkinson's disease', in *Behavioural Brain Research*. Elsevier, pp. 109–125. doi: 10.1016/S0166-4328(01)00309-6.

Seppi, K. *et al.* (2019) 'Update on treatments for nonmotor symptoms of Parkinson's disease—an evidence-based medicine review', *Movement Disorders*. John Wiley and Sons Inc., 34(2), pp. 180–198. doi: 10.1002/mds.27602.

Shi, N. *et al.* (2017) 'Interaction between the gut microbiome and mucosal immune system.', *Military Medical Research*, 4, p. 14. doi: 10.1186/s40779-017-0122-9.

Sitte, H. H. *et al.* (2017) 'Dopamine and noradrenaline, but not serotonin, in the human claustrum are greatly reduced in patients with Parkinson's disease: possible functional implications', *European Journal of Neuroscience*. Edited by P. Bolam. Wiley/Blackwell (10.1111), 45(1), pp. 192–197. doi: 10.1111/ejn.13435.

Söderbom, G. (2020) 'Chapter Six - Status and future directions of clinical trials in Parkinson's disease', in Söderbom, G. et al. (eds) *Metabolic and Bioenergetic Drivers of Neurodegenerative Disease: Neurodegenerative Disease Research and Commonalities with Metabolic Diseases*. Academic Press, pp. 153–188. doi: https://doi.org/10.1016/bs.irn.2020.02.009.

Song, S. *et al.* (2019) 'Noradrenergic dysfunction accelerates LPS-elicited inflammation-related ascending sequential neurodegeneration and deficits in non-motor/motor functions', *Brain, Behavior, and Immunity*. Academic Press Inc., 81, pp. 374–387. doi: 10.1016/j.bbi.2019.06.034.

Sorg, B. A. *et al.* (2016) 'Casting a Wide Net: Role of Perineuronal Nets in Neural Plasticity.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*. Society for Neuroscience, 36(45), pp. 11459–11468. doi: 10.1523/JNEUROSCI.2351-16.2016.

Spielman, L. J., Gibson, D. L. and Klegeris, A. (2018) 'Unhealthy gut, unhealthy brain: The role of the intestinal microbiota in neurodegenerative diseases.', *Neurochemistry international*. England, 120, pp. 149–163. doi: 10.1016/j.neuint.2018.08.005.

Spillantini, M. G. *et al.* (1997) 'α-synuclein in Lewy bodies', *Nature*. Nature Publishing Group, 388(6645), pp. 839–840. doi: 10.1038/42166.

Srivanitchapoom, P., Pitakpatapee, Y. and Suengtaworn, A. (2018) 'Parkinsonian syndromes: A review', *Neurology India*. Medknow Publications, pp. S15–S25. doi: 10.4103/0028-3886.226459.

Srivastav, S. *et al.* (2019) 'Probiotics mixture increases butyrate, and subsequently rescues the nigral dopaminergic neurons from MPTP and rotenone-induced neurotoxicity.', *The Journal of nutritional biochemistry*. United States, 69, pp. 73–86. doi: 10.1016/j.jnutbio.2019.03.021.

Stefanis, L. (2012) 'α-Synuclein in Parkinson's disease.', *Cold Spring Harbor perspectives in medicine*. Cold Spring Harbor Laboratory Press, 2(2), p. a009399. doi: 10.1101/cshperspect.a009399.

Steiner, H. and Kitai, S. T. (2000) 'Regulation of rat cortex function by D1 dopamine receptors in the striatum.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*. Society for Neuroscience, 20(14), pp. 5449–60. doi: 10.1523/JNEUROSCI.20-14-05449.2000.

Stettler, D. D. and Axel, R. (2009) 'Representations of Odor in the Piriform Cortex', *Neuron*. Elsevier Ltd, 63(6), pp. 854–864. doi: 10.1016/j.neuron.2009.09.005.

Stevenson, T. J. *et al.* (2020) 'α-synuclein inclusions are abundant in non-neuronal cells in the

anterior olfactory nucleus of the Parkinson's disease olfactory bulb', *Scientific Reports*. Nature Research, 10(1). doi: 10.1038/s41598-020-63412-x.

Strauch, C. and Manahan-Vaughan, D. (2018) 'In the Piriform Cortex, the Primary Impetus for Information Encoding through Synaptic Plasticity Is Provided by Descending Rather than Ascending Olfactory Inputs', *Cerebral Cortex*, 28(2), pp. 764–776. doi: 10.1093/cercor/bhx315.

