Investigating the potential of targeting the CB_2 receptor for anti-inflammatory disease modification in Parkinson's disease

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Declaration

I declare that the work presented in this thesis has not been submitted for any degree or diploma at this, or any other university and that the work described herein is my own with the following exceptions:

- Mr. Tommy Patton carried out the immunohistochemical stain for aggregate-specific α-synuclein in the AAV-A53T-α-synuclein study in Chapter 4.

Signed:…… Date:……..

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Abstract

Parkinson's disease is a multifaceted neurodegenerative disorder that currently has no cure. Chronic neuroinflammation is thought to play a crucial role in the progression of the disease, with neuroinflammation and neurotoxicity driving each other in a self-sustaining cycle that is detrimental to the survival of the neurons. Subsequently, the concept of anti-inflammatory disease therapy for the treatment of Parkinson's disease has emerged in recent years. The cannabinoid CB_2 receptor is an attractive target for anti-neuroinflammatory therapy due to its location on microglia and its immunomodulatory effect on these cells. Thus, the overarching aim of this body of research was to investigate the potential of targeting the CB_2 receptor for Parkinson's disease therapy.

Firstly, we assessed the temporal expression of the CB_2 receptor in a genetic model of Parkinson's disease that was induced by administering an adeno-associated viral (AAV) vector which expressed human α-synuclein with the A53T mutation to the rat brain. Following on from this, we endeavoured to enhance the AAV-α-synuclein model using a small molecule α-synuclein aggregator to aid in further studies of the condition. Finally, we investigated the potential of targeting the CB_2 receptor for anti-inflammatory disease modification by determining if the pharmacological targeting of this receptor could provide functional neuroprotection in inflammatory rat models.

In the AAV-α-synuclein genetic model of Parkinson's disease, we did not detect any alterations in CB_2 gene expression despite a profound upregulation in human α-synuclein expression in the substantia nigra and the striatum. However, there was a decrease in the striatal levels of the endocannabinoid 2-arachidonylglycerol (2-AG) and of the related *N*-acylethanolamine *N*-oleoyl ethanolamide (OEA), indicating a dysregulation of the cannabinoid system in this model. Interestingly, an overall decrease in the expression of microglial and astrocytic markers was observed in the substantia nigra, but these alterations were not accompanied by dopaminergic degeneration.

Further to this, we sought to enhance the AAV-α-synuclein model of Parkinson's disease by combining AAV-mediated α-synuclein overexpression with FN075-mediated α-synuclein aggregation. The α-synuclein aggregating molecule FN075 combined with the AAV-α-synuclein vectors exhibited a significant increase in the pathogenic

phosphorylated form of α-synuclein, with visible anomalous accumulations. However, there was no significant degeneration of the nigrostriatal dopamine neurons in any group.

Finally, we investigated if CB_2 agonism produced anti-inflammatory or neuroprotective effects in a viral priming model and in an inflammatory LPS model. We did not observe a significant anti-inflammatory response, but such a role cannot be dismissed, as previous studies have shown the type of model and the particular agonist used results in disparate outcomes.

To conclude, dysregulation of the endocannabinoid system may precede nigrostriatal degeneration, with alterations in the levels of 2-AG and OEA observed in a prodromal AAV-α-synuclein model. However, further studies are required to determine the viability of targeting the CB_2 receptor for anti-inflammatory disease modification in Parkinson's disease. Furthermore, this body of research suggests that, with refinement, the combination of AAV-α-synuclein and FN075 may be a promising novel animal model in which to study Parkinson's disease in the future.

Research Dissemination

Peer-Reviewed Published Manuscripts

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List of commonly used abbreviations

2-AG	2-arachidonylglycerol
6-OHDA	6-hydroxydopamine
A53T	alanine to threonine point-mutation
AAV	adeno-associated virus
ACN	acetonitrile
ACREC	Animal Care and Research Ethics Committee
AD	Alzheimer's disease
AEA	*N*-arachidonyl ethanolamide (anandamide)
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
AP	anterior posterior
APP	amyloid precursor protein
Arg1	arginine 1
ATP	adenosine triphosphate
Aβ	amyloid-β
BBB	blood-brain barrier
BCP	beta-caryophyllene
CB_1	cannabinoid receptor 1
CB_2	cannabinoid receptor 2
CBD	cannabidiol
CD	cluster of differentiation
CD11b	integrin alpha-M/CR3
cDNA	complementary DNA
cm	centimetre
CNS	central nervous system
COMT	catechol-O-methyltransferase
COX2	cyclooxygenase 2
Ct	cycle threshold
D_1	dopamine receptor 1
D_2	dopamine receptor 2
DAB	diaminobenzidine tetrahydrochloride
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DAT	dopamine transporter
DBS	deep brain stimulation
DMSO	dimethyl sulfoxide
DOPA	dihydroxyphenylalanine
DV	dorsoventral
EAE	experimental autoimmune encephalomyelitis

FAAH	fatty acid amide hydrolase
g	gram
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GPe	global pallidus *pars externa*
GPi	global pallidus *pars interna*
GWAS	genome-wide association study
H_2O_2	hydrogen peroxide
HD	Huntington's disease
HLA	human leukocyte antigen
hr	hours
i.p.	intraperitoneal
IFN-γ	interferon-γ
IHC	immunohistochemistry
IL	interleukin
iNOS	inducible nitric oxide synthase
LC-MS/MS	liquid chromatography mass spectrometry
L-DOPA	L-3,4-dihydroxyphenylalanine / levodopa
LPS	lipopolysaccharide
LRRK2	leucine-rich repeat kinase 2
LV	lentivirus
MAGL	monoacylglycerol lipase
MAO	monoamine oxidase
mg	milligram
mg/kg	milligram per kilogram
MHC	major histocompatibility complex
min	minutes
ml	millilitre
ML	mediolateral
mm	millimetre
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
MSN	medium spiny neuron
NArPE	*N*-arachidonoyl-phosphatidylethanolamine
NAPE-PLD	*N*-acyl-phosphatidylethanolamine phospholipase D
ng	nanogram
NMDA	*N*-methyl-D-aspartate
NSAID	non-steroidal anti-inflammatory drug

O_2	oxygen
OD	optical density
OEA	*N*-oleoyl ethanolamide
OX-42	integrin alpha-M
PBS	phosphate-buffered saline
PEA	*N*-palmitoyl ethanolamide
PGK	phosphoglycerate kinase
PINK1	PTEN-induced kinase 1
Poly I:C	polyinosinic:polycytidylic acid
PPAR	peroxisome proliferator-activated receptor
pS129	phosphorylated at serine 129 residue
qRT-PCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species
SEM	standard error of the mean
sec	seconds
SN	substantia nigra
SNP	single nucleotide polymorphism
SNpc	substantia nigra *pars compacta*
SNpr	substantia nigra *pars reticulata*
STN	subthalamic nucleus
TBS	tris-buffered saline
TGF-β	transforming growth factor-β
TH	tyrosine hydroxylase
Th1	T helper 1
Th2	T helper 2
THC	Δ9-tetrahydrocannabinol
THCV	tetrahydrocannabivarin
TLR	toll-like receptor
TNF-α	tumour necrosis factor-α
vg	vector genomes
VL	ventrolateral nucleus
VMAT2	vesicular monoamine transporter 2
VTA	ventral tegmental area
WT	wild-type
µg	microgram
µl	microlitre
°C	degrees Celsius

Most abbreviations, other than commonly used expressions, are also defined at the first point of occurrence in the text.

Table of contents

Chapter 1: General Introduction

1.1 PARKINSON'S DISEASE

Parkinson's disease is a chronic, progressive neurodegenerative disorder that affects 1% of the population over 65, making it the second most common neurodegenerative disorder after Alzheimer's disease (Nussbaum and Ellis, 2003). Symptomatically, it manifests primarily as a motor disorder, with patients presenting with tremor, bradykinesia, and gait and balance disturbances (Sveinbjornsdottir, 2016). Pathologically, it is characterised by the degeneration of dopamine neurons in the nigrostriatal pathway of the brain and the formation of abnormal intracellular protein aggregates termed Lewy bodies (Kalia and Lang, 2015). By the time symptoms emerge, about 50% of the neurons in the nigrostriatal pathway have already been lost (Fearnley and Lees, 1991). Current therapies offer no neuroprotection to prevent the loss of the remaining neurons and are merely symptomatic, replacing the absent dopamine. Undoubtedly, there is an unmet clinical need for therapies in this condition that could impede or curtail the dopaminergic neuron loss.

In the past decades, it has become increasingly evident that chronic neuroinflammation plays a crucial role in the progression of Parkinson's disease. Neuroinflammation and neurotoxicity drive each other in a self-sustaining cycle which is detrimental to the survival of the neurons (Frank-Cannon *et al.*, 2009; Tansey and Goldberg, 2010). Therefore, the concept of anti-inflammatory therapy for Parkinson's disease emerged to break this cycle and potentially slow the progression of the disease. The immunomodulatory cannabinoid CB_2 receptor is present on microglia (Benito *et al.*, 2003; Gómez-Gálvez *et al.*, 2016), the resident immune cells of the brain, and thus represents an attractive target for potential anti-inflammatory effects.

With this in mind, the research described in this thesis sought to investigate the potential of the CB_2 receptor as a target for anti-inflammatory disease modification in Parkinson's disease. This work also involved the development of a new model in which to better study Parkinson's disease and its links to the cannabinoid system, as currently available animal models do not fully recapitulate the disease condition. This introductory chapter will provide a background for the work presented here, including an overview of Parkinson's

disease, the role of the endocannabinoid system in neuroinflammatory conditions, as well as outlining the currently existing Parkinsonian animal models.

1.1.1 A HISTORICAL PERSPECTIVE

Parkinson's disease was first described medically in the early nineteenth century by James Parkinson in his 'An Essay on the Shaking Palsy' (Parkinson, 1817). In this seminal paper, Parkinson, based on observations of six patients on the streets of his native London, reports on the motor symptoms of a disease he refers to as 'paralysis agitans': resting tremors, a shuffling gait, festination, flexed posture and falls. Over 50 years later, Jean-Martin Charcot and his colleagues at the Salpêtrière Hospital in Paris were more thorough in their description of the condition, and identified bradykinesia as a cardinal symptom of the disease, allowing it to be distinguished from other neurological disorders such as multiple sclerosis (Charcot and Vulpian, 1861; Charcot, 1877). In the subsequent years, many individuals provided clinical descriptions of the disease convening on the multi-faceted understanding we have of the disorder today. The slight male predominance of the disease was noted by William Gowers in his 'Manual of Diseases of the Nervous System' (Gowers, 1888), and Babinski commented on the motor fluctuations characteristic of the disease (Babinski *et al.*, 1921). Classification of Parkinson's disease came with the publication of the seminal paper of Hoehn and Yahr in 1967 (Hoehn and Yahr, 1967). Here they first introduced their time-honoured staging system of the progression of Parkinson's disease, making the important distinction between unilateral and bilateral disease.

The unravelling of the brain pathophysiology underlying Parkinson's disease commenced in the late nineteenth century. Blocq and Marinesco reported a patient with a left-sided Parkinsonian tremor, who, at autopsy, had a tuberculoma in the right substantia nigra (Blocq and Marinesco, 1893) and similar cases were described by Moritz Benedikt (Benedikt, 1889). Based upon these findings, in 1895 Édouard Brissaud postulated that the substantia nigra was involved in the regulation of muscle tone and that it was damage to this structure that caused Parkinson's disease (Brissaud, 1895). In 1919, Konstantin Trétiakoff noted the presence of intracellular and extracellular pathological inclusions in the substantia nigra in six cases of Parkinson's disease patients (Trétiakoff, 1919). He named these inclusions 'Lewy bodies' as Fritz Jakob Heinrich Lewy had described

similar inclusions in other brain areas in Parkinson's disease several years earlier (Lewy, 1912).

However, it was not until the late 1950s that it became evident that dopamine played a key role in the pathology of Parkinson's disease. In his studies in rabbits, Arvid Carlsson demonstrated that administration of reserpine, a molecule that depletes dopamine, induced motor impairments in the animals similar to those observed in Parkinson's disease patients. Administration of the dopamine precursor, L-DOPA, resulted in an amelioration of this motor dysfunction (Carlsson *et al.*, 1957). A few years later, Ehringer and Hornykiewicz detected a depletion of striatal dopamine in Parkinson's disease and post-encephalitic parkinsonism in their examinations of patients at *post mortem* (Ehringer and Hornykiewicz, 1960). These seminal discoveries led to the first administrations of L-DOPA intravenously to Parkinsonian patients in 1961 by Hornykiewicz and the physician Birkmayer, which produced dramatic but short-lived anti-akinetic effects (Birkmayer and Hornykiewicz, 1961). However, the major clinical breakthrough occurred in 1967, when George Cotzias developed a novel technique that entailed gradually increasing the dose of L-DOPA, negating the gastrointestinal adverse effects which had previously impeded the usage of higher doses (Cotzias *et al.*, 1967). This regimen was well tolerated by patients, who achieved dramatic benefits once therapeutic doses were reached. While new L-DOPA formulations and the use of peripherally restricted DOPA decarboxylase inhibitors have improved the therapy, these early discoveries have been unrivalled and L-DOPA administration remains the primary treatment for Parkinson's disease to this day.

1.1.2 PATHOPHYSIOLOGY

1.1.2.1 Nigrostriatal degeneration

1.1.2.1.1 The basal ganglia

Although there is growing recognition of the significance of a multitude of non-motor symptoms, Parkinson's disease is still predominantly characterised by the debilitating motor symptoms. The basal ganglia is an intricately wired circuitry of subcortical nuclei that is primarily responsible for the control of voluntary motor movement, in addition to motor learning and emotions. These nuclei include the caudate nucleus and caudate putamen (which together make up the striatum), the global pallidus *pars interna* (GPi),

the global pallidus *pars externa* (GPe), the substantia nigra *pars reticulata* (SNpr), the substantia nigra *pars compacta* (SNpc), and the subthalamic nucleus (Figure 1.1) (Lanciego *et al.*, 2012). In the striatum, medium spiny neurons (MSNs) which use γ-aminobutyric acid (GABA) as a primary neurotransmitter are highly abundant, comprising about 90% of all striatal neurons. These MSNs express both D_1 and D_2 dopamine receptors, and the stimulation of these receptors controls the direct and indirect pathways of movement (Gerfen *et al.*, 1990).

The direct pathway is a result of the action of striatal dopamine on excitatory dopamine D_1 receptors on MSNs which project to the SNpr and the GPi. This results in an inhibition of GABAergic projections from the SNpr and the GPi to the thalamus, thereby disinhibiting thalamocortical projections and activating cortical neurons, facilitating movement. Conversely, the indirect pathway is an inhibitory pathway where the activation of D_2 receptors on striatal MSNs inhibit the GABAergic GPe neurons, thereby resulting in a disinhibition of glutamatergic neurons in the subthalamic nucleus. This leads to an increased inhibition of thalamocortical projections and thus an inhibition of movement (Blandini *et al.*, 2000; Kreitzer and Malenka, 2008).

The role of dopamine in the basal ganglia is crucial, as it modulates neuronal firing in both the excitatory direct pathway and the inhibitory indirect pathway. Overall, motor control is executed by a very delicate balance between these two pathways. If this balance is disrupted or becomes skewed, there may be consequent motor dysfunction, as is the case in Parkinson's disease.

1.1.2.1.2 Loss of nigrostriatal dopaminergic neurons

The motor symptoms of Parkinson's disease can be attributed to the loss of dopamine neurons in the basal ganglia which project from the substantia nigra to the striatum. The death of these neurons commences years before there are any clinical signs of the disease and by the time motor symptoms manifest, the dopaminergic cell loss is approximately 50%, and consequently the levels of dopamine in the striatum is about 80% lower than basal levels (Bernheimer *et al.*, 1973; Fearnley and Lees, 1991).

When the dopamine neurons in the nigrostriatal pathway start to degenerate, this prompts the execution of numerous compensatory responses. In the surviving neurons, there is an

upregulation of dopamine release, turnover and transmission (Zigmond *et al.*, 1990; Lee *et al.*, 2000). Additionally, there is evidence that there is a reduction in dopamine uptake by the dopamine transporter (DAT), as downregulation of DAT mRNA has been reported in Parkinson's disease patients (Uhl *et al.*, 1994; Joyce *et al.*, 1997; Adams *et al.*, 2005), and carriers of the risk gene leucine-rich repeat kinase 2 (*LRRK2)* have reduced DAT binding (Wile *et al.*, 2017). Furthermore, dopamine denervation is associated with alterations in expression levels of dopamine receptors, namely an increase in the density of D_2 receptors on the MSNs (Decamp *et al.*, 1999; Chefer *et al.*, 2008; Sun *et al.*, 2013). Nigrostriatal neurons also form highly branched and very dense axonal arborisations in the striatum, resulting in the release of dopamine from many neurons at the same striatal site (Matsuda *et al.*, 2009). However, with progressive neuronal loss, these compensatory measures inevitably fail and patients begin to contend with impairments of motor function.

Figure 1.1 Basal ganglia circuitry in the normal and Parkinsonian brain. In the healthy brain (A), the direct and indirect circuits exert opposing functions to control movement. In the Parkinsonian state (B), the dopamine deficit leads to increased activity in the indirect circuit and decreased activity in the direct circuit, ultimately reducing movement. Green arrows indicate excitatory activity and red arrows indicate inhibitory activity. GPe = globus pallidus *pars externa*; GPi = globus pallidus *pars interna*; SNc = substantia nigra *pars compacta*; SNr = substantia nigra *pars reticulata*; STN = subthalamic nucleus; VL = ventrolateral nucleus. Image adapted from Rodriguez-Oroz *et al.* (2009).

1.1.2.2 Lewy bodies and α-synuclein

In addition to the degeneration of the nigrostriatal dopaminergic neurons, Parkinson's disease is also characterised by the presence of Lewy bodies and Lewy neurites. Lewy bodies are intraneuronal eosinophilic inclusions consisting mainly of insoluble α-synuclein protein, although they also contain ubiquitin, ubiquitinated protein, and neurofilaments (Kuzuhara *et al.*, 1988; Schmidt *et al.*, 1991; Spillantini *et al.*, 1997). Disparately, Lewy neurites are more abundant than Lewy bodies and are usually found in the striatum of Parkinson's disease patients, and they consist of α-synuclein filaments and granular material (Braak *et al.*, 1999; Duda *et al.*, 2002). Lewy bodies have been found in the brains of both sporadic and familial Parkinson's disease patients, suggesting that they play an important role in the progression of the disease (Goedert *et al.*, 2013). Remarkable work was undertaken by Braak and colleagues through analyses of brains of *post mortem* Parkinson's disease patients (Braak *et al.*, 2002). These experimenters observed an apparent correlation between the amount of insoluble α-synuclein, its location and the stage of the disease, and from these observations proposed six stages of Parkinson's disease pathology. These proposed anatomical stages concur with the development of disease symptoms. Clearly, these intraneuronal inclusions are intricately linked to Parkinson's disease progression and pathology.

As previously mentioned, Lewy bodies are named after Fritz Lewy, who was the first person to report the presence of these proteinaceous inclusions in the brains of Parkinson's disease patients in the early 20th century (Lewy, 1912). However, it was not until over 80 years later that the α-synuclein protein was identified as the major component of these pathological aggregates (Spillantini *et al.*, 1997). This is a 140 amino acid protein that is abundantly expressed in the brain and is found extensively in presynaptic terminals. While the normal physiological role of α-synuclein remains to be definitively elucidated, its location in nerve terminals and results from animal studies suggest that it has a role in synaptic vesicle trafficking and dopamine signalling (reviewed in Burré, 2015).

Several mutations in the *SNCA* gene that encodes α-synuclein have been associated with familial inherited forms of Parkinson's disease, as has the carrying of multiple copies of the gene such as duplications and triplications (Polymeropoulos *et al.*, 1997; Krüger *et al.*, 1998; Zarranz *et al.*, 2004; Appel-Cresswell *et al.*, 2013; Kiely *et al.*, 2013; Book *et*

al., 2018). In addition, the conformation of α-synuclein appears to be important in its toxicity. The protein undergoes extensive conformational transitions and misfolds to form oligomers, which aggregate into protofibrils and subsequently amyloid fibrils which form the basis of Lewy bodies (Ghosh *et al.*, 2017). The post-translational modification of the protein, for example phosphorylation or ubiquitination, has been implicated in α-synuclein aggregation and neurotoxicity (Kim *et al.*, 2014). Moreover, α-synuclein was detected in the cells of foetal transplant grafts in Parkinson's disease patients at *post mortem*, suggesting that α-synuclein is self-propagating, transmitting from cell-to-cell in a prion-like fashion (Li *et al.*, 2008). Clearly, this protein is integral to the pathology of Parkinson's disease.

The reason for the increased susceptibility of nigrostriatal dopamine neurons to α-synuclein induced toxicity remains to be fully elucidated, although evidence suggests that cytosolic dopamine oxidation and calcium-related mitochondrial dysfunction may be contributing factors (Post *et al.*, 2018). However, extensive research is yet required to determine the exact mechanism of the α-synuclein protein and the role of Lewy bodies in Parkinson's disease, and perhaps with this knowledge, we may understand how we could control the toxic effects.

1.1.2.3 Neuroinflammation

Neuroinflammation was first suggested to play a role in neurodegenerative disease as early as the beginning of the 20th century. In 1907, the same year Alzheimer published his paper first describing the disease that was to become his namesake (Alzheimer, 1907), Oskar Fischer described plaque formation in patients with senile dementia. Fischer later stated that the plaque deposition was a result of an inflammatory reaction, but he could not find the morphological characteristics of an inflammatory reaction (Fischer, 1907; Fischer, 1910). Thus, the idea of inflammation playing a role in neurodegenerative disease largely lost favour for many years as it was a widespread belief in the scientific community at the time that the central nervous system (CNS) was an immune-privileged site, separated from the immune system by the blood-brain barrier. The discovery of microglia as the resident immune cells of the brain led to a breakdown of this notion, and though it took several decades for a rebirth of research into the role of inflammatory responses in neurodegenerative disease, it is now broadly accepted to contribute to the progression of these diseases, including Parkinson's disease.

Activation of an acute inflammatory response after an injury to the CNS, such as trauma, ischemia, or infection, is a necessary physiological response to curtail injury and to repair damage. This should be of a relatively short duration and result in the elimination of the offending cell or damage, and initiation of a tissue repair response. However, if this inflammatory response persists, it can induce tissue damage, as is the case in neurodegenerative diseases. Microglia are activated in these conditions, and their role as scavengers to help clear the debris of the dying neurons is critical (DiSabato *et al.*, 2016). However, when the microglia are activated chronically such as in the neurodegenerative disease state, they can release an excess of cytotoxic factors that can further contribute to neuronal death, and thus a self-sustaining cycle of neuroinflammation and neurodegeneration is initiated (Figure 1.2) (Frank-Cannon *et al.*, 2009; Tansey and Goldberg, 2010). While microglial cells are found abundantly throughout the brain, the substantia nigra is one of the most densely populated regions, with 4-5 times the number of microglia compared to some other regions (Lawson *et al.*, 1990), rendering the dopamine neurons in this region particularly susceptible to microglial activation.

Figure 1.2 Reactive microgliosis helps to drive a self-perpetuating cycle of neurotoxicity. Inflammatory triggers and direct neurotoxic insults can instigate a cycle of neuroinflammation and neuronal damage, leading to progressive neuronal degeneration. Image taken from Block *et al.* (2007).

1.1.2.3.1 Clinical evidence

One of the first definitive pieces of evidence that neuroinflammation is important in the pathology of Parkinson's disease came from McGeer and colleagues, who observed elevated numbers of microglia in the substantia nigra of *post mortem* Parkinson's disease patients (McGeer *et al.*, 1988). These were identified as microglia due to the presence of human leukocyte antigen – DR (HLA-DR) on the surface of the cells, which is a receptor belonging to the major histocompatibility complex class II (MHC class II). These results were later confirmed by other groups using different microglial markers such as ICAM-1 or CD68 (Banati *et al.*, 1998; Imamura *et al.*, 2003). Furthermore, a large scale genome-wide association study (GWAS) of patients with late-onset sporadic Parkinson's disease carried out by Hamza and colleagues identified a single nucleotide polymorphism (SNP) in the *HLA-DR* gene expressed on microglia that was a genetic risk factor for the disease, providing evidence in living patients that microglia may contribute to the advancement of the disease (Hamza *et al.*, 2010). Since the discovery of this SNP in 2010, several other common genetic variants associated with an increased risk of Parkinson's disease have been identified in the *HLA*s corresponding to MHC class II (Hill-Burns *et al.*, 2011; Ahmed *et al.*, 2012; Wissemann *et al.*, 2013).

However, microglia are not the only cells that may contribute to the chronic neuroinflammation observed in Parkinson's disease. In Parkinson's disease brains, an increased density of reactive astrocytes has been observed in the substantia nigra (Damier *et al.*, 1993), and in addition, several genes that have been associated with an increased risk of Parkinson's disease have been identified to play a role in astrocyte function (reviewed in Booth *et al.*, 2017). Although the exact contribution of these cells to the pathogenesis of the disease remains to be elucidated, they may contribute to neuroprotective mechanisms via free radical scavenging or secretion of neurotrophic factors (Hirsch and Hunot, 2009). Furthermore, in addition to identifying increased microglial numbers in the substantia nigra of Parkinson's disease patients, McGeer and co-workers also noted the presence of $CD8^+$ lymphocytes (McGeer *et al.*, 1988), a finding which was corroborated in a mouse model (Kurkowska-Jastrzebska *et al.*, 1999). In the absence of mature T lymphocytes in two different immunodeficient mouse strains, dopaminergic degeneration induced by the toxin 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP) was markedly attenuated, indicating the adaptive immune system may also contribute to Parkinson's disease pathology (Brochard *et al.*, 2009).

The presence of neuroinflammatory processes has also been confirmed on a molecular basis. Mogi and colleagues reported increased levels of numerous cytokines in both the substantia nigra and the striatum at *post mortem* (Mogi *et al.*, 1994a; Mogi *et al.*, 1994b; Mogi *et al.*, 2007). Some of these cytokines can induce the expression of enzymes such as inducible nitric oxide synthase (iNOS) which can produce toxic reactive species. *In vivo* studies in patients have also indicated alterations in inflammatory processes in the disease. Increased levels of pro-inflammatory cytokines and chemokines such as interleukin-2 (IL-2), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and chemokine ligand 2 (CCL2) have been detected in blood samples of Parkinson's disease patients (Stypuła *et al.*, 1996; Dobbs *et al.*, 1999; Grozdanov *et al.*, 2014). Furthermore, men with elevated IL-6 levels in their plasma were reported to be at an increased risk of developing the disease, although these results may need confirmation due to the small size of this study (Chen *et al.*, 2008). Moreover, genetic studies have identified polymorphisms in neuroinflammation-related genes such as *TNF-α*, *IL-1β*, and *IL-6* that are associated with an increased risk of the disease (Krüger *et al.*, 2000; McGeer *et al.*, 2002; Håkansson *et al.*, 2005; Wahner *et al.*, 2007). Other genetic variants may affect the clinical progression of the disease, such as allele 122 of interferon-γ (IFN-γ), which is less common in early-onset than late-onset Parkinson's disease (Mizuta, 2001).

While there is clearly extensive evidence that neuroinflammation plays a role in the progression and pathology of Parkinson's disease, the most pertinent question is whether the modulation of the neuroinflammatory processes has potential as a disease-modifying therapy. Numerous epidemiological studies have investigated the risk of Parkinson's disease in chronic users of non-steroidal anti-inflammatory drugs (NSAIDs), with contradictory results. Initially, it was reported that the chronic use of non-aspirin NSAIDs was associated with a decreased risk (Chen *et al.*, 2003), and a subsequent study by the same group identified a reduced risk with ibuprofen, but not with any other NSAIDs (Chen *et al.*, 2005). Another study investigating the link between non-aspirin NSAIDs and Parkinson's disease risk noted an increased risk for women, but a decreased risk for men (Hernán *et al.*, 2006). Contrastingly, some studies did not identify any relationship between chronic NSAID use and the incidence of Parkinson's disease (Ton *et al.*, 2006;

Hancock *et al.*, 2007; Etminan *et al.*, 2008; Manthripragada *et al.*, 2011). A meta-analysis of studies between 1966 and 2008 indicated that overall as a class, NSAIDs do not modify the risk of Parkinson's disease, but ibuprofen may have a slight protective effect (Samii *et al.*, 2009). This conflicting data does not answer definitively whether anti-inflammatory therapy is a valid therapeutic approach, and perhaps more selective targets need to be identified.

1.1.2.3.2 Pre-clinical evidence

In addition to the extensive body of evidence from studies in patients as discussed above, pre-clinical experiments also provide indications that neuroinflammatory processes are involved in Parkinson's disease. In animals, models of Parkinson's disease can be induced by the administration of neurotoxins including the catecholaminergic toxin 6-hydroxydopamine (6-OHDA) (Akiyama and McGeer, 1989; Depino *et al.*, 2003; Rodrigues *et al.*, 2003), the complex I inhibitor MPTP (Liberatore *et al.*, 1999; McGeer *et al.*, 2003; Barcia *et al.*, 2004), and the pesticide rotenone (Gao *et al.*, 2002; Sherer *et al.*, 2003a), which all have been shown to result in pronounced microglial activation. Moreover, a number of pro-inflammatory stimuli have been seen to induce dopaminergic death. Injection of the bacterial endotoxin lipopolysaccharide (LPS) results in the activation of microglial cells and the death of dopaminergic neurons in the nigrostriatal pathway (Choi *et al.*, 2009; Hoban *et al.*, 2013). Similarly, administration of the viral mimetic polyinosinic:polycytidylic acid (poly I:C) in combination with other neurotoxins such as 6-OHDA or paraquat resulted in an additive inflammatory effect (Deleidi *et al.*, 2010; Bobyn *et al.*, 2012).

In addition to neuroinflammation being reported in neurotoxic models of Parkinson's disease, it has also been observed in genetic models of the disease. Microglial activation has been described in α-synuclein transgenic models and α-synuclein overexpression models, although it has been observed more consistently in overexpression models (Su *et al.*, 2008; Su *et al.*, 2009; Watson *et al.*, 2012; Cebrián *et al.*, 2014). Alterations in pro-inflammatory cytokines and increases in toll-like receptor expression have also been reported in these models (Su *et al.*, 2008; Theodore *et al.*, 2008; Watson *et al.*, 2012). Other genetic models which involve mutations or knockdowns of genes that are implicated in the risk of developing Parkinson's disease have also provided evidence that

neuroinflammatory processes contribute to the pathology of the disease, although their exact role has yet to be fully elucidated. Deletion or pharmacological inhibition of *LRRK2* led to a decreased production of pro-inflammatory mediators (Russo *et al.*, 2015), and an *LRRK2* knockout rat model was resistant to neuronal degeneration induced by LPS exposure or α-synuclein overexpression, an effect that was attributed to reduced microglial activation (Daher *et al.*, 2014). Mice with a knockout of parkin (*PARK2*) exhibited lower astrocytic and higher microglial numbers, and increased expression of pro-apoptotic proteins (Solano *et al.*, 2008).

Overall, it is clear that neuroinflammation is an important pathophysiological feature in Parkinson's disease, and thus is an attractive target for potential disease-modifying therapies. However, the contribution of these processes to the causation and progression of the disease remains to be fully deciphered, and further research into this area is required to confer more knowledge to the aetiology of Parkinson's disease and to identify specific targets that could potentially be manipulated to provide a therapeutic effect.

1.1.2.4 Oxidative stress and mitochondrial dysfunction

Reactive oxygen species (ROS) are constantly being produced by the body in all tissues as a normal product of cellular metabolism. However, if there is an imbalance between ROS production and the antioxidant activity of cells, oxidative stress can occur, as is the case in Parkinson's disease. Mitochondria are central players in this management of oxidative stress, as they are the main cellular source of free radicals and have key roles in electron transport and oxidative phosphorylation.

There is a growing body of evidence that aberrant mitochondrial function is central to the pathophysiology of Parkinson's disease. Several molecules have been identified that interfere with complex I, an important component of the mitochondrial electron transport chain. These toxins have been associated with an increased risk of the disease or are used pre-clinically to induce disease models. One of the first pieces of evidence to this was when several individuals in California inadvertently self-administered MPTP and developed levodopa-responsive Parkinsonism, which was due to the effect of the toxic metabolite MPP^+ on the mitochondrial complex I (Langston *et al.*, 1983). Subsequently, MPTP has been used to model Parkinson's disease in non-human primates and rodents as it replicates many of the pathological features of the disease, including the degeneration

of dopamine neurons and the formation of abnormal intracellular protein aggregates (Forno *et al.*, 1993; Meredith and Rademacher, 2011; Porras *et al.*, 2012). Similarly, the pesticide rotenone interferes with complex I and has been used successfully to model the disease in rodents (Betarbet *et al.*, 2000; Sherer *et al.*, 2003b; Sherer *et al.*, 2003a), and it has also been positively associated with an increased risk of developing the disease (Tanner *et al.*, 2011). The herbicide paraquat has a striking structural similarity to MPP^+, but despite this, it does not appear to interfere with complex I. This molecule has also been linked to an increased risk of developing Parkinson's disease (Tanner *et al.*, 2011), and has been used to model the disease in rodents (Brooks *et al.*, 1999; McCormack *et al.*, 2002), but its primary mechanism of toxicity appears to be due to increased oxidative stress, mediated by redox cycling (Day *et al.*, 1999; Bonneh-Barkay *et al.*, 2005).