Stuart, S. *et al.* (2018) 'Cortical activity during walking and balance tasks in older adults and in people with Parkinson's disease: A structured review', *Maturitas*. Elsevier Ireland Ltd, pp. 53–72. doi: 10.1016/j.maturitas.2018.04.011.

Stuart, S. *et al.* (2019) 'Pre-frontal Cortical Activity During Walking and Turning Is Reliable and Differentiates Across Young, Older Adults and People With Parkinson's Disease', *Frontiers in Neurology*. Frontiers Media S.A., 10(MAY), p. 536. doi: 10.3389/fneur.2019.00536.

Sui, X. *et al.* (2019) 'Hyposmia as a Predictive Marker of Parkinson's Disease: A Systematic Review and Meta-Analysis'. doi: 10.1155/2019/3753786.

Surmeier, D. J. *et al.* (2017) 'Calcium and Parkinson's disease', *Biochemical and Biophysical Research Communications*, 483(4), pp. 1013–1019. doi: 10.1016/j.bbrc.2016.08.168.

Surmeier, D. J. and Schumacker, P. T. (2013) 'Calcium, Bioenergetics, and Neuronal Vulnerability in Parkinson's Disease', *Journal of Biological Chemistry*, 288(15), pp. 10736–10741. doi: 10.1074/jbc.R112.410530.

Suttkus, A. *et al.* (2014) 'Aggrecan, link protein and tenascin-R are essential components of the perineuronal net to protect neurons against iron-induced oxidative stress', *Cell Death and Disease*, 5(3), p. e1119. doi: 10.1038/cddis.2014.25.

Suzuki, N. and Bekkers, J. M. (2007) 'INHIBITORY INTERNEURONS IN THE PIRIFORM CORTEX', *Clinical and Experimental Pharmacology and Physiology*. Blackwell Publishing Asia, 34(10), pp. 1064–1069. doi: 10.1111/j.1440-1681.2007.04723.x.

Suzuki, N. and Bekkers, John M (2010) 'Distinctive classes of GABAergic interneurons provide layer-specific phasic inhibition in the anterior piriform cortex.', *Cerebral cortex (New York, N.Y. : 1991)*. Oxford University Press, 20(12), pp. 2971–84. doi: 10.1093/cercor/bhq046.

Suzuki, N. and Bekkers, John M. (2010) 'Inhibitory neurons in the anterior piriform cortex of the mouse: Classification using molecular markers', *The Journal of Comparative Neurology*, 518(10), pp. 1670–1687. doi: 10.1002/cne.22295.

Svensson, E. *et al.* (2015) 'Vagotomy and subsequent risk of Parkinson's disease', *Annals of Neurology*. John Wiley & Sons, Ltd, 78(4), pp. 522–529. doi: https://doi.org/10.1002/ana.24448.

Swick, T. J. (2012) 'Parkinson's disease and sleep/wake disturbances.', *Parkinson's disease*. Hindawi Limited, 2012, p. 205471. doi: 10.1155/2012/205471.

Tadaiesky, M. T. *et al.* (2008a) 'Emotional, cognitive and neurochemical alterations in a premotor stage model of Parkinson's disease', *Neuroscience*, 156(4), pp. 830–840. doi: 10.1016/j.neuroscience.2008.08.035.

Tadaiesky, M. T. *et al.* (2008b) 'Emotional, cognitive and neurochemical alterations in a premotor stage model of Parkinson's disease', *Neuroscience*, 156(4), pp. 830–840. doi: 10.1016/j.neuroscience.2008.08.035.

Taguchi, T. *et al.* (2020) 'a-Synuclein BAC transgenic mice exhibit RBD-like behaviour and hyposmia: a prodromal Parkinson's disease model', *Brain*. doi: 10.1093/brain/awz380.

Tamtaji, O. R. *et al.* (2019) 'Clinical and metabolic response to probiotic administration in people with Parkinson's disease: A randomized, double-blind, placebo-controlled trial', *Clinical Nutrition*, 38(3), pp. 1031–1035. doi: 10.1016/j.clnu.2018.05.018.

Tan, A. H., Lim, S.-Y., *et al.* (2020) 'Probiotics for constipation in Parkinson's disease: A randomized placebo-controlled study', *Neurology*, p. 10.1212/WNL.0000000000010998. doi: 10.1212/WNL.0000000000010998.

Tan, A. H., Hor, J. W., *et al.* (2020) 'Probiotics for Parkinson's disease: Current evidence and future directions', *JGH Open*, pp. 1–6. doi: 10.1002/jgh3.12450.

Tanner, J. J. *et al.* (2015) 'Temporal lobe and frontal-subcortical dissociations in non-demented Parkinson's disease with verbal memory impairment', *PLoS ONE*. Public Library of Science, 10(7). doi: 10.1371/journal.pone.0133792.

Taylor, T. N., Greene, J. G. and Miller, G. W. (2010) 'Behavioral phenotyping of mouse models of Parkinson's disease', *Behavioural Brain Research*. Elsevier, pp. 1–10. doi: 10.1016/j.bbr.2010.03.004.

Thiele, S. L., Warre, R. and Nash, J. E. (2012) 'Development of a unilaterally-lesioned 6-OHDA mouse model of Parkinson's disease.', *Journal of visualized experiments : JoVE*. MyJoVE Corporation, (60). doi: 10.3791/3234.

Thumm, P. C. *et al.* (2018) 'Treadmill walking reduces pre-frontal activation in patients with Parkinson's disease', *Gait and Posture*. Elsevier B.V., 62, pp. 384–387. doi: 10.1016/j.gaitpost.2018.03.041.

Tillerson, J. L. *et al.* (2006) 'Olfactory discrimination deficits in mice lacking the dopamine transporter or the D2 dopamine receptor.', *Behavioural brain research*. Netherlands, 172(1), pp. 97–105. doi: 10.1016/j.bbr.2006.04.025.

Tong, J. *et al.* (2015) 'Low levels of astroglial markers in Parkinson's disease: relationship to α-synuclein accumulation.', *Neurobiology of disease*. NIH Public Access, 82, pp. 243–253. doi: 10.1016/j.nbd.2015.06.010.

Tong, Y. *et al.* (2009) 'R1441C mutation in LRRK2 impairs dopaminergic neurotransmission in mice', *Proceedings of the National Academy of Sciences*, 106(34), pp. 14622–14627. doi: 10.1073/pnas.0906334106.

Tooney, P. A. and Chahl, L. A. (2004) 'Neurons expressing calcium-binding proteins in the prefrontal cortex in schizophrenia', *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 28(2), pp. 273–278. doi: https://doi.org/10.1016/j.pnpbp.2003.10.004.

Tordo, J. *et al.* (2018) 'A novel adeno-associated virus capsid with enhanced neurotropism corrects a lysosomal transmembrane enzyme deficiency.', *Brain : a journal of neurology*. Oxford University Press, 141(7), pp. 2014–2031. doi: 10.1093/brain/awy126.

Del Tredici, K. and Braak, H. (2016) 'Review: Sporadic Parkinson's disease: development and distribution of *α* -synuclein pathology', *Neuropathology and Applied Neurobiology*. Wiley/Blackwell (10.1111), 42(1), pp. 33–50. doi: 10.1111/nan.12298.

Turovsky, E. A. *et al.* (2018) 'Calcium-Binding Proteins Protect GABAergic Neurons of the Hippocampus from Hypoxia and Ischemia in vitro', *Biochemistry (Moscow), Supplement Series A: Membrane and Cell Biology*, 12(1), pp. 74–84. doi: 10.1134/S1990747818010105.

Ubeda-Bañon, I., Saiz-Sanchez, D., de la Rosa-Prieto, C., Mohedano-Moriano, A., *et al.* (2010) 'Staging of α-synuclein in the olfactory bulb in a model of Parkinson's disease: Cell types involved', *Movement Disorders*, 25(11), pp. 1701–1707. doi: 10.1002/mds.23197.

Ubeda-Bañon, I., Saiz-Sanchez, D., de la Rosa-Prieto, C., Argandoña-Palacios, L., *et al.*

(2010) ‘α-Synucleinopathy in the human olfactory system in Parkinson’s disease: involvement of calcium-binding protein- and substance P-positive cells’, *Acta Neuropathologica*, 119(6), pp. 723–735. doi: 10.1007/s00401-010-0687-9.

Ubeda-Bañon, I. *et al.* (2017) ‘Differential Effects of Parkinson’s Disease on Interneuron Subtypes within the Human Anterior Olfactory Nucleus.’, *Frontiers in neuroanatomy*. Frontiers Media SA, 11, p. 113. doi: 10.3389/fnana.2017.00113.