At *post mortem*, decreased activity of complex I was reported in the substantia nigra of Parkinsonian patients (Schapira *et al.*, 1990). This deficiency is not restricted to the substantia nigra but has also been observed in other brain regions (Mizuno *et al.*, 1989; Parker *et al.*, 2008), as well as skeletal muscle (Blin *et al.*, 1994) and platelets (Yoshino *et al.*, 1992; Haas *et al.*, 1995), suggesting that this effect may be systemic. In addition, several genes that have been associated with familial inherited forms of Parkinson's disease have been reported to have effects on mitochondrial function and oxidative stress, including *LRRK2*, *Parkin*, *PINK1* and *DJ-1* (reviewed in Henchcliffe and Beal, 2008). The α-synuclein protein, encoded for by the *SNCA* gene, which is the major component of the Lewy body pathological inclusions present in the disease, has also been indicated to be intricately linked with oxidative stress and mitochondria. Treatment of cells with a mitochondrial complex I inhibitor was reported to result in an increase in α-synuclein aggregates and a reduction in adenosine triphosphate (ATP) (Lee *et al.*, 2002a). Furthermore, a transgenic mouse model expressing the mutant A53T form of α-synuclein exhibited mitochondrial DNA damage (Martin *et al.*, 2006). Mice that were deficient in α-synuclein had increased resistance to the mitochondrial toxin MPTP (Schlüter *et al.*, 2003), whereas transgenic mice that overexpressed α-synuclein had swollen, morphologically abnormal mitochondria after MPTP treatment (Song *et al.*, 2004).

The high levels of dopamine in the neurons of the nigrostriatal pathway are considered to be critical for the vulnerability of these cells to oxidative stress. Dopamine is an unstable molecule and can undergo auto-oxidation in the cytoplasm to form ROS. Under normal

conditions, monoamine oxidase A (MAO-A) is one of the major enzymes that regulates dopamine levels through oxidative metabolism. However, in cases of ageing and neuronal degeneration, levels of monoamine oxidase B (MAO-B) increase and it becomes the predominant enzyme for dopamine metabolism (Fowler *et al.*, 1980; Saura *et al.*, 1997). One of the products of MAO-B mediated metabolism is hydrogen peroxide (H_2O_2) which can be converted to the hydroxyl radical in the presence of reduced metals such as ferric iron (Fe^{3+}) (Nagatsu and Sawada, 2006). Parkinson's disease patients have been reported to have higher levels of iron in the substantia nigra when compared to controls (Dexter *et al.*, 1989; Dexter *et al.*, 1991; Kosta *et al.*, 2006), indicating that in this disease the neurons of this region are more susceptible to the toxic effects of hydroxyl radicals. In addition, the vesicular monoamine transporter 2 (VMAT2) usually keeps cytoplasmic dopamine levels under control through an active transport process but if the activity of this transporter is impaired, it can result in the formation of ROS and subsequently contribute to dopaminergic degeneration (Uhl *et al.*, 2000; Caudle *et al.*, 2007). Indeed, defects in this transporter have been reported at *post mortem* in Parkinson's disease patients (Pifl *et al.*, 2014).

To summarise, it is apparent from both pre-clinical studies and clinical research that mitochondrial function and oxidative stress are intricately intertwined and undeniably contribute to the pathology and progression of Parkinson's disease.

1.1.3 AETIOLOGY

1.1.3.1 Age

Age is the biggest risk factor for the development of Parkinson's disease, with a sharp increase in the prevalence of the disease after 60 years of age (de Lau and Breteler, 2006; Driver *et al.*, 2009; Pringsheim *et al.*, 2014). With our ever-increasing elderly population, the incidence of Parkinson's disease is set to escalate, and a study in some of the world's most populous nations predicted that the number of cases in these fifteen countries could rise to over nine million in 2030, double the number it was in 2005 (Dorsey *et al.*, 2007).

In the irreversible and inevitable process of ageing, a number of changes in physiological function occur that contribute to an increased risk of neurodegenerative disease. With age, there is a chronic progressive increase in the inflammatory status in a phenomenon known as 'inflammaging' (reviewed in Franceschi *et al.*, 2018), and as previously detailed,

neuroinflammation is an important component of Parkinson's disease pathology. Many of the other age-related changes, such as mitochondrial dysfunction, loss of proteostasis, DNA damage, and telomere attrition, have also been associated with the pathogenesis of Parkinson's disease (reviewed in Hou *et al.*, 2019).

The substantia nigra is primarily affected in Parkinson's disease, and it appears that the dopaminergic neuronal population of this brain region is particularly vulnerable to the natural ageing process. A study of elderly individuals (mean age 88.5 years) without clinically diagnosed Parkinson's disease showed that about a third of these participants exhibited mild to severe neuronal loss within the substantia nigra (Buchman *et al.*, 2012). This increased vulnerability has been attributed to the distinct environment of the substantia nigra. The dopaminergic neurons that populate this region are pigmented, show pacemaking activity and are under additional oxidative stress related to the metabolism of dopamine. These neurons are also thought to be particularly sensitive to mitochondrial dysfunction. All of these characteristics contribute to an increased susceptibility leading to cell death (reviewed in Reeve *et al.*, 2014). Although age is the single largest contributing factor associated with the risk of developing Parkinson's disease, it is likely to be due to a combination of factors, including age, genetics, and environment.

1.1.3.2 Genetic predisposition

While the majority of cases of Parkinson's disease are sporadic with an unclear aetiology, 15% of Parkinson's disease patients have a family history, and 5-10% of patients suffer from a monogenic form of the disease exhibiting a classical Mendelian type of inheritance (Lesage and Brice, 2009; Kalinderi *et al.*, 2016). The first Parkinson's disease gene mutation discovered was a point mutation in the α-synuclein gene, which is the major component of Lewy bodies, and resulted in an autosomal dominant form of the disease (Polymeropoulos *et al.*, 1997). Since then, other mutations in this gene leading to an inherited form of the disorder have since been identified (Table 1.1), including point mutations and whole-locus multiplications (Krüger *et al.*, 1998; Ibáñez *et al.*, 2004; Zarranz *et al.*, 2004).

The leucine-rich repeat kinase 2 (*LRRK2*) gene was the second causal gene linked to autosomal dominant inherited Parkinson's disease (Paisán-Ruíz *et al.*, 2004; Zimprich *et al.*, 2004). This gene encodes for a large multi-domain protein, and although its properties

have yet to be fully elucidated, it has been associated with mitochondrial function and vesicle trafficking (reviewed in Wallings *et al.*, 2015). Mutations in genes that result in an autosomal recessive inherited form of the disease have also been discovered, including *Parkin* (E3 ubiquitin ligase), *PINK1* (PTEN-induced kinase 1) and *DJ-1* (protein deglycase) (Kitada *et al.*, 1998; Bonifati *et al.*, 2003; Valente *et al.*, 2004). To date, at least 23 loci and 19 genes have been linked to a Parkinson's disease phenotype (Deng *et al.*, 2018). While the majority of this research is primarily focused on variations in the motor phenotypes of the disease, polymorphisms have also been identified that affect the risk of developing non-motor symptoms, such as a cannabinoid CB_1 receptor gene polymorphic triplet which has been associated with a reduction in the prevalence of depression in Parkinson's disease (Barrero *et al.*, 2005). Genome-wide association studies and meta-analyses have been key at identifying these genes which are associated with an altered risk of Parkinson's disease. However, the loci that have been identified do not account for all of the phenotypic variability in the disease, and while such studies are a useful tool for identifying the most common variants, a great number of variants of small effect remain to be discovered.

Gene	Gene product	Inheritance	Pathology	Clinical phenotype	Reference
SNCA	α-synuclein	Autosomal dominant	Nigral degeneration with Lewy bodies	Early-onset, aggressive course	(Polymeropoulos *et al.*, 1997)
LRRK2	Leucine-rich repeat kinase 2	Autosomal dominant	Variable α-synuclein and tau pathology	Late-onset, typical course	(Paisán-Ruíz *et al.*, 2004; Zimprich *et al.*, 2004)
VPS35	Vacuolar protein sorting 35	Autosomal dominant	No pathology reported	Late-onset, typical course	(Vilariño-Güell *et al.*, 2011; Zimprich *et al.*, 2011)
GBA	Lysosomal glucocerebrosidase	Autosomal dominant	Nigral degeneration with Lewy bodies	Late-onset, typical course	(Neudorfer *et al.*, 1996; Aharon-Peretz *et al.*, 2004)
Parkin	E3 ubiquitin ligase	Autosomal recessive	Nigral degeneration without Lewy bodies	Early-onset, slow course	(Kitada *et al.*, 1998)
PINK1	PTEN-induced mitochondrial serine/threonine kinase	Autosomal recessive	Nigral degeneration with Lewy bodies	Early-onset, slow course	(Valente *et al.*, 2004)
DJ-1	Redox-dependent molecular chaperone in mitochondria	Autosomal recessive	No pathology reported	Early-onset, slow course	(Bonifati *et al.*, 2003)
ATP13A2	Neuronal P-type ATPase	Autosomal recessive	Pallidopyramidal degeneration	Juvenile onset, atypical	(Ramirez *et al.*, 2006)
PLA2G6	Phospholipase A2	Autosomal recessive	Spheroid body accumulation, Lewy body formation	Juvenile onset, atypical	(Paisan-Ruiz *et al.*, 2009)
FBX07	E3 ubiquitin protein ligase	Autosomal recessive	Parkinsonism and pyramidal tract signs	Juvenile onset, atypical	(Shojaee *et al.*, 2008; Di Fonzo *et al.*, 2009)

Table 1.1 Genes implicated in the aetiology of Parkinson's disease. Adapted from Funke *et al.* (2013); Bonifati (2014).

1.1.3.3 Environmental factors

The aetiology of Parkinson's disease is widely accepted to be multi-factorial and numerous environmental determinants have been implicated in the onset of this disease, with some associated with causing an increased risk and others being protective.

Beginning in 1917 and persisting through the 1920s, an epidemic of encephalitis lethargica spread across the globe, providing the first suggestion of a link between viral infection and Parkinson's disease (reviewed in Hoffman and Vilensky, 2017). This malady is classically characterised by acute and chronic phases. The chronic phase could occur months or years later and was most commonly associated with Parkinsonian features. In the years following the epidemic, it was estimated that as many as 50% of Parkinsonian cases were post-encephalitic (Krusz *et al.*, 1987). Links have since been identified between Parkinson's disease risk and several other infections, including herpes simplex virus (HSV), influenza virus A and mumps (Marttila *et al.*, 1977; Marttila and Rinne, 1978; Harris *et al.*, 2012; Vlajinac *et al.*, 2013; Olsen *et al.*, 2018).

One of the most persuasive pieces of evidence that environmental factors other than infection play a major role in the aetiology of Parkinson's disease came in the 1980s when several intravenous drug users in California developed marked Parkinsonism seemingly overnight. These individuals had all inadvertently self-administered the meperidine analogue MPTP which was present in a contaminated batch of heroin. These patients all had a dramatic response to treatment with L-DOPA (Langston *et al.*, 1983). The toxic metabolite of MPTP, MPP^+, has a striking structural similarity to the herbicide paraquat (Sandy *et al.*, 1988), and thus an interest was incited in the potential role of herbicides and pesticides in Parkinson's disease pathogenesis.

To date, numerous epidemiological studies have investigated the link between Parkinson's disease and pesticides, indicating a correlation between pesticide use and disease development (Brown *et al.*, 2006). However, the majority of these studies rely on the self-recollection of lifetime pesticide exposure, and the common exposure of individuals to more than one pesticide also adds confoundation to the identification of a singular causative agent. Tanner and colleagues (Tanner *et al.*, 2011) found that specifically the herbicide paraquat and the insecticide rotenone led to an increased risk of Parkinson's disease. These compounds are now used to model Parkinson's disease in

animals and cause degeneration of the nigrostriatal dopamine neurons by different mechanisms: paraquat by inducing oxidative stress (McCormack *et al.*, 2002), and rotenone by inhibition of mitochondrial complex I (Betarbet *et al.*, 2000).

Conversely, some environmental factors have been found to be protective against the risk of developing Parkinson's disease. An inverse relationship between smoking and Parkinson's disease has been demonstrated in several studies (Ritz *et al.*, 2007; Li *et al.*, 2015). This relationship has been shown to be dose-dependent, with a lower risk with increasing numbers of pack-years, and also temporal, with more years of smoking and fewer years since quitting associated with a decreased Parkinson's disease risk (Hernán *et al.*, 2001; Thacker *et al.*, 2007). β-adrenoceptor agonists such as salbutamol have also been associated with a decreased Parkinson's disease risk, and conversely, chronic use of β-blockers are associated with an increased risk of the disease (reviewed in Hopfner *et al.*, 2020). Caffeine is an adenosine A2A receptor antagonist, and consumption of caffeine has been seen to have a potential protective effect (Ross *et al.*, 2000; Ascherio *et al.*, 2001).

To summarise, there is no single specific cause of Parkinson's disease, and the development of the disease is most likely due to an interplay of age, genetic factors and environmental influences.

1.1.4 CURRENT PHARMACOLOGICAL TREATMENTS

1.1.4.1 L-DOPA

The development of high-dose levodopa (L-DOPA) therapy in 1967 by Cotzias and colleagues was revolutionary for the treatment of Parkinson's disease (Cotzias *et al.*, 1967), and presently, over 50 years later, this treatment has remained the 'gold standard' for the management of the motor symptoms of the disease. L-DOPA rapidly traverses the blood-brain barrier and is converted to active dopamine by the enzyme DOPA decarboxylase, thus replacing the striatal dopamine that has been lost due to the degeneration of the nigrostriatal neurons. It is usually administered alongside a peripherally restricted DOPA decarboxylase inhibitor such as carbidopa or benserazide in order to maximise central dopamine levels and thus maximise the therapeutic effect. This approach also avoids the potential gastrointestinal and cardiovascular side effects associated with elevated circulating catecholamines peripherally (Fahn, 2008).

1.1.4.2 Dopamine agonists

Dopamine agonists exert anti-Parkinsonian effects by working directly on postsynaptic dopamine receptors, thereby bypassing the degenerating nigrostriatal neurons. These can be classified into ergoline agonists, such as bromocriptine, pergolide and cabergoline, or non-ergoline agonists such as ropinirole and pramipexole (Brooks, 2000). There is now a shift away from using drugs in the ergoline class, which were the first generation of dopamine agonists, as these drugs have been associated with a higher risk of fibrosis and valvular heart disease than their non-ergoline counterparts (Reichmann *et al.*, 2006; Zanettini *et al.*, 2007; Tran *et al.*, 2015).

Dopamine agonist therapy was originally introduced as an adjunct to levodopa treatment for patients who were exhibiting side effects dyskinesias or motor fluctuations (see in further detail in section 1.1.4.6). This permitted a modest reduction in the dose of L-DOPA for patients and thus an amelioration of these motor complications. Dopamine agonist monotherapy has also been successful in the treatment of newly diagnosed patients of a younger age of onset (<60 years). This approach delays the requirement of levodopa and thus the onset of the motor side effects of levodopa therapy (Przuntek *et al.*, 1996; Stowe *et al.*, 2008; Connolly and Lang, 2014).

1.1.4.3 MAO-B/COMT inhibitors

Another strategy to treat the deficiency of dopamine in Parkinson's disease is by the inhibition of its metabolism. Monoamine oxidase isoform B (MAO-B) and catechol-O-methyltransferase (COMT) are two enzymes that are involved in the breakdown of dopamine, and drugs that inhibit these enzymes have significant effects on striatal dopamine levels. Selective irreversible MAO-B inhibitors, such as selegiline and rasagiline, can be used as a monotherapy in earlier Parkinson's disease when some dopamine neurons are still intact (Parkinson Study Group, 1993; Biglan *et al.*, 2006). However, they are mainly used as an adjunct to levodopa therapy in patients with more advanced disease who are experiencing motor fluctuations (Golbe *et al.*, 1988; Heinonen and Rinne, 1989; Waters *et al.*, 2004). COMT inhibitors can be peripherally acting (e.g. entacapone) or CNS-active (e.g. tolcapone) and both types can be beneficial in Parkinson's disease therapy, although peripheral inhibitors are usually preferred due to the severe hepatic adverse effects associated with tolcapone (Borges, 2005). These drugs are used as an adjunct to combined levodopa and DOPA decarboxylase inhibitor therapy

and work by inhibiting the systemic metabolism of L-DOPA thus prolonging its half-life (Rivest *et al.*, 1999).

1.1.4.4 Non-dopaminergic pharmacological therapies

In the 19th century, many years before the discovery of the neurotransmitter dopamine, anticholinergic drugs were used as a treatment for Parkinson's disease. The credit of the observation of anticholinergic efficacy has been attributed to Jean-Martin Charcot, whose intern Leopold Ordenstein wrote his thesis on the treatment of Parkinsonian tremor with belladonna alkaloids (Ordenstein, 1868; Goetz, 2011). Later it was understood that these drugs affect the dopaminergic/cholinergic balance in the striatum, hence their therapeutic effect. Anticholinergics are still used clinically to treat Parkinson's disease to this day, for monotherapy or as an adjunct to levodopa, and are especially used in cases in which tremors are resistant to levodopa therapy (Brocks, 1999).