Uc, E. Y. *et al.* (2006) ‘Impaired visual search in drivers with Parkinson’s disease’, *Annals of Neurology*. Ann Neurol, 60(4), pp. 407–413. doi: 10.1002/ana.20958.

Ulusoy, A. *et al.* (2013) ‘Caudo-rostral brain spreading of α-synuclein through vagal connections’, *EMBO molecular medicine*. 2013/05/23. Blackwell Publishing Ltd, 5(7), pp. 1119–1127. doi: 10.1002/emmm.201302475.

Ulusoy, A. *et al.* (2017) ‘Brain-to-stomach transfer of α-synuclein via vagal preganglionic projections’, *Acta neuropathologica*. 2016/12/23, 133(3), pp. 381–393. doi: 10.1007/s00401-016-1661-y.

Unger, M. M. *et al.* (2016) ‘Short chain fatty acids and gut microbiota differ between patients with Parkinson’s disease and age-matched controls.’, *Parkinsonism & related disorders*. England, 32, pp. 66–72. doi: 10.1016/j.parkreldis.2016.08.019.

Vaillancourt, D. E. *et al.* (2013) ‘Dopamine overdose hypothesis: Evidence and clinical implications’, *Movement Disorders*. Mov Disord, pp. 1920–1929. doi: 10.1002/mds.25687.

Valls-Solé, J. (2000) ‘Neurophysiological characterization of parkinsonian syndromes’, *Neurophysiologie Clinique*. Elsevier Masson SAS, pp. 352–367. doi: 10.1016/S0987-7053(00)00236-7.

Ventorp, F. *et al.* (2017) ‘Exendin-4 treatment improves LPS-induced depressive-like behavior without affecting pro-inflammatory cytokines’, *Journal of Parkinson’s Disease*, 7(2), pp. 263–273. doi: 10.3233/JPD-171068.

Vermeiren, Y. *et al.* (2018) ‘Serotonergic Dysfunction in Amyotrophic Lateral Sclerosis and Parkinson’s Disease: Similar Mechanisms, Dissimilar Outcomes’, *Frontiers in Neuroscience*. Frontiers Media S.A., 12(MAR), p. 185. doi: 10.3389/fnins.2018.00185.

Vermetten, E. *et al.* (2007) ‘PTSD and Vietnam Veterans’, *Science*. American Association for the Advancement of Science (AAAS), 315(5809), pp. 184b-187b. doi: 10.1126/science.315.5809.184b.

Versace, V. *et al.* (2017) ‘Impaired cholinergic transmission in patients with Parkinson’s disease and olfactory dysfunction’, *Journal of the Neurological Sciences*. Elsevier B.V., 377, pp. 55–61. doi: 10.1016/j.jns.2017.03.049.

Vilas, D. *et al.* (2020) ‘Olfaction in LRRK2 Linked Parkinson’s Disease: Is It Different from Idiopathic Parkinson’s Disease?’, *Journal of Parkinson’s Disease*. IOS Press, pp. 1–8. doi: 10.3233/jpd-201972.

Vingill, S., Connor-Robson, N. and Wade-Martins, R. (2018) ‘Are rodent models of Parkinson’s disease behaving as they should?’, *Behavioural Brain Research*. Elsevier, 352, pp. 133–141. doi: 10.1016/J.BBR.2017.10.021.

Vreugdenhil, M. *et al.* (2003) ‘Parvalbumin-Deficiency Facilitates Repetitive IPSCs and Gamma Oscillations in the Hippocampus’, *Journal of Neurophysiology*, 89(3), pp. 1414–1422. doi: 10.1152/jn.00576.2002.

Wang, C.-H. *et al.* (2013) ‘Oxidative stress response elicited by mitochondrial dysfunction: Implication in the pathophysiology of aging’, *Experimental Biology and Medicine*, 238(5), pp.

450–460. doi: 10.1177/1535370213493069.

Wang, J. *et al.* (2011) 'Association of Olfactory Bulb Volume and Olfactory Sulcus Depth with Olfactory Function in Patients with Parkinson Disease', *American Journal of Neuroradiology*, 32(4), pp. 677–681. doi: 10.3174/ajnr.A2350.

Wang, Q., Liu, Y. and Zhou, J. (2015a) 'Neuroinflammation in Parkinson's disease and its potential as therapeutic target.', *Translational neurodegeneration*. BioMed Central, 4, p. 19. doi: 10.1186/s40035-015-0042-0.

Wang, Q., Liu, Y. and Zhou, J. (2015b) 'Neuroinflammation in Parkinson's disease and its potential as therapeutic target', *Translational Neurodegeneration*. BioMed Central, 4(1), p. 19. doi: 10.1186/s40035-015-0042-0.

Watson, G. S. and Leverenz, J. B. (2010) 'Profile of cognitive impairment in parkinson's disease', in *Brain Pathology*. NIH Public Access, pp. 640–645. doi: 10.1111/j.1750-3639.2010.00373.x.

Wattendorf, E. *et al.* (2009) 'Olfactory Impairment Predicts Brain Atrophy in Parkinson's Disease', *Journal of Neuroscience*, 29(49), pp. 15410–15413. doi: 10.1523/JNEUROSCI.1909-09.2009.

Weintraub, D. *et al.* (2011) 'Neurodegeneration across stages of cognitive decline in Parkinson disease', *Archives of Neurology*. American Medical Association, 68(12), pp. 1562–1568. doi: 10.1001/archneurol.2011.725.

Weis, S. *et al.* (2019) 'Effect of Parkinson's disease and related medications on the composition of the fecal bacterial microbiota', *npj Parkinson's Disease*, 5(1), p. 28. doi: 10.1038/s41531-019-0100-x.

Wen, T. H. *et al.* (2018) 'The Perineuronal "Safety" Net? Perineuronal Net Abnormalities in Neurological Disorders', *Frontiers in Molecular Neuroscience*. Frontiers, 11, p. 270. doi: 10.3389/fnmol.2018.00270.

Whitton, P. S. (2007) 'Inflammation as a causative factor in the aetiology of Parkinson's disease.', *British journal of pharmacology*, 150(8), pp. 963–76. doi: 10.1038/sj.bjp.0707167.

Wilson, D. A. (2009) 'Olfaction as a model system for the neurobiology of mammalian short-term habituation', *Neurobiology of Learning and Memory*, 92(2), pp. 199–205. doi: 10.1016/j.nlm.2008.07.010.

Winner, B. *et al.* (2006) 'Striatal deafferentation increases dopaminergic neurogenesis in the adult olfactory bulb', *Experimental Neurology*. Academic Press, 197(1), pp. 113–121. doi: 10.1016/J.EXPNEUROL.2005.08.028.

Wintergerst, E. S., Faissner, A. and Celio, M. R. (1996) 'The proteoglycan DSD-1-PG occurs in perineuronal nets around parvalbumin-immunoreactive interneurons of the rat cerebral cortex.', *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience*, 14(3), pp. 249–55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8842802 (Accessed: 7 August 2017).

Wirdefeldt, K. *et al.* (2011) 'Epidemiology and etiology of Parkinson's disease: a review of the evidence', *European Journal of Epidemiology*. Springer Verlag, 26(S1), pp. 1–58. doi: 10.1007/s10654-011-9581-6ï.

Xie, Z. *et al.* (2018) 'Exendin-4 attenuates neuronal death via GLP-1R/PI3K/Akt pathway in early brain injury after subarachnoid hemorrhage in rats', *Neuropharmacology*, 128, pp. 142–151. doi: 10.1016/j.neuropharm.2017.09.040.

Xu, Q. *et al.* (2011) 'Diabetes and Risk of Parkinson's Disease', *Diabetes Care*, 34(4), pp. 910

LP – 915. doi: 10.2337/dc10-1922.

Xue, Y.-X. *et al.* (2014) 'Depletion of Perineuronal Nets in the Amygdala to Enhance the Erasure of Drug Memories', *Journal of Neuroscience*, 34(19), pp. 6647–6658. doi: 10.1523/JNEUROSCI.5390-13.2014.

Yahiaoui-Doktor, M. *et al.* (2019) 'Olfactory function is associated with cognitive performance: results from the population-based LIFE-Adult-Study', *Alzheimer's Research & Therapy*, 11(1), p. 43. doi: 10.1186/s13195-019-0494-z.

Yamada, J. and Jinno, S. (2013) 'Spatio-temporal differences in perineuronal net expression in the mouse hippocampus, with reference to parvalbumin', *Neuroscience*. Pergamon, 253, pp. 368–379. doi: 10.1016/J.NEUROSCIENCE.2013.08.061.

Yamada, M. *et al.* (2004) 'Neurogenesis in olfactory bulb identified by retroviral labeling in normal and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated adult mice', *Neuroscience*, 124(1), pp. 173–181. doi: 10.1016/j.neuroscience.2003.10.040.