Amantadine was originally in use as an anti-viral drug when it was coincidentally discovered to improve the symptoms of Parkinson's disease (Hubsher *et al.*, 2012). It is a non-competitive antagonist at the *N*-methyl-D-aspartate (NMDA) receptor, a glutamate receptor and ion channel found in neurons. Alterations in glutamate signalling have been implicated in Parkinson's disease pathophysiology and in the development of levodopa-induced dyskinesias, and the therapeutic effects of amantadine have been attributed to attenuation of the glutamatergic drive to the direct pathway (Crosby *et al.*, 2003; Vanle *et al.*, 2018).

1.1.4.5 Deep brain stimulation

Deep brain stimulation (DBS) is the most common non-pharmacological procedure used to treat Parkinson's disease. This therapy involves the surgical implantation of electrodes that can stimulate specific brain regions. The most common regions targeted in the treatment of Parkinson's disease are the subthalamic nucleus and the internal globus pallidus. The stimulation of these areas has been proven to be effective at reducing the tremor, bradykinesia, rigidity and gait disturbances from which Parkinson's disease patients suffer (Deep-Brain Stimulation for Parkinson's Disease Study Group *et al.*, 2001; Weaver *et al.*, 2005). As it is an invasive surgical procedure, DBS is usually only considered when a patient's symptoms can no longer be sufficiently controlled by dopaminergic therapy and further refinement of the pharmacological treatment regime is

not possible. Furthermore, there are a number of exclusion criteria which means patient candidacy is limited by surgical risk factors such as age and co-morbidities (Perlmutter and Mink, 2006; Groiss *et al.*, 2009).

1.1.4.6 Limitations of current treatments

Despite the profound therapeutic effects associated with initial levodopa treatment, these effects are short-lived. Long-term levodopa treatment is very commonly associated with motor fluctuations and dyskinesias, which have been reported in 70-90% of patients within 10 years of therapy (López *et al.*, 2010). With time, the duration of symptomatic control of L-DOPA becomes shorter as plasma levels of L-DOPA swing between peaks and troughs. This results in oscillations in motor functionality and a re-emergence of Parkinsonian symptoms in the patient. Dyskinesias, or hyperkinetic involuntary movements, are also frequent side effects with chronic levodopa treatment (Salat and Tolosa, 2013). Several factors have been identified that can contribute to the development of dyskinesias, such as earlier age at onset of Parkinson's disease, longer duration of levodopa treatment and overall higher exposure, as well as some genetic factors (Fabbrini *et al.*, 2007).

As previously mentioned, dopamine agonists, inhibitors of dopamine metabolism or other pharmacological treatments have been developed in an effort to delay the requirement for levodopa and to reduce the debilitating side effects of chronic levodopa treatment. Dopamine agonist monotherapy is often recommended in the early stages of Parkinson's disease when the loss of dopaminergic neurons is less extensive, to prolong the time until levodopa therapy is required. However, these drugs can impose risks for patients with a history of cardiovascular diseases, and they have also been linked to the development of impulse control disorders in numerous studies (Weintraub *et al.*, 2006; Voon *et al.*, 2011; Borovac, 2016). These impulse control disorders include pathological gambling, hypersexuality, and compulsive eating and buying, and can have a devastating impact on a patient's life and on the lives of their families.

MAO-B inhibitors are usually well tolerated and side effects are usually relatively minor gastrointestinal issues, but their therapeutic effect is modest in comparison to levodopa treatment (Dezsi and Vecsei, 2017). In the case of COMT inhibitors, drugs in this class have been associated with diarrhoea which sometimes necessitates discontinuation of the

drug. The use of tolcapone, a centrally acting COMT inhibitor, has been severely restricted by the FDA after being linked to several deaths of Parkinson's disease patients due to hepatic toxicity, and severe monitoring criteria need to be fulfilled if it is prescribed (Colosimo, 1999; Rezak, 2007).

Anticholinergic drugs are fraught with adverse effects, particularly in the elderly, hence their reservation for tremors that are resistant to dopamine therapy. These adverse effects include peripheral anticholinergic effects such as blurred vision, constipation and urinary retention, and centrally-mediated effects such as mental confusion (Brocks, 1999). The anti-viral agent amantadine is generally associated with a low adverse effect profile, but it can cause dizziness, peripheral oedema, dry mouth and hallucinations. Livedo reticularis, or a mottled reticulated discolouration of the skin, is a less common side effect, but amantadine is one of the best-known drugs to cause this condition (Crosby *et al.*, 2003; Dragašević-Mišković *et al.*, 2019).

While deep brain stimulation can be highly effective at managing some of the motor symptoms of Parkinson's disease, it has little effect on the cognitive and affective symptoms. There are also large capital costs associated with the procedure, and it requires an expert multidisciplinary team. In addition, the advanced age of the general Parkinson's disease population means that many patients are not eligible for the surgery, due to an increased risk of pre-existing comorbidities in addition to the inherent risk of age (Groiss *et al.*, 2009; Lozano *et al.*, 2019).

Although the therapies that have been developed to treat Parkinson's disease in the last 50 years have been hugely beneficial at treating the motor symptoms of the disease and giving patients back their quality of life, there is still a major unmet clinical need. All currently available pharmacological therapies fail to treat the underlying pathological cause of the disease, which is the death of the nigrostriatal dopamine neurons. The death of these neurons progresses while the treatment merely alleviates symptoms, and thus there is a clear therapeutic need for treatments that are neuroprotective or disease-modifying in some way. If such a therapy was developed that could slow or halt the progression of the disease, this would be hugely beneficial in maintaining a patient's quality of life.

1.2 THE ENDOCANNABINOID SYSTEM

The endocannabinoid system is a relatively newly discovered system in neuropharmacology. However, research into this field has grown exponentially in recent decades and it is now recognized that the endocannabinoid system plays important physiological and pathological roles in neuroinflammation and neurodegeneration, alongside numerous other functions such as in pain, mood, and appetite.

1.2.1 OVERVIEW OF THE ENDOCANNABINOID SYSTEM

The endocannabinoid system is a complex signalling system, composed of receptors, enzymes, transport proteins and endogenous ligands. The term 'cannabinoid' refers to any compound whose effects are mediated through the modulation of components of this system. This includes molecules found in the two main *Cannabis* plant subspecies, *Cannabis sativa* and *Cannabis indica*, as well as endogenous and synthetic compounds. The potential of cannabinoids as therapeutic agents has been acknowledged from as early as the third millennium B.C. in China, where texts have been discovered that observe the beneficial effects of cannabinoids or cannabis in the relief of rheumatic pain (Mechoulam, 1986). Throughout the subsequent millennia, cannabinoids remained in use both medicinally and recreationally. To date, there are over 100 cannabinoids that have been isolated from cannabis, including cannabidiol (CBD) identified in 1963 (Mechoulam and Shvo, 1963) and the main psychoactive component, Δ9-tetrahydrocannabinol (THC) identified in 1965 by Mechoulam and Gaoni, which constituted the first major breakthroughs in modern times (Mechoulam and Gaoni, 1965). It would be over two decades before the first THC binding site was discovered (Matsuda *et al.*, 1990), but since then research into this field has grown expeditiously.

1.2.1.1 Cannabinoid receptors

The effects of cannabinoids are primarily mediated through two major receptors, type 1 and type 2, CB_1 and CB_2 respectively. The receptor now known as the CB_1 receptor was identified first (Devane *et al.*, 1988; Matsuda *et al.*, 1990) and in recent years the structure has been further elucidated (Hua *et al.*, 2017; Shao *et al.*, 2019). This receptor is widely distributed in the central nervous system, particularly in the cerebellum, cortex, basal ganglia, and hippocampus (Herkenham *et al.*, 1990; Glass *et al.*, 1997). Within the CNS,

it has been shown to be predominantly expressed on the axons and terminals of neurons. CB_1 receptors are also present at lower but still functionally relevant levels in many peripheral tissues and organs, including adipose tissue (Cota *et al.*, 2003), liver (Osei-Hyiaman *et al.*, 2005), pancreas (Nakata and Yada, 2008), and skeletal muscle (Cavuoto *et al.*, 2007).

Cloning of the CB_2 receptor came in 1993, three years following the identification of the CB_1 receptor and the crystal structure of the receptor has subsequently been reported at a high resolution (Munro *et al.*, 1993; Li *et al.*, 2019). Originally, CB_2 was informally referred to as the 'peripheral receptor' as it was largely found in immune tissues (with the highest mRNA levels in the tonsils and spleen) and in peripheral immune cells, and it did not appear to be present in the brain (Galiegue *et al.*, 1995). However, it is now widely accepted that the expression of this receptor is upregulated on activated microglia in the central nervous system (Carlisle *et al.*, 2002; Maresz *et al.*, 2005). The presence of the CB_2 receptor on neurons in the CNS remained a debate for years, with much contrasting evidence being published. It is now thought that the receptor is expressed on particular subsets of neurons at functionally relevant levels, although the different techniques used to identify CB_2 expression can exhibit varying results (reviewed in Atwood and Mackie, 2010). The human, mouse, and rat protein sequences of the CB_2 receptor have been found to differ quite substantially in the C-terminus, with the mouse sequence being 13 amino acids shorter and the rat sequence 50 amino acids longer compared to the human protein (Brown *et al.*, 2002). Therefore, caution must be taken in extrapolating the results from animal studies to the effects of CB_2 activation in humans. Furthermore, one of the greatest challenges currently in the field is the lack of reliable and specific antibodies against CB_2 (Baek *et al.*, 2013; Marchalant *et al.*, 2014), which has contributed to the conflicting reports on CB_2 receptor distribution.

Both CB_1 and CB_2 receptors belong to the $G_{i/o}$ family of seven transmembrane G protein-coupled receptors, although the identity between the two receptors in humans is remarkably low, with only 44% homology overall and 68% in the transmembrane helices, which contain the putative binding sites for cannabinoids (Lutz, 2002). Both receptors inhibit the enzyme adenylyl cyclase, resulting in reduced synthesis of cyclic adenosine monophosphate, and activate the mitogen-activated protein kinase pathway by signalling through $G_{i/o}$ proteins (Figure 1.3). Furthermore, CB_1 activation can modulate certain

voltage-gated calcium channels and inwardly rectifying potassium currents (Howlett *et al.*, 2002). The CB_2 receptor also affects additional pathways, such as activation of phospholipase C, leading to calcium release (Sugiura *et al.*, 2000), and activation of the phosphatidylinositol 3-kinase pathway (Viscomi *et al.*, 2009).

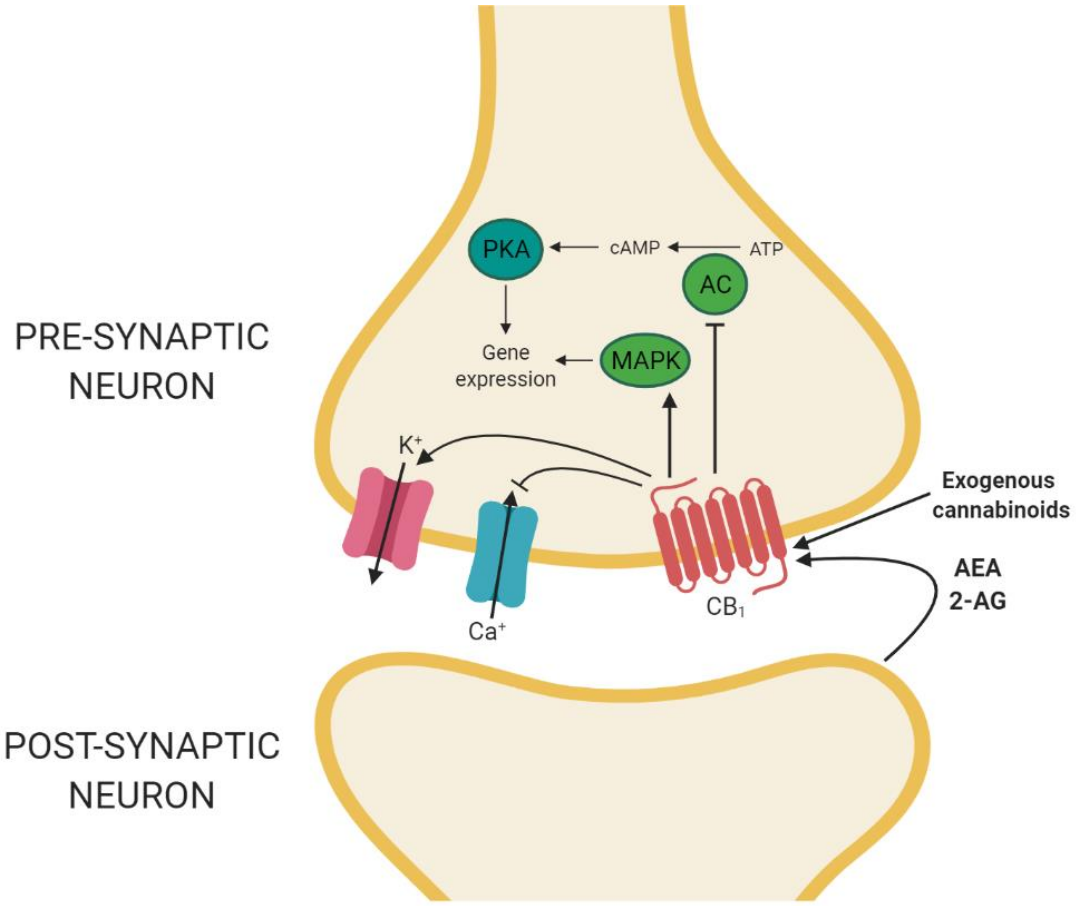

Figure 1.3 Signalling pathways involved in cannabinoid receptor activation. Activation of cannabinoid receptors by exogenous cannabinoids or endogenous cannabinoids such as the retrograde neurotransmitters AEA and 2-AG stimulates $G_{i/o}$ proteins. These proteins are coupled to inhibition of adenylate cyclase (AC) with subsequent inactivation of the protein kinase A (PKA) pathway and to stimulation of mitogen-activated protein kinase (MAPK) which induces gene expression. Stimulation of CB_1 receptors also induces inhibition of voltage-activated Ca^{2+} channels and stimulation of inwardly rectifying K^+ channels in neurons. cAMP = cyclic adenosine monophosphate; ATP = adenosine triphosphate.

1.2.1.2 Cannabinoid ligands

Equipped with the rationale that receptors would not be present in the body without the existence of an endogenous ligand, following the discovery of the cannabinoid receptors researchers began the search for these 'endocannabinoids'. The two best characterised are

arachidonoyl ethanolamide (usually referred to as anandamide or AEA) and 2-arachidonoyl glycerol (2-AG). Unlike most classical neurotransmitters, anandamide and 2-AG are not stored in vesicles but are synthesized 'on demand' (usually by membrane depolarization or activation of certain G-protein coupled receptors) from their lipid membrane precursors. Upon their postsynaptic release, they act as retrograde messengers, suppressing transmitter release from neurons in either a transient or long-term manner, roles that have been well established (Freund *et al.*, 2003; Chevaleyre *et al.*, 2006; Kano *et al.*, 2009). These two endocannabinoids possess distinct properties with regard to their interactions with the cannabinoid receptors. 2-AG works as a full agonist at both receptors with low-to-moderate affinity. Anandamide, on the other hand, has very low activity at the CB_2 receptor but is a high-affinity, partial agonist at the CB_1 receptor (Gonsiorek *et al.*, 2000). Anandamide also has affinity at transient receptor potential vanilloid type 1 (TRPV1) channels (Smart *et al.*, 2000), although the strength of its agonism seems to be strongly dependent on the level of receptor reserve in the tissue (Andersson *et al.*, 2002; Ross, 2003). Besides the aforementioned receptors, endocannabinoids and phytocannabinoids also interact with the orphan receptor G protein-coupled receptor 55 (GPR55) (Ryberg *et al.*, 2007) and peroxisome proliferator-activated receptors (PPARs) (Sun and Bennett, 2007; Pertwee *et al.*, 2010).

As well as having disparate intrinsic efficacies, anandamide and 2-AG also have distinct biosynthesis and metabolism pathways. Anandamide is primarily formed through cleavage of the phospholipid *N*-arachidonoyl-phosphatidylethanolamine (NArPE) by a specific phosphodiesterase enzyme known as *N*-acyl phosphatidylethanolamine-specific phospholipase D or NAPE-PLD (Di Marzo *et al.*, 1994; Okamoto *et al.*, 2004). Ultimately, the inactivation of anandamide is carried out by an enzyme named fatty acid amide hydrolase (FAAH) through hydrolysis of the amide bond to form ethanolamine and arachidonic acid (Di Marzo *et al.*, 1994). 2-AG is a monoacylglycerol and is formed through the hydrolysis of diacylglycerols (DAGs) by diacylglycerol lipases (DAGLs) α and β, with the two isoforms differentially expressed in developing and adult nervous tissue (Bisogno *et al.*, 2003). 2-AG can be metabolized by several different chemical reactions. It is mainly degraded by monoacylglycerol lipase (MAGL) to arachidonate and glycerol, although the αβ-hydrolases ABHD6 and ABHD12 can also catalyse its hydrolysis (Blankman *et al.*, 2007).

In addition to 2-AG and anandamide, other endogenous molecules have also been discovered that are in some way related to the cannabinoid system. This includes molecules that interact in some way with the endocannabinoid system (e.g. 2-arachidonyl glyceryl ether (noladin ether) (Hanus *et al.*, 2001)), and endocannabinoid-like lipids that do not act via cannabinoid receptors (e.g. *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA) (Rodriguez de Fonseca *et al.*, 2001; Re *et al.*, 2007)). However, the importance of these molecules in relation to the endocannabinoid system has as of yet not been as well characterised as the two primary endocannabinoids.

Many synthetic cannabinoid compounds have now been developed, including specific agonists, antagonists, and inverse agonists, which have different affinities and efficacies at the cannabinoid receptors (Le Boisselier *et al.*, 2017). Molecules that act on enzymes or transporter proteins involved in the cannabinoid system have also been generated. The creation of these molecules and the isolation of the vast array of cannabinoid compounds found naturally in the *Cannabis* plants allows researchers to better investigate the physiological functions of the cannabinoid system, and thus advance potential therapies for neurological disorders.

1.2.2 THE CANNABINOID SYSTEM IN INFLAMMATION AND IMMUNE MODULATION

Mounting evidence indicates that the cannabinoid system has a major function in the modulation of the immune response and inflammation, both centrally and peripherally. Therefore, this system has the potential to be manipulated in order to provide therapeutic benefits in diseases with an inflammatory component.

The presence of both the CB_1 receptor and the CB_2 receptor on immune cells was one of the first pieces of evidence to indicate that the endocannabinoid system might play a role in the immune response (Galiegue *et al.*, 1995). Results from subsequent *in vitro* and *in vivo* studies suggest that cannabinoids execute their immunomodulatory effects in numerous ways: by induction of apoptosis; by suppression of cell proliferation; by modulation of immune cell migration; by increased anti-inflammatory cytokine production and inhibited production of pro-inflammatory cytokines and chemokines; and by modulation of the expansion of regulatory T cells (Klein *et al.*, 1998; Klein *et al.*, 2003). Cannabinoid compounds have been seen to cause alterations in immune function from as early as the 1980s, a decade before the cannabinoid receptors were even

characterized. Tindall and co-workers detected a more rapid progression from HIV infection to AIDS in marijuana smokers compared to those who did not use the drug (Tindall *et al.*, 1988). HIV-positive individuals who used marijuana also had an increased risk of bacterial pneumonia, opportunistic infections, and Kaposi's sarcoma (Newell *et al.*, 1985; Caiaffa *et al.*, 1994). Alveolar macrophages obtained from the lungs of habitual marijuana smokers who were otherwise healthy individuals showed an impaired phagocytic ability, decreased cytotoxicity, and reduced cytokine production (Baldwin *et al.*, 1997). Clearly, exogenous cannabinoids affect the immune system and if this effect could be manipulated, it could be beneficial in the treatment of a vast number of conditions with an immune component.

As described in the previous section, in the brain, CB_1 receptors are predominantly found on the terminals of neurons, where they play a role in neurotransmitter release. However, as they are also present on immune cells, albeit in relatively low quantities, ergo they can have an effect on immune modulation. mRNA analyses showed that with regard to human peripheral immune cells, the highest levels of CB_1 expression were seen in B cells, followed by natural killer cells, and with varying expression in several other blood cell types including monocytes and T cells (Galiegue *et al.*, 1995). Multiple sources of evidence suggest that the CB_1 receptor on immune cells could be a potential target for the regulation of inflammation. Indeed, much evidence exists for a role of the CB_1 receptor in the chronic demyelinating disease multiple sclerosis (MS), which is an immune-mediated disease involving the demyelination of neurons by $CD4^+$ T cells. In *post mortem* brain tissue from MS patients, CB_1 staining co-localized with $CD68^+$ macrophages and $CD3^+$ T cells in areas of active lesions (i.e. areas with activated microglia) (Benito *et al.*, 2007). As expected, this study also reported CB_1 staining in $MAP2^+$ neurons and MBP^+ oligodendrocyte cells. Animal models of MS such as the experimental autoimmune encephalomyelitis (EAE) model found immune modulation or disease amelioration through CB_1 receptor agonism (Arevalo-Martin *et al.*, 2003; Maresz *et al.*, 2007; Rossi *et al.*, 2011; De Lago *et al.*, 2012). Furthermore, anandamide, through a CB_1-dependent mechanism, inhibited Theiler's virus-induced vascular cell adhesion molecule-1 (VCAM-1) expression in mice. This molecule is involved in leukocyte transmigration across the blood-brain barrier, which contributes to the pathology in MS (Mestre *et al.*, 2011). Apart from these immunomodulatory effects, CB_1 can also have beneficial roles

in neuroprotection by the inhibition of excitotoxicity. A number of observations suggest that excitotoxicity contributes to the pathology of MS (Werner *et al.*, 2001; Srinivasan *et al.*, 2005; Kostic *et al.*, 2013). The CB_1 receptor can be found presynaptically and can modulate glutamate release (van der Stelt *et al.*, 2001), and thus has a critical role for excitotoxicity control in neurological conditions. However, despite the immunomodulatory and anti-excitotoxic effects associated with targeting the CB_1 receptor, research has largely focused on the CB_2 receptor as a potential target in the endocannabinoid system to modulate the immune response and inflammation. This is based on the undesired psychoactive effects of CB_1 activation, and the associated safety concerns. In addition, the therapeutic window to target the CB_1 receptor following acute injury is likely to be relatively short, as most neuroprotective drugs in response to acute brain injury need to be administered within 6 hours of injury onset (Ashton and Glass, 2007). As the CB_2 receptor is thought to be devoid of psychoactivity due to its restricted presence on CNS neurons, and as it is expressed at vastly greater numbers on immune cells and tissues than the CB_1 receptor, the CB_2 receptor has been the focus of much research investigating its potential as an immunomodulatory or anti-inflammatory target.

CB_2 receptors are expressed in high levels in peripheral immune tissues, such as the spleen and the tonsils, and at levels in immune cells that are 10–100 times the levels of expression of the CB_1 receptor. The immune cells express the CB_2 receptor to different extents, with the rank order being B cells > natural killer cells > monocytes > neutrophils > CD8 lymphocytes > CD4 lymphocytes (Galiegue *et al.*, 1995). Dendritic cells have also been shown to express CB_2 receptors (Matias *et al.*, 2002). However, the level of expression of this receptor on immune cells is dependent on both the activation state of the cell and the nature of the activating stimulus. Lee and co-workers found that stimulation with LPS substantially downregulated CB_2 mRNA expression in splenocyte cultures in a dose-dependent manner (Lee *et al.*, 2001). Contrastingly, stimulation with antibodies against cluster of differentiation 40 (CD40), which is a costimulatory molecule expressed by antigen-presenting cells, upregulated CB_2 expression. This CD40-mediated upregulation was also seen in peripheral blood and tonsillar B cells (Carayon *et al.*, 1998). CB_2 upregulation has also been reported in response to IFN-γ in both microglia and macrophages (Carlisle *et al.*, 2002).

As previously mentioned, one way cannabinoids execute their immunomodulatory role is by affecting the apoptosis of immune cells. As early as 1994 Schwarz and colleagues (Schwarz *et al.*, 1994) demonstrated that high concentrations of THC or anandamide could induce apoptosis of B and T lymphocytes. Zhu *et al.* (Zhu *et al.*, 1998) later demonstrated that THC induced apoptosis in both lymphocytes and macrophages, and that Bcl-2 and caspase-1 were involved. It was noted that fragmentation preceded membrane damage, suggesting that THC was inducing apoptosis rather than necrosis of cells. This THC-induced immune suppression via T cell apoptosis was later exhibited *in vivo* in C57BL/6 mice (McKallip *et al.*, 2002b). The use of CB_2 antagonists, but not CB_1 antagonists, blocked apoptosis in these cells, indicating that THC induced apoptosis in a CB_2 receptor-dependent manner. This suggests that targeting the CB_2 receptor may be a promising approach to treating inflammatory and autoimmune diseases. Evidence to confirm this included the use of the synthetic CB_2 agonist, JWH015, which in a dose-dependent manner both inhibited proliferation and induced apoptosis of splenocytes and thymocytes (Lombard *et al.*, 2007). Cannabinoids have also been observed to induce the apoptosis of malignant immune cells (McKallip *et al.*, 2002a; Lombard *et al.*, 2005; McKallip *et al.*, 2006), insinuating that CB_2 receptor activation could also be a novel therapeutic modality against immune system malignancies such as lymphomas and leukaemias.

In addition to affecting apoptosis, cannabinoids also influence the proliferation of immune cells. Anandamide has been demonstrated to inhibit mitogen-induced proliferation of B and T lymphocytes, at concentrations relevant to the regulation of neuronal responses. Δ8-tetrahydrocannabinol and the non-selective cannabinoid agonist CP55,940 also inhibited lymphocyte proliferation, but to a lesser extent (Schwarz *et al.*, 1994). 2-AG was noted to have an effect on the proliferation of mouse splenocytes in culture, an effect that seems to be dependent in part on cell density (Lee *et al.*, 1995). Cannabinoids have been observed to have a biphasic role with regard to both B cells and T cells. In B cells, increased proliferation was demonstrated in response to THC at low nanomolar concentrations (Derocq *et al.*, 1995), whereas another study showed that THC caused a reduction in LPS-induced proliferation of B cells (Klein *et al.*, 1985). A similar effect was seen in T cells, with high doses of THC being inhibitory and low doses being

stimulatory (Patrini *et al.*, 1997). This biphasic response should be taken into consideration when examining the effects of cannabinoids on immune cell proliferation.

Additionally, cannabinoid receptor activation has been shown to modulate the migration of both central and peripheral immune cells, which is an important element to acknowledge when studying diseases with an inflammatory component. The synthetic cannabinoid CP55,940 decreased the *in vitro* migration of rat macrophages, an effect that was attributed to both cannabinoid receptors, although CB_2 had a more substantial effect (Sacerdote *et al.*, 2000). In addition to affecting macrophage migration, the CB_2 receptor also regulates the migration of neutrophils, natural killer cells, T cells, and B cells, with different agonists seen to cause varying effects. Endocannabinoids, phytocannabinoids, and synthetic cannabinoids can differentially modulate second messenger pathways in a phenomenon known as 'functional selectivity' or 'agonist trafficking' (Shoemaker *et al.*, 2005). This phenomenon is thought to be relevant for CB_2-induced cell migration (reviewed in Miller and Stella, 2008), but undoubtedly further studies are required in order to make targeting this process a feasible therapeutic approach.

T helper cells are important enforcers of cell-mediated (Th1) and humoral (Th2) adaptive immunity. Cannabinoids have been demonstrated to regulate the balance of T helper 1 (Th1) and T helper 2 (Th2) cytokines in murine studies, with a downregulation of Th1-associated cytokines such as IFN-γ, IL-2, and IL-12, and an increase in levels of Th2-associated cytokines such as IL-4, IL-10, and transforming growth factor-β (TGF-β) (Ouyang *et al.*, 1998; Klein *et al.*, 2000; Smith *et al.*, 2000; Zhu *et al.*, 2000). These effects are thought to be modulated to a considerable extent by the CB_2 receptor, as evidenced by the blockade of the majority of these effects by the CB_2 receptor antagonist, SR144528 (Zhu *et al.*, 2000; Yuan *et al.*, 2002). Th1 cytokines have been implicated in the pathogenesis of a number of conditions, including MS (Panitch *et al.*, 1987), rheumatoid arthritis (Kusaba *et al.*, 1998), and primary sclerosing cholangitis (Bo *et al.*, 2001). The suppression of Th1 responses has been effective in inhibition in animal models of inflammatory disease such as rheumatoid arthritis (Mageed *et al.*, 1998; Malfait *et al.*, 2000) and EAE (Nizri *et al.*, 2009; Lu *et al.*, 2013), suggesting cannabinoid manipulation of this response could be a helpful therapeutic agent for inflammatory disease.

Cannabinoids are well established as modulators of the immune system, affecting a variety of immune functions in humans and animals. With further research, this might be exploited in future therapies for numerous disorders, such as rheumatic disease, atherosclerosis, allergic asthma, and neurodegenerative diseases.

1.2.3 THE CANNABINOID SYSTEM AND NEUROINFLAMMATION

As is the case with immune cells in the periphery, the activity of immune cells in the brain and spinal cord can also be modified by cannabinoids. Extensive research has been carried out on microglial cells as they are the first line of defence in the CNS, and because there is evidence that microglia possess a complete endocannabinoid system and that the expression or production of some of the components of this system is altered in neuropathological states.

In the CNS, endocannabinoids are produced by both neurons and glial cells such as microglia (Salzet *et al.*, 2000; Freund *et al.*, 2003). Microglial cells in culture produce both 2-AG and anandamide, with calcium ionophores and ATP selectively and substantially increasing 2-AG production (Walter *et al.*, 2003; Carrier *et al.*, 2004). This ATP-induced increase in 2-AG production has been shown to be due to the activation of P2X purinoceptor 7 (P2X7) ionotropic receptors, which are highly permeable to calcium. The subsequent sustained increase in intracellular calcium induced by the activation of these receptors increases DAGL activity and therefore production of 2-AG while reducing 2-AG degradation by inhibiting MAGL activity (Witting *et al.*, 2004). It is suggested that microglia may be the main producers of endocannabinoids under neuroinflammatory conditions. This is supported by studies that show that microglia produce approximately 20-fold more endocannabinoids than neurons and astrocytes in culture (Walter *et al.*, 2003; Stella, 2009). In addition, 2-AG production is significantly diminished in P2X7 knockout mice in an EAE model (Witting *et al.*, 2006). As this receptor is only expressed by activated microglia, this supports the hypothesis that the synthesis of endocannabinoids is closely linked with the microglial activation state. Mecha *et al.* found that different *in vitro* microglial phenotypes were associated with an altered synthesis of endocannabinoids, with IL-4 and IL-13-stimulated microglia (a regeneration and repair subtype) selectively producing 2-AG, and TGF-β-stimulated microglia (an acquired-deactivation subtype) producing anandamide (Mecha *et al.*, 2015).

Microglia in culture also express enzymes of endocannabinoid biosynthesis and degradation such as FAAH and DAGL, which can similarly be manipulated by microglial activation states (Witting *et al.*, 2004; Muccioli *et al.*, 2007). Primary microglia stimulated with IL-4 and IL-13 were shown to induce a time-dependent rise in *DAGL-α* gene expression while TGF-β stimulation induced the accumulation of NAPE-PLD and a reduction in FAAH mRNA, consequently producing 2-AG and anandamide respectively (Mecha *et al.*, 2015). The activity of the serine hydrolase ABHD6 is also important for the regulation or inactivation of cannabinoids in the BV2 microglial cell line (Blankman *et al.*, 2007; Marrs *et al.*, 2010). The existence of this novel enzyme expressed by microglia is promising with regard to a means of enhancement of endocannabinoid signalling, and selective inhibition of cannabinoid-degrading enzymes could be used as a potential therapy in neuroinflammation. Recognizing that microglial activation states are more complex *in vivo*, it is clear more studies are necessary to understand the role of endocannabinoids in microglial function.

In addition to producing endocannabinoids, microglia also express functional cannabinoid receptors. As is the case for macrophages and other peripheral immune cells, the expression of the cannabinoid receptors is related to their activation state. It is thought that in the healthy brain, resting microglia do not express the CB_2 receptor, as the mRNA encoding for these receptors is undetectable or only detectable in trace amounts (Munro *et al.*, 1993; Schatz *et al.*, 1997; Griffin *et al.*, 1999). This is contrary to microglial cells in primary culture, which seem to be intrinsically 'primed', probably because of the methods used to transfer them into culture (Becher and Antel, 1996). Numerous laboratories have shown that these 'primed' microglia prepared from murine or human tissue express CB_2 receptors (Carlisle *et al.*, 2002; Klegeris *et al.*, 2003; Rock *et al.*, 2007). The expression levels of the receptors seem to vary depending on stimulus exposure, with LPS inducing a downregulation in both the CB_1 and CB_2 receptor on microglia, and with IL-4 and IL-13 or TGF-β inducing an upregulation in the two receptors (Mecha *et al.*, 2015). However, it is generally accepted that a massive upregulation of the CB_2 receptor on microglia occurs in response to inflammation or injury, as evidenced by animal models of a variety of diseases and species, from simian immunodeficiency virus-induced encephalitis in macaques, to stroke in mice, and paclitaxel-induced neuropathy in rats (Benito *et al.*, 2005; Maresz *et al.*, 2005; Ashton *et*

al., 2007; Naguib *et al.*, 2012; Zarruk *et al.*, 2012). *In vivo*, the phenotype of the microglia and the upregulation of CB_2 receptors have been shown to vary depending on the neuropathology or type of insult. For example, an increase in CB_2 receptor expression and microglial activation was observed in the rat spinal cord in a chronic model of neuropathic pain, but not a peripheral inflammatory pain model (Zhang *et al.*, 2003).

The CB_1 receptor is controversial with regard to the effect of its activation on microglia. This receptor has been reported in microglial cultures from mice, rats, and molluscs, but not humans. Disparate consequences have been observed after CB_1 activation in different species, with increased nitric oxide production seen in molluscs but decreased production seen in rats (Stefano *et al.*, 1996; Waksman *et al.*, 1999). Therefore, the CB_1 receptor is not a major focus of interest for researchers examining the potential of cannabinoids on microglial modulation in the context of neuroinflammatory diseases.

Multiple lines of evidence demonstrate the role of cannabinoids in the regulation of microglial cytokine production. Early reports that involved antagonism of the CB_2 receptors on microglia in culture showed increased mRNA levels of pro-inflammatory cytokines such as IL-1α, IL-6, and TNF-α, suggesting that agonism of these receptors would induce a reduction in pro-inflammatory cytokines (Puffenbarger *et al.*, 2000). Follow-up studies confirmed that either the non-selective cannabinoid receptor agonist, CP55,940 or the CB_2 selective agonist JWH015 lead to a dose-dependent reduction in LPS-induced production of TNF from rat primary cortical microglia (Facchinetti *et al.*, 2003; Romero-Sandoval *et al.*, 2009). Similar blockade of microglial TNF production following fibrillar Aβ incubation was observed after treatment with non-selective CB receptor agonists HU-210 or WIN55,212-2, and with CB_2 selective agonist JWH133 (Ramirez *et al.*, 2005). More recently, reports have highlighted the ability of cannabinoids to increase microglial anti-inflammatory cytokine signalling. Correa and co-workers demonstrated that microglia isolated from the forebrain of neonate mice and treated with either JWH133 (a CB_2 receptor selective agonist) or AEA further enhanced the LPS/IFN-γ-induced expression of IL-10 (Correa *et al.*, 2010). Similarly, 2-AG and AEA increased primary rat microglia expression of Arg1 (arginase 1) towards an *in vitro* protective phenotype, suggesting endocannabinoids can regulate microglia by amplifying the wound healing profile and restraining the pro-inflammatory effects of microglia (Mecha *et al.*, 2015). The anti-inflammatory response of Arg1 was suggested to be mediated by CB_2

receptors, as demonstrated by reduced Arg1 expression in IL-4 and IL-13-stimulated microglia from CB_2 receptor-deficient mice. Interestingly, another class of drugs known as CB_2 receptor inverse agonists, such as SMM-189, have demonstrated similar reductions in pro-inflammatory cytokines from stimulated human microglia as seen from CB_2 agonists (Reiner *et al.*, 2015). The increased phosphorylation and translocation of cAMP Response Element-Binding Protein (CREB) have been proposed as the cellular pathways that promote the CB_2 inverse agonist anti-inflammatory effects. Inhibitors of cannabinoid degradation have also been observed to modulate cytokine production. URB597, a selective FAAH inhibitor, increased the levels of anandamide and attenuated the LPS-induced increase in IL-1β expression *in vivo* (Kerr *et al.*, 2012). Furthermore, JZL184, an inhibitor of the MAGL enzyme, attenuated LPS-induced increases in IL-1β, IL-6 and TNF-α (Kerr *et al.*, 2013). This evidence suggests another potential avenue for the use of cannabinoids in the regulation of neuroinflammation.

Cannabinoid signalling has also been recognised to play a role in the functional behaviour of microglia, including their phagocytic and migratory activity. Primary microglia deficient in CB_2 receptor expression engulfed significantly fewer fluorescent microspheres than wild-type microglia following IL-4 and IL-13 stimulation, suggesting an impact of CB_2 receptors on the phagocytic function of microglia (Mecha *et al.*, 2015). In addition to affecting the chemotaxis of peripheral immune cells, cannabinoid receptors have also been reported to modulate microglial migration. 2-AG was reported to be a very efficacious ligand regarding this response in the BV2 mouse microglial cell line, an effect that was prevented by cannabinol and cannabidiol, by blocking CB_2 and cannabidiol-sensitive receptors respectively (Walter *et al.*, 2003). Another study corroborated that 2-AG-induced BV2 migration relies on CB_2 receptors, as demonstrated by the blockade of microglia recruitment after treatment with the CB_2 inverse agonist SR144528 (Offertáler *et al.*, 2003). Anandamide increased BV2 cell migration in a concentration-dependent manner, as did the two putative endocannabinoids homo-γ-linolenylethanolamide (HEA) and docosatetraenylethanolamide (DEA), whereas another putative endocannabinoid, PEA, which does not act on CB_1 or CB_2 receptors, had no effect (Walter *et al.*, 2003). However, immortalized BV2 cells have been shown to produce different migration and cytokine responses than primary microglia, including higher migratory rates in immortalized microglia cell lines compared to primary cells (Horvath *et al.*, 2008). To

that point, rat primary microglia treated with a CB_2 receptor agonist, JWH015, decreased LPS-induced chemotaxis, which is incongruent with cell line data given the same reduced migratory response was found in BV2 cells when CB_2 receptors were blocked instead of activated (Romero-Sandoval *et al.*, 2009).

Due to all the modulatory effects cannabinoids have on microglia, in addition to the massive upregulation of the CB_2 receptor on microglia in neuroinflammatory states, there is an acute interest in harnessing these immune-modulatory effects for therapy in neurodegenerative diseases. Yet, translatability to *in vivo* remains questionable due to the varying responses and behaviours of the different microglia preparations (cell lines vs. primary), and thus more research is needed to understand cannabinoid specific immune-related functions and how they act on neuroinflammation in model systems.

1.3 THE ENDOCANNABINOID SYSTEM IN PARKINSON'S DISEASE

As previously stated, in recent years it has become apparent that in neurodegenerative disease there is a self-sustaining cycle of neuroinflammation and neuronal death, with dying neurons activating microglia, which then can release cytotoxic factors that cause further neuronal death (Frank-Cannon *et al.*, 2009; Tansey and Goldberg, 2010; Sanchez-Guajardo *et al.*, 2013). The concept of pharmacologically targeting the cannabinoid system in Parkinson's disease is predicated on the upregulation of cannabinoid receptors in Parkinson's disease patients identified in both the substantia nigra and the hippocampus, providing a neuroanatomical basis (Gómez-Gálvez *et al.*, 2016; Navarrete *et al.*, 2018). The *in vitro* evidence supporting the anti-inflammatory regulation of cannabinoid receptors on microglia suggests that cannabinoids may have the potential to modulate microglial dysfunction in Parkinson's disease, and thus slow or even prevent the dopaminergic degeneration.

Although this thesis is focused on the role of cannabinoids in neuroinflammation, when discussing the cannabinoid system in the context of Parkinson's disease it is important to note that the CB_1 receptor is highly expressed in the basal ganglia (Herkenham *et al.*, 1991; Mailleux and Vanderhaeghen, 1992), indicating this receptor may play a part in motor control. Although the density is highest in the substantia nigra, it is not the dopaminergic nigrostriatal neurons that express the receptor. Instead, it is the medium

spiny GABAergic neurons that project to the substantia nigra from the striatum that are responsible for this high expression (Hohmann and Herkenham, 2000). These neurons co-express the dopaminergic D_1 and D_2 receptors with CB_1 receptors in the striatum (Hermann *et al.*, 2002). CB_1 knockout mice have more severe motor deterioration and neurodegeneration, as well as a reduced incidence of L-DOPA-induced dyskinesias (Perez-Rial *et al.*, 2011), highlighting the importance that this receptor has in motor control and its link to the dopaminergic system. Researchers are investigating the potential use of cannabinoids for therapy in Parkinson's disease from a number of aspects: for alleviation of motor symptoms, alleviation of drug-induced side effects, and disease-modifying effects such as effects on α-synucleinopathy and through direct neuroprotection. Indeed, a number of clinical trials have been carried out examining the therapeutic potential of cannabinoids with regard to these aspects of Parkinson's disease (reviewed in Concannon *et al.*, 2015a). However, these facets are outside the scope of this thesis.

1.3.1 THE CB_2 RECEPTOR AS AN ANTI-INFLAMMATORY TARGET IN NEURODEGENERATIVE DISEASE

1.3.1.1 Clinical evidence

In 2003, one of the first pieces of evidence that alterations in the endocannabinoid system relevant to neuroinflammation occur in the neurodegenerative disease state came from a study by Benito and colleagues (Benito *et al.*, 2003). They found that in the hippocampus and entorhinal cortex of Alzheimer's disease (AD) patients, there was a substantial and specific overexpression of the CB_2 receptor on microglia in the neuritic plaques, while CB_1 receptor expression was not altered. This upregulation of the CB_2 receptor on microglia surrounding senile plaques has been confirmed with additional studies (Solas *et al.*, 2013). One study found a correlation between expression levels of the CB_2 receptor and senile plaque score and Aβ42 levels, which are two major pathological molecular markers of AD (Solas *et al.*, 2013). In addition to an upregulation in AD, a substantial increase in CB_2 receptor expression has been found in human CNS tissue in a number of disorders associated with neuroinflammation, including MS, Down syndrome, and amyotrophic lateral sclerosis (ALS) (Yiangou *et al.*, 2006; Benito *et al.*, 2007; Nunez *et al.*, 2008). This elevation of CB_2 expression on microglia has also been confirmed to be

present in *post mortem* Parkinson's disease brains (Gómez-Gálvez et al., 2016). It has been suggested that the upregulation and activation of the CB_2 receptor in neurodegenerative disease may be part of a type of negative feedback loop in response to physiological stress, with the aim of limiting the inflammatory process (reviewed in Bie *et al.*, 2018).

Alterations in the expression levels of enzymes in the endocannabinoid system have also been observed in clinical neurodegenerative disease. In AD, increased expression of FAAH, the primary enzyme responsible for the metabolism of anandamide, was noted in astrocytes associated with senile plaques (Benito *et al.*, 2003). Anandamide is converted to arachidonic acid by FAAH, and the abundance of this enzyme in astrocytes in this disease suggests that astrocytes could be a major source of arachidonic acid and related pro-inflammatory compounds in the vicinity of these plaques. An increase in FAAH gene expression was also observed in peripheral blood mononuclear cells of late-onset AD patients (D'Addario *et al.*, 2012), indicating a potential new biomarker of this disease. In another study, increased DAGL was noted in the brains of AD-affected patients (Farooqui *et al.*, 1988).

1.3.1.2 The CB_2 receptor in models of neurodegeneration

Studies using *in vitro* and *in vivo* models have illustrated the potential of the CB_2 receptor as a therapeutic target in neurodegenerative diseases such as Parkinson's disease. In concurrence with clinical evidence, upregulation of the CB_2 receptor has been identified in models of numerous neurodegenerative diseases, including AD (Esposito *et al.*, 2007; Wu *et al.*, 2013), Huntington's disease (HD) (Sagredo *et al.*, 2009), MS (Maresz *et al.*, 2005), and indeed Parkinson's disease (García *et al.*, 2011; Concannon *et al.*, 2015b; Concannon *et al.*, 2016). Alterations in CB_2 receptor expression have been observed in animal models of Parkinson's disease induced by a wide variety of stimuli including 6-OHDA, rotenone, LPS, and poly I:C, with all these neurotoxins causing a marked upregulation of CB_2 receptor expression in the rat striatum (Concannon *et al.*, 2015b; Concannon *et al.*, 2016). Interestingly, a more pronounced upregulation was observed in response to the bacterial and viral inflammagens, LPS and poly I:C respectively, compared to the direct neurotoxins, highlighting the link between the CB_2 receptor and the inflammatory aspect of the pathology of Parkinson's disease. In several of these models, CB_2 receptor expression correlated strongly with expression of the microglial

marker CD11b. Similar co-expression of CD11b (Mac1) and CB_2 immunostaining was observed in the ventral midbrain of mice three days after treatment with MPTP, a dopamine-selective neurotoxin (Price *et al.*, 2009). These results are consistent with the upregulation of CB_2 expression on microglia which has been confirmed to be present in *post mortem* Parkinson's disease brains (Gómez-Gálvez *et al.*, 2016).

Studies in neurodegenerative disease models in which the expression of the CB_2 receptor has been ablated or altered lend further evidence to the key role this receptor plays in these conditions. In a transgenic mouse model of Alzheimer's disease, soluble Aβ42 and plaque deposition was reported to be increased in CB_2-deficient mice (Koppel *et al.*, 2013). Genetic deletion of CB_2 receptors in the slowly progressing BACHD Huntington's disease model resulted in an accelerated disease onset and an exacerbated severity (Bouchard *et al.*, 2012). In the R6/2 HD mouse model, CB_2 receptor ablation resulted in an exacerbated severity, and also an increase in microglial activation and a reduction in the lifespan of the mice (Palazuelos *et al.*, 2009). With regard to Parkinson's disease, an exacerbation of MPTP-induced toxicity has been described in CB_2 knockout mice (Price *et al.*, 2009). An aggravation of inflammation and neuronal death due to genetic inactivation of the CB_2 receptors has also been reported in the LPS model, but not in the 6-OHDA model (García *et al.*, 2011; Gómez-Gálvez *et al.*, 2016). In contrast, mice overexpressing CB_2 receptors present significantly less motor impairment compared to wild-type mice following intra-caudate 6-OHDA administration, as well as reduced microgliosis and astrocytosis (Ternianov *et al.*, 2012).

1.3.1.3 Pharmacological targeting of the CB_2 receptor in models of neurodegeneration

Activation of the CB_2 receptor has been observed to have anti-inflammatory effects in various animal models of acute and chronic neuronal diseases in which inflammation is involved, including stroke, traumatic brain injury, MS, and AD. In the case of stroke and traumatic brain injury, many different categories of cannabinoids were demonstrated to have neuroprotective effects (Belayev *et al.*, 1995; Shohami *et al.*, 1997; Zhang *et al.*, 2009; Elliott *et al.*, 2011; Suzuki *et al.*, 2012; Reiner *et al.*, 2015). However, some of the studies researching the potential of cannabinoid compounds for acute brain injury have been conducted with the administration of the cannabinoid before injury, which is a scenario that is not possible in a clinical context, and therefore the results of these studies

and their translatability to potential therapies should be approached with caution. The number of clinical trials with cannabinoids in these diseases is limited, with one of the most relevant being a multicentre placebo-controlled phase III trial investigating dexanabinol in traumatic brain injury (Maas *et al.*, 2006). Dexanabinol is a synthetic cannabinoid derivative that does not have cannabinoid activity but instead acts as an NMDA antagonist. Despite its promising pre-clinical effects (Belayev *et al.*, 1995; Shohami *et al.*, 1995; Shohami *et al.*, 1997), the trial found it was safe but not efficacious in traumatic brain injury.

Due to the slow progression of chronic neurodegenerative diseases, there is a much greater opportunity for anti-inflammatory therapeutic intervention with cannabinoids compared to acute brain injury conditions. With regard to AD, cannabinoids, including the CB_2 selective agonist JWH133, were demonstrated to block Aβ peptide-induced activation of microglia *in vitro*, including a reduction in the release of the pro-inflammatory cytokine TNF-α (which is associated with a cytotoxic microglial phenotype) and a reduction in mitochondrial activity (Ramirez *et al.*, 2005). In co-cultures with neurons, the cannabinoids also prevented microglial-mediated neurotoxicity after Aβ exposure. In a transgenic Tg2576 mouse model of AD, which overexpresses a mutant form of amyloid precursor protein (APP), WIN55,212-2 and JWH133 both reduced the APP-induced increase in TNF-α and Aβ levels. In addition, JWH133 reduced cognitive defects as measured by the novel object recognition test, and decreased microglial cell density (Martín-Moreno *et al.*, 2012). Cannabinoid-induced beneficial effects were also seen in a rat AD model treated with WIN55,212-2, where improvements in memory functions and reductions in the elevation of pro-inflammatory markers such as TNF-α and nuclear factor κB (NFκB) were observed (Fakhfouri *et al.*, 2012). In the Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) model of MS, treatment with WIN 55,212-2, ACEA (a CB_1 agonist), or JWH015 (a CB_2 agonist) caused a marked reduction in microglial activation visible in the cell morphology, as well as functional recovery in the rotarod test (Arevalo-Martin *et al.*, 2003). In the EAE model, administration of 2-AG ameliorated acute and chronic phases of the disease, which was accompanied by the polarization of macrophages towards a non-cytotoxic, protective phenotype (Lourbopoulos *et al.*, 2011). In an HD model, cannabinoids were shown to protect striatal projections from malonate-induced death (Sagredo *et al.*, 2009). These

beneficial anti-inflammatory and neuroprotective effects of CB_2 activation have been attributed to the presence of the receptor on microglia, and are associated with suppression of microglial activation (Ehrhart *et al.*, 2005; Ashton and Glass, 2007), and hence inhibition of the release of cytotoxic factors that cause neuronal damage.

1.3.1.4 Pharmacological targeting of the CB_2 receptor in Parkinson's disease models

The neuroprotective and anti-inflammatory effects of cannabinoids have been demonstrated across numerous animal models of Parkinson's disease (Table 1.2). One of the first indications that cannabinoids may have potential neuroprotective effects in Parkinson's disease came in 2005 when Lastres-Becker and co-workers exposed the cannabinoid agonist, HU-210, to a cerebellar granule cell culture which was treated with 6-OHDA (Lastres-Becker *et al.*, 2005). HU-210 increased cell survival when the cells were directly exposed, even more so when the neuronal cultures were exposed to conditioned media from mixed glial cell cultures that had been treated with HU-210. This suggests that the drug exerted its neuroprotective effect largely by regulating the glial influence on neurons. Of note, the mixed glial cultures were comprised of 70% astrocytes and 30% microglia, highlighting the desirable effects of cannabinoids on glia in general. Further studies by another group using the non-selective agonists WIN55,212-2 and HU-210 in the MPTP mouse and the LPS rat demonstrated profound anti-inflammatory effects, with reduced $CD11b^+$ microglial activation and reduced expression of pro-inflammatory cytokines, which was also associated with neuroprotective effects (Chung *et al.*, 2011; Chung *et al.*, 2012). The inhibition of microglial activation and the observed neuroprotection was reserved upon treatment with CB_1 selective antagonists, suggesting that the CB_1 receptor is involved. Although several studies do suggest the anti-inflammatory potential of the CB_1 receptor, the majority of research is now focused on the CB_2 receptor in Parkinson's disease. This is due both to the profound upregulation of the CB_2 receptor on activated microglia, and the results from numerous *in vitro* studies that demonstrate that activation of the microglial CB_2 receptor changes the activation state of the microglia, reducing their release of pro-inflammatory cytokines and increasing the release of anti-inflammatory cytokines (reviewed in Benito *et al.*, 2008).

The first study to provide solid evidence that the anti-inflammatory effects of cannabinoid drugs were mediated by the CB_2 receptor was carried out in an MPTP mouse model. The

non-selective cannabinoid agonist WIN55,212-2 reduced MPTP-induced microglial CD11b marker upregulation in the ventral midbrain, and this effect was blocked by the CB_2 antagonist JTE-907 (Price *et al.*, 2009). In the decade since the publication of this paper, several studies investigating CB_2 receptor agonism in animal models of Parkinson's disease have reported an anti-inflammatory effect. García and colleagues found that chronic administration of Δ9-THCV (tetrahydrocannabivarin) attenuated the loss of dopaminergic neurons in both the 6-OHDA and LPS models. This effect was also elicited in the LPS model by the CB_2 selective agonist HU-308, suggesting that the effect was CB_2 receptor-mediated (García *et al.*, 2011). However, HU-308 did not induce a significant neuroprotective effect in 6-OHDA lesioned rats (García-Arencibia *et al.*, 2007). This could be due to the lower inflammatory response induced by 6-OHDA, which is a direct neurotoxin, compared to LPS, an endotoxin expressed in the outer membrane of gram negative bacteria that evokes an immune response. Treatment with the naturally occurring CB_2 agonist β-caryophyllene has also been demonstrated to reduce rotenone-induced dopaminergic degeneration and microglial activation (Javed *et al.*, 2016).

Together these results demonstrate that CB_2 receptors play an important role in neurodegenerative diseases such as Parkinson's disease. However, much remains to be elucidated regarding the link between this receptor and the pathology of the disease, and regarding the full ramifications of targeting this receptor *in vivo*. This research is impeded partly by the lack of appropriate animal models of the disease. The following section will outline the animal models of the disease which are currently available, their advantages and their limitations.

Species	Inflammagen	Cannabinoid Treatment	Treatment Timeline	Inflammation Effects	Neuroprotective Effects	Reference
C57BL/6 mice	MPTP (4 × 20 mg/kg every 2 hrs)	HU-210 or WIN55,212-2	2 d before MPTP and again 12 hrs after MPTP and continue for 3 d (microglia analysis) and 7 d (neuron analysis)	↓ MPTP-induced nigral Mac1 (CD11b) activation and production of O_2^- (ethidium accumulation) ↓ MPTP-induced 8-OHdG suggesting reduced protein oxidative damage ↓ MPTP-induced nigral *TNF*, *IL-1β*, and *iNOS* gene expression and TNF and IL-1β protein expression	↑ MPTP-induced TH^+ stereological nigral cell count ↑ MPTP-induced rotarod latency to fall and striatal dopamine content	(Chung *et al.*, 2011)
Neuron-enriched VM cultures	MPP^+ for 48 hours	+AM251 (microglia/ neuron) or SR141716A (neuron)	30 min prior to non-selective agonists	↑ Agonist-induced nigral Mac1 (CD11b) activation and production of O_2^- (ethidium accumulation) ↑ Agonist-induced 8-OHdG suggesting oxidative damage ↑ Agonist-induced nigral *TNF*, *IL-1β*, and *iNOS* gene expression and TNF and IL-1β protein expression	↓ Agonist-induced TH^+ stereological nigral cell count (no difference with MPTP alone group) when treated with CB_1 antagonists ↓ Agonist-induced rotarod latency to fall and striatal dopamine content (no difference with MPTP alone group) when treated with AM251	
$CB_2^{-/-}$ (C57BL/6 background)	Intra-striatal LPS	--	--	↑ CD68-immunoreactivity in the nigra of KO mice compared to WT	Not evaluated	(Gómez-Gálvez *et al.*, 2016)
C57BL/6 mice	Intra-striatal LPS	HU-308	Daily injections for 2 weeks starting 16 hr after LPS	↓ LPS-induced nigral CD68-immunoreactivity ↓ LPS-induced striatal *iNOS* gene expression	↑ LPS-induced nigral TH-immunoreactivity	
C57BL/6 P1 glia and cerebellar neural cultures	6-OHDA	HU-210 directly to cultures	Neurons +/− conditioned media from glia culture 24 hrs after HU-210	Not directly evaluated but neuroprotective effects from glia conditioned media suggest CB_1 and CB_2-mediated glial effects	↑ cerebellar granule cell survival with direct HU-210 to neurons and greater protection when neurons treated with glia conditioned media treated with HU-210	(Lastres-Becker *et al.*, 2005)

Sprague Dawley rats	Intra-nigral LPS	HU-210 or WIN55,212-2	ICV injections 1 hr prior to LPS	↓ LPS-induced nigral CD11b activation and production of O_2^- (ethidium accumulation) ↓ TNF and IL-1β after WIN55,212-2 and ↓ IL-1β after HU-210 24 hrs after LPS as measured by ELISA ↓ p67phox and p47phox subunits in cytosol and membrane nigral fractions 12 hrs after LPS by western, suggesting reduced translocation of NADPH oxidase which was specific to $CD11b^+$ cells	↑ TH^+ stereological nigral cell count	(Chung *et al.*, 2012)
C57BL/6 mice	MPTP (4 × 20 mg/kg every 2 hrs)	JWH015 (microglia) or WIN55,212-2 (microglia/ neuron)	Daily for 3d (microglia analysis) or 5 d (neuron analysis) starting 1 d after MPTP	↓ MPTP-induced nigral Mac1 (CD11b) protein with WIN55,212-2	↑ MPTP-induced nigral TH^+ stereological neuron counts (dose-dependent) ↑ MPTP-induced midbrain dopamine levels after WIN55,212-2 treatment	(Price *et al.*, 2009)
		WIN55,212-2 +JTE907	20 min before WIN55,212-2	↑ Agonist-induced Mac1 (CD11b) when administered alone or in conjunction with WIN55,212-2 agonist	Not evaluated	
Sprague Dawley rats	ICV 6-OHDA	Δ9-THCV or enriched CBD	Daily for 14 d starting 16 hrs after 6-OHDA	↓ 6-OHDA-induced nigral OX-42-immunoreactivity with either Δ9-THCV or enriched CBD	↑ 6-OHDA-induced nigral TH-immunoreactivity when treated with CBD but not Δ9-THCV	(García *et al.*, 2011)
C57BL/6 mice	Intra-striatal LPS	Δ9-THCV or HU-308	Daily for 14 d starting 16 hrs after LPS	Not evaluated	↑ LPS-induced nigral TH-immunoreactivity when treated with HU-308 or Δ9-THCV	
Sprague Dawley rats	Unilateral MFB injection of 6-OHDA	CBD	Daily for 2 weeks, starting 16 hr after 6-OHDA	↑ 6-OHDA induced striatal *Cu,Zn-SOD* gene expression suggesting protection from endogenous oxidative stress	Not evaluated	(García-Arencibia *et al.*, 2007)
		HU-308	Daily for 2 weeks, starting 16 hr after 6-OHDA	Not evaluated	Did not alter striatal TH activity by HPLC or nigral TH mRNA levels compared to 6-OHDA + vehicle	

Wistar rats	Rotenone i.p. once daily for 4 weeks	β-caryophyllene (BCP) ± AM630	Daily for 4 weeks and 30 min prior to rotenone	↓ Rotenone-induced striatal Iba1^{+} activated microglia with BCP and blocked by AM630 ↓ Rotenone-induced striatal GFAP activated astrocytes with BCP and blocked by AM630 ↓ Rotenone-induced midbrain pro-inflammatory cytokines IL-1β, TNF, and IL-6 with BCP and blocked by AM630 ↓ Rotenone-induced striatal NFκB p65, COX2, iNOS with BCP and blocked by AM630	↑ Rotenone-induced striatal and nigral TH-immunoreactivity with BCP and blocked by AM630	(Javed *et al.*, 2016)
CB2xP mice (over-expression of mouse CB_2)	Unilateral striatal 6-OHDA	--	7 weeks	↓ 6-OHDA-induced striatal GFAP expression vs. WT. Overexpression of CB_2 altered striatal Iba1 immunoreactivity, but not striatal levels of iNOS and COX2 vs. matched WT ↓ Striatal malonyldialdehyde (lipid peroxidation product) vs. WT at basal and 6-OHDA conditions ↓ 6-OHDA induced striatal ratio of oxidized GSSG:glutathione (oxidative stress marker) vs. WT	↓ 6-OHDA-induced apomorphine rotations vs. WT ↑ 6-OHDA-induced time in open arms of elevated plus maze suggesting CB_2 role in anxiety-like behaviour vs. WT ↑ 6-OHDA-induced memory impairment in step-down inhibitory avoidance task vs. WT ↑ 6-OHDA-induced striatal and nigral TH immunostaining vs. WT	(Ternianov *et al.*, 2012)

Table 1.2 Cannabinoid-mediated effects on inflammation and neuroprotection in models of Parkinson's disease. ↑, increase. ↓, decrease. 8-OHdG, 8-hydroxy-2-deoxyguanosine. TH, tyrosine hydroxylase. VM, ventral mesencephalic. LPS, lipopolysaccharide. KO, knockout. ICV, intracerebroventricular. MFB, medial forebrain bundle. i.p., intraperitoneal. COX2, cyclooxygenase 2. WT, wild-type. $CB_{1/2}$ non-selective agonist: WIN55,212-2, HU-210. CB_1 antagonist: AM251, SR141716A. CB_2 selective agonist: HU-308, JWH015. CB_2 selective antagonist: JTE907, AM630.

1.4 PRE-CLINICAL MODELS OF PARKINSON'S DISEASE

As previously stated, Parkinson's disease is the second most common neurodegenerative disorder, and currently available treatments merely address the symptoms and not the underlying pathophysiology. Accurate animal models of the disease are fundamental for the testing of potential therapeutic interventions that could slow down the progression of the disease or even cure it. However, the success at which pre-clinical research translates into clinical relevance is highly dependent on the extent to which the experimental model emulates the pathology, progression and symptoms of the disease in patients. No Parkinson's disease model that is currently available displays all of these features. Despite these limitations, animal models have contributed significantly to our understanding of the pathology of Parkinson's disease and the potential of proposed therapeutic interventions. In this section, the mechanisms of action, benefits and drawbacks of some of the most commonly used models are outlined.

1.4.1 NEUROTOXIC MODELS

1.4.1.1 6-OHDA

The neurotoxin 6-hydroxydopamine (6-OHDA) is one of the most widely used toxins to produce animal models of Parkinson's disease. This is a highly oxidisable dopamine analogue that is capable of killing catecholaminergic neurons as it has a high affinity for the dopamine transporter and the noradrenergic transporter, and hence is selectively taken up into these cells and causes damage (Jonsson, 1980; Luthman *et al.*, 1989). While the exact mechanisms by which 6-OHDA exerts its cytotoxic effects remains to be fully elucidated, several bodies of evidence have indicated oxidative stress plays a major role. Oxidation of 6-OHDA results in the production of hydrogen peroxide and triggers the generation of reactive oxygen species, ultimately leading to oxidative-stress mediated cytotoxicity (Saner and Thoenen, 1971; Graham, 1978; Blum *et al.*, 2001). Trace elements such as manganese have been seen to work as catalysts and to accelerate these toxic reactions (Garner and Nachtman, 1989).

6-OHDA does not cross the blood-brain barrier (BBB), and therefore requires stereotaxic administration directly into the brain. Unilateral injections are more frequently used in the case of 6-OHDA, as bilateral infusion results in such a severe neuronal degeneration

that the animals need intensive care for survival (Sakai and Gash, 1994; Ferro *et al.*, 2005). Usually, administration at one of three sites along the nigrostriatal pathway is used to induce this model: at the substantia nigra, the medial forebrain bundle, or the striatum. The site of injection affects the extent and characteristics of the neuronal degeneration (Agid *et al.*, 1973; Przedbroski *et al.*, 1995; Tieu, 2011). Lamentedly, this lesion model fails to replicate Lewy bodies, the pathological intracellular protein inclusions that are present in the human condition (Lane and Dunnett, 2008).

In addition to the marked neurodegeneration, 6-OHDA also induces severe deficits in both spontaneous and drug-induced motor function. The most commonly used motor test to assess the extent of a 6-OHDA lesion is the amphetamine-induced rotation test. Amphetamine is a dopamine-releasing compound, which causes an imbalance in dopamine between the lesioned and intact sides of the brain. Administration of this drug to a unilaterally lesioned animal will thus result in an abnormal circling behaviour which can be quantified and is correlated with the extent of neuronal loss (Ungerstedt and Arbuthnott, 1970; Olds *et al.*, 2006).

1.4.1.2 MPTP

MPTP is another neurotoxin that is very commonly used to model Parkinson's disease. As previously mentioned (section 1.1.3.3), in the early 1980s in California several drug users developed Parkinsonian symptoms after injecting themselves intravenously with an illicit drug that had been contaminated with MPTP (Langston *et al.*, 1983). The publishing of the case reports of these patients led to an instantaneous excitement in the scientific community about the potential of this molecule for modelling the disease in animals (Langston, 2017), and presently MPTP animal models are among some of the most established models.

MPTP is highly lipophilic, and upon systemic injection will rapidly cross the blood-brain barrier (Riachi *et al.*, 1989). In astrocytes, the enzyme MAO-B converts MPTP to MPP^+, an active toxic cation, which is then released into the extracellular space before being taken up into dopamine neurons via the dopamine transporter (Ransom *et al.*, 1987; Cui *et al.*, 2009). MPP^+ induces death of these neurons by inhibiting complex I of the electron transport chain, resulting in a reduction in ATP and increased oxidative stress (Nicklas *et al.*, 1985; Mizuno *et al.*, 1987; Banerjee *et al.*, 2009).

To date, various mammalian species have been administered MPTP to model Parkinson's disease, including rats, guinea pigs and sheep, although non-human primates and mice are the most commonly used (Chiueh *et al.*, 1984; Baskin *et al.*, 1994; Meredith and Rademacher, 2011; Porras *et al.*, 2012). In monkeys, MPTP consistently induces neurodegeneration of the nigrostriatal pathway, which results in many motor impairments that are characteristic of the disease, such as bradykinesia and rigidity (Porras *et al.*, 2012). Notably, these motor deficits have been demonstrated to be reversible upon treatment with drugs that are used clinically to treat Parkinson's disease such as levodopa and dopamine agonists. Furthermore, intraneuronal inclusions resembling the early stages of Lewy body formation have been reported (Forno *et al.*, 1993; Kowall *et al.*, 2000). For these reasons, the MPTP monkey model is the paragon for the pre-clinical testing of therapeutic strategies for the disease. However, although their motor impairments are less distinct relevant to primates, mouse models are very commonly used by many experimenters, due to their reduced cost and requirement for expertise. Rats are used less frequently, as they are not as sensitive to the neurotoxic effects of MPTP as mice, which has been proposed to be due to differential sequestration of MPP^+ (Giovanni *et al.*, 1994).

In summary, the MPTP model, although it does not explicitly imitate all the features of Parkinson's disease, is an important tool in the study of the disease and will remain so in the years to come until an improved model is devised.

1.4.2 PESTICIDE MODELS

1.4.2.1 Rotenone

Rotenone is a compound that is found naturally occurring in the roots of several plants including *Lonchocarpus* or *Derris* plants and was broadly used for many years as an insecticide and to kill nuisance fish in lakes (Soloway, 1976). Due to its high lipophilicity, it easily crosses the blood-brain barrier and therefore can be administered either systemically or locally (Talpade *et al.*, 2008). The mechanism of toxicity of rotenone is similar to that of MPTP: it accumulates in mitochondria and interferes with complex I of the electron transport chain (Sherer *et al.*, 2003b), inducing oxidative stress.

Rotenone models of Parkinson's disease were first used as early as 1985 (Heikkila *et al.*, 1985), but it was not until 15 years later when Greenamyre and colleagues developed a chronic low-dose regimen that the rotenone model garnered attention (Betarbet *et al.*,

2000). By their method of subcutaneous infusion using an osmotic mini-pump, rotenone produced specific nigrostriatal degeneration and the formation of α-synuclein-positive intracellular inclusions. This generated substantial interest, as rotenone is capable of entering all cells of the brain, indicating that the nigrostriatal dopamine neurons have an inherent vulnerability or susceptibility to this toxicity. Microglial activation has also been seen after rotenone administration, which is consistent with the neuroinflammation observed in Parkinson's disease patients (Sherer *et al.*, 2003a; Tansey and Goldberg, 2010; Concannon *et al.*, 2016). In addition to the neuropathological features, rotenone-intoxicated animals have also exhibited Parkinsonian-like motor impairments such as hypokinesia and alterations in posture (Betarbet *et al.*, 2000; Nehru *et al.*, 2008).

The main drawback of the systemic rotenone model is the lack of reproducibility with regard to the location and magnitude of the lesion, as well as of the other neuropathological features (Fleming *et al.*, 2004; Lapointe *et al.*, 2004; Zhu *et al.*, 2004). The high mortality of this model due to the systemic effects of rotenone is a contributing factor and therefore some experimenters have used a direct intracerebral injection to combat this (Ravenstijn *et al.*, 2008; Xiong *et al.*, 2009; Mulcahy *et al.*, 2011). Although the rotenone model is not as well established as some other models of Parkinson's disease, its replication of the nigrostriatal neurodegeneration and of the cytoplasmic inclusions reminiscent of Lewy bodies mean it can provide valuable insights in pre-clinical investigations of Parkinson's disease.

1.4.2.2 Paraquat

Paraquat (1,1'-dimethyl-4,4'-bipyridinium) is a widely used herbicide that bears remarkable structural similarity to MPP^+, the active metabolite of MPTP, and therefore it was correctly proposed to be a potential neurotoxin and a contributing factor to the development of Parkinson's disease. Despite being a divalent cation, paraquat appears to be capable of crossing the brain-blood barrier, and can thus be administered systemically as well as intracerebrally. There is evidence to suggest that this ability to penetrate the BBB is mediated by the neutral amino acid transporter (Shimizu *et al.*, 2001; McCormack and Di Monte, 2003). The primary mode of toxicity of paraquat is mediated by redox cycling with cellular diaphorase, resulting in the production of superoxide (Day *et al.*, 1999; Bonneh-Barkay *et al.*, 2005). Despite its striking structural similarity to MPP^+,

paraquat does not appear to directly interfere with complex I of the electron transport chain as MPP^+ does (Richardson *et al.*, 2005).

In mice, administration of paraquat has been observed to induce nigral degeneration and motor dysfunction in a dose-dependent manner (Brooks *et al.*, 1999; McCormack *et al.*, 2002). However, this effect on degeneration of the dopamine neurons has not been consistently reproduced, and even in studies where neuronal loss does occur, paraquat does not affect striatal dopamine levels (Thiruchelvam *et al.*, 2000b; McCormack *et al.*, 2002; Cicchetti *et al.*, 2005). Following treatment with paraquat, there have also been reports of aggregates of α-synuclein redolent of Lewy bodies (Manning-Bog *et al.*, 2002; Naudet *et al.*, 2017). Additionally, degeneration of noradrenergic neurons in the locus coeruleus has been noted, a region that has been implicated in cognitive impairment in Parkinson's disease (Fernagut *et al.*, 2007; Hou *et al.*, 2017; Peterson and Li, 2018). Paraquat is sometimes administered with the fungicide maneb, which results in a more significant nigral degeneration and a trend in striatal dopamine loss (Thiruchelvam *et al.*, 2000a; Thiruchelvam *et al.*, 2000b; Tinakoua *et al.*, 2015). Notwithstanding, even with this revised paraquat/maneb protocol, it remains unclear how the impairments in motor function can be ascribed to the very modest reduction in striatal dopamine.

In addition, exposure to paraquat has been linked to an increased risk of Parkinson's disease in numerous epidemiological studies (Hertzman *et al.*, 1990; Kamel *et al.*, 2006; Costello *et al.*, 2009), making it a highly relevant molecule with which to model the disease. However, the inconsistencies in nigrostriatal degeneration and the meagre reductions in striatal dopamine limit the relevance of this model to assess potential neuroprotective therapeutic strategies.

1.4.3 INFLAMMATORY MODELS

1.4.3.1 LPS

As previously detailed, inflammatory processes have been implicated as one of the key active contributors to the development and progression of Parkinson's disease, as chronic activation of microglia can perpetuate a self-sustaining cycle of neuroinflammation and neurotoxicity (Frank-Cannon *et al.*, 2009; Tansey and Goldberg, 2010). Lipopolysaccharide (LPS) is an endotoxin found in the outer membrane of gram negative bacteria and can stimulate the toll-like receptor 4 (TLR4) (Lu *et al.*, 2008). Activation of

this receptor by LPS results in microglial activation, the production of reactive oxygen species and an altered cytokine profile. As formerly stated, the density of microglia surrounding the nigrostriatal dopamine neurons is high relative to other areas of the brain, rendering these neurons extremely vulnerable to inflammation-driven neurotoxicity such as that induced by LPS (Lawson *et al.*, 1990; Kim *et al.*, 2000).

The first *in vivo* study involving the direct administration of LPS to model Parkinson's disease was published in 1998 (Castaño *et al.*, 1998). These experimenters reported that after stereotaxic injection of LPS into the substantia nigra, there was a significant microglial response and a degeneration of the nigrostriatal neurons. This degeneration was specific to the dopamine neurons, with serotoninergic neurons and GABAergic neurons remaining unaffected (Castaño *et al.*, 1998; Herrera *et al.*, 2000). Since then, other researchers have also used the intra-nigral LPS model and noted a rapid microglial activation, followed by nigrostriatal degeneration and a reduction in striatal dopamine (Lu *et al.*, 2000; Arimoto *et al.*, 2007; Hernández-Romero *et al.*, 2008). Impairments in motor function have also been observed in LPS-treated animals (Choi *et al.*, 2009; Hoban *et al.*, 2013); however, there have been no reports of any intracellular inclusions resembling Lewy bodies, or of extranigral pathology. Other routes and sites of administration have been used for LPS, including stereotactically into the striatum or medial forebrain bundle, or systemically by intraperitoneal injection, with varying effects on the rate of nigrostriatal degeneration (Qin *et al.*, 2007; Machado *et al.*, 2011).

Given the wealth of evidence that neuroinflammation is a contributing factor in the progression of Parkinson's disease, the LPS inflammatory model is a valuable tool in many respects. However, it is limited by its relatively rapid disease course and the lack of α-synuclein pathology.

1.4.3.2 Poly I:C

As previously detailed, there is mounting evidence that viral infections play a role in the aetiology of Parkinson's disease. Animal models are required in order to elucidate the link between viruses, viral-induced inflammation and the aetiology of Parkinson's disease. Clinical evidence has observed the link between viruses and Parkinson's disease, and pre-clinical studies have also supported this. Jang and colleagues found that administration of the highly pathogenic H5N1 influenza virus to mice resulted in

microglial activation, α-synuclein aggregation and nigrostriatal degeneration (Jang *et al.*, 2009). However, this is not a feasible model by which to study the effect of viruses on the development of Parkinson's disease as approximately half of the animals developed neurological symptoms so severe they had to be euthanized.

Polyinosinic:polycytidylic acid (poly I:C) is a synthetic analogue of a double-stranded RNA virus that binds to and activates the toll-like receptor 3 (TLR3). However, the effects of this viral mimetic on inducing inflammation and neurodegeneration are as of yet poorly characterised. Several researchers have investigated the ability of poly I:C to induce neuroinflammation and prime the neurons to subsequent exposure to a second toxin. These studies have shown that prior administration of poly I:C does make the neurons more 'sensitive' to the neurotoxic effects of paraquat or 6-OHDA (Deleidi *et al.*, 2010; Bobyn *et al.*, 2012). In a study combining poly I:C with the administration of FN075, a novel molecule that accelerates the aggregation of α-synuclein, priming with the viral mimetic significantly increased the α-synuclein aggregate-induced neuropathological and behavioural effects (Olsen *et al.*, 2019). However, no study has thoroughly investigated the effect of this inflammagen alone on the integrity of the neurons of the nigrostriatal pathway.

1.4.4 GENETIC MODELS

As previously detailed, genetics play important roles in the pathogenesis of Parkinson's disease, with at least 19 genes having been shown to be linked to a Parkinson's disease phenotype (Deng *et al.*, 2018). The *SNCA* gene was the first gene to be associated with a familial inherited form of this disease, and the α-synuclein protein which is encoded for by this gene is integral in the pathology of the disease, as evidenced by its presence in Lewy bodies (Polymeropoulos *et al.*, 1997; Spillantini *et al.*, 1997). Therefore, the majority of research into genetic models of Parkinson's disease have focused on α-synuclein-based models. Genetic models of other genes linked to Parkinson's disease risk such as *LRRK2* and *Parkin* have also been developed with varying degrees of success at mimicking the pathology of the disease, but for the sake of succinctness they have not been elaborated upon here (reviewed in Lim and Ng, 2009; Konnova and Swanberg, 2018).

1.4.4.1 Transgenic α-synuclein models

The first transgenic mouse model that overexpressed wild-type human α-synuclein was developed in 2000 by Masliah and co-workers (Masliah *et al.*, 2000). They observed the presence of intracellular inclusions in the dopaminergic neurons of the substantia nigra which stained positively for α-synuclein, but these inclusions did not resemble Lewy bodies with regard to their fibrillary composition. Furthermore, although there was some depletion of dopaminergic terminals in the striatum, there was no apparent loss of neurons in the substantia nigra. Other transgenic mouse models have followed which differ from each other in terms of the promotor and the variant of the α-synuclein transgene used. However, most of these models fail to properly recapitulate the pathology of the disease. Models that use less specific promotors such as the prion promotor can result in widespread extranigral pathology such as motor neuron degeneration, which is not representative of the human disease (Van Der Putten *et al.*, 2000; Giasson *et al.*, 2002; Lee *et al.*, 2002b; Gomez-Isla *et al.*, 2003). Several groups have utilised a TH promotor which results in expression specifically in catecholaminergic neurons. However, by this method, using wild-type, A53T or A30P α-synuclein transgenes, mice failed to display substantial dopaminergic neuron loss and did not always present with α-synuclein positive inclusions (Matsuoka *et al.*, 2001; Richfield *et al.*, 2002). An exception is a model which uses a TH promotor and expresses a doubly mutated form of α-synuclein with both the A30P and A53T polymorphisms. This model did not produce discernable intraneuronal aggregates, but exhibited a loss of dopamine neurons in the substantia nigra and a decline in motor function (Thiruchelvam *et al.*, 2004). However, as no such double mutation has been reported in familial inherited Parkinson's disease thus far, the relevance of this model is dubious.

In recent years, a more advanced transgenic model involves a tetracycline (Tet)-regulated transgenic switch, as this technique permits temporal on/off control of transgene induction. These conditional models represent an important advancement in α-synuclein Parkinson's disease modelling, as the role of this protein in the progression of the disease can be investigated by reducing or switching off its expression (Nuber *et al.*, 2008; Lee *et al.*, 2012). However, none of these α-synuclein transgene rodent models succeed in fully replicating the pathology of the human condition of Parkinson's disease.

1.4.4.2 Viral vector-mediated α-synuclein models

In addition to the transgene models, extensive effort has gone into the development of α-synuclein overexpression models that are mediated by viral vectors. This approach has the significant benefit that genes can be delivered locally, and thus specific brain regions can be targeted. Deniz Kirik and colleagues (Kirik *et al.*, 2002) were amongst the first to attempt this approach to develop an α-synuclein model, and they utilised adeno-associated virus (AAV) vectors which expressed either wild-type or A53T mutated human α-synuclein. After injection into the substantia nigra of rats, they observed a significant loss of dopaminergic neurons in the substantia nigra and a subsequent reduction in striatal dopamine. This was accompanied by motor dysfunction in rats where a critical threshold of 50-60% loss of neurons was reached. Furthermore, they also noted the presence of intracellular aggregates which stained positive for α-synuclein and dystrophic neurites similar to those seen in Parkinson's disease patients. Subsequently, AAV vectors have been used successfully to produce both mouse (St Martin *et al.*, 2007; Ip *et al.*, 2017) and primate models (Kirik *et al.*, 2003; Eslamboli *et al.*, 2007). Lentiviral vectors (LV) have also been utilised, but they are not as efficacious as AAV vectors at expressing the transgene in the dopamine neurons of the brain and they do not produce as substantial a level of neuronal death (Lo Bianco *et al.*, 2002; Lauwers *et al.*, 2003).

This viral vector-mediated α-synuclein overexpression model is one of the best currently available with regard to recapitulating the pathophysiology of the disease. The gradual progression of this model more accurately portrays the normal disease course compared to neurotoxin-based models, which typically induce rapid degeneration of the nigrostriatal dopamine neurons. Furthermore, most neurotoxin models do not incorporate α-synuclein pathology, which is a key aspect of the pathophysiology of Parkinson's disease. The validity of this model means it is a fitting model in which to carry out studies investigating the potential of targeting the endocannabinoid system for disease modification in Parkinson's disease.

1.5 AIM OF THIS PROJECT

As outlined above, it is clear that the cannabinoid system, particularly the CB_2 receptor, is intricately linked to the neuroinflammatory aspect of Parkinson's disease. The α-synuclein protein contributes to the pathology of Parkinson's disease by various mechanisms, including affecting the neuroinflammatory component of the disease by modulating microglial activation. Animal models induced by viral vector-mediated overexpression of α-synuclein encompass many of the pathophysiological features of Parkinson's disease, but are still limited by their variability and slow development.

Thus, our hypotheses were three-fold:

- That levels of components of the endocannabinoid system and neuroinflammatory markers would be altered by the viral vector-mediated overexpression of α-synuclein.
- That the viral vector-mediated α-synuclein overexpression rat model would be enhanced by combination with a small molecule α-synuclein aggregator.
- That agonism of the immunomodulatory CB_2 receptor would provide anti-inflammatory disease modification in Parkinson's disease animal models.

Specifically, our aims were to:

- Examine alterations in components of the cannabinoid system in the highly relevant AAV-α-synuclein model of Parkinson's disease.
- Attempt to improve the AAV-α-synuclein model by combination with an α-synuclein aggregator, in order to provide a more reliable animal model for future studies of the endocannabinoid system.
- Investigate if agonism of the CB_2 receptor could reduce the neuroinflammatory response and confer functional neuroprotection in a viral priming model and in an inflammatory LPS model.

Chapter 2: Materials and Methods

2.1 ETHICAL STATEMENT

All procedures involving the use of animals were approved by the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland, Galway, and were carried out under a project licence issued to Prof. Eilís Dowd by the Irish Health Products Regulatory Authority (AE19125/P078), and under an Individual Authorisation issued to Ms. Rachel Kelly (AE19125/I199). These procedures were carried out in compliance with the European Union Directive 2010/63/EU and S.I. No. 543 of 2012. Any experiments involving the use of viral vectors were performed under licence from the Irish Environmental Protection Agency (GMO Register Number 290).

2.2 GLOBAL EXPERIMENTAL DESIGN

The overarching aim of this project was to ascertain if pharmacological targeting of the CB_2 receptor could suppress neuroinflammation in animal models of Parkinson's disease, and to determine if this anti-inflammatory effect was sufficient to protect nigrostriatal integrity and motor function in these animals. Firstly, we examined alterations in components of the cannabinoid system in the highly relevant AAV-α-synuclein model of Parkinson's disease. Furthermore, we sought to improve the AAV-α-synuclein model by combination with a small molecule α-synuclein aggregator, in order to provide a more reliable model for future studies of Parkinson's disease and the cannabinoid system. Finally, we examined if pharmacological targeting of the CB_2 receptor could reduce neuroinflammation and thus confer functional neuroprotection in inflammatory Parkinsonian animal models.

In the first results chapter