Yang, D. *et al.* (2019) 'The Role of the Gut Microbiota in the Pathogenesis of Parkinson's Disease', *Frontiers in Neurology*. Frontiers Media S.A., p. 1155. doi: 10.3389/fneur.2019.01155.

Yang, M. and Crawley, J. N. (2009) 'Simple behavioral assessment of mouse olfaction', *Current protocols in neuroscience*, Chapter 8, p. Unit-8.24. doi: 10.1002/0471142301.ns0824s48.

Yang, X. *et al.* (2017) 'Microglia P2Y6 receptor is related to Parkinson's disease through neuroinflammatory process.', *Journal of neuroinflammation*. BioMed Central, 14(1), p. 38. doi: 10.1186/s12974-017-0795-8.

Yedlapudi, D. *et al.* (2016) 'Inhibition of alpha-synuclein aggregation by multifunctional dopamine agonists assessed by a novel in vitro assay and an in vivo Drosophila synucleinopathy model.', *Scientific reports*. Nature Publishing Group, 6, p. 38510. doi: 10.1038/srep38510.

Yoneyama, N. *et al.* (2018) 'Severe hyposmia and aberrant functional connectivity in cognitively normal Parkinson's disease', *PLoS ONE*, 13(1), pp. 1–17. doi: 10.1371/journal.pone.0190072.

Youdim, M. B. H., Ben-Shachar, D. and Riederer, P. (1991) 'Iron in Brain Function and Dysfunction with Emphasis on Parkinson's Disease', *European Neurology*, 31(1), pp. 34–40. doi: 10.1159/000116719.

Yue, M. *et al.* (2015) 'Progressive dopaminergic alterations and mitochondrial abnormalities in LRRK2 G2019S knock-in mice', *Neurobiology of Disease*. Academic Press, 78, pp. 172–195. doi: 10.1016/J.NBD.2015.02.031.

Yulug, B., Ozansoy, M. and Cankaya, S. (2019) 'A different view on the pathophysiology of Parkinson's disease: a descendent neurochemical hypothesis?', *Neural Regeneration Research*. Wolters Kluwer Medknow Publications, 14(10), p. 1717. doi: 10.4103/1673-5374.257527.

Zadrozna, M. *et al.* (2011) 'Different pattern of changes in calcium binding proteins immunoreactivity in the medial prefrontal cortex of rats exposed to stress models of depression', *Pharmacological Reports*, 63(6), pp. 1539–1546. doi: 10.1016/S1734-1140(11)70718-6.

Zaltieri, M. *et al.* (2015) 'Mitochondrial Dysfunction and α-Synuclein Synaptic Pathology in Parkinson's Disease: Who's on First?', *Parkinson's disease*. Hindawi Limited, 2015, p. 108029. doi: 10.1155/2015/108029.

Zgaljardic, D. *et al.* (2006) 'An examination of executive dysfunction associated with frontostriatal circuitry in Parkinson's disease', *Journal of Clinical and Experimental Neuropsychology*. J Clin Exp Neuropsychol, 28(7), pp. 1127–1144. doi: 10.1080/13803390500246910.

Zhang, H. *et al.* (2016) 'Intranasal Delivery of Exendin-4 Confers Neuroprotective Effect Against Cerebral Ischemia in Mice.', *The AAPS journal*. Springer, 18(2), pp. 385–94. doi: 10.1208/s12248-015-9854-1.

Zhao, Y. Z. *et al.* (2014) 'Gelatin nanostructured lipid carriers-mediated intranasal delivery of basic fibroblast growth factor enhances functional recovery in hemiparkinsonian rats', *Nanomedicine: Nanotechnology, Biology, and Medicine*. Elsevier Inc., 10(4), pp. 755–764. doi: 10.1016/j.nano.2013.10.009.

Zimprich, A. *et al.* (2004) 'Mutations in LRRK2 Cause Autosomal-Dominant Parkinsonism with Pleomorphic Pathology', *Neuron*. Cell Press, 44(4), pp. 601–607. doi: 10.1016/J.NEURON.2004.11.005.

Zündorf, G. and Reiser, G. (2011) 'Calcium dysregulation and homeostasis of neural calcium in the molecular mechanisms of neurodegenerative diseases provide multiple targets for neuroprotection.', *Antioxidants & redox signaling*. Mary Ann Liebert, Inc., 14(7), pp. 1275–88. doi: 10.1089/ars.2010.3359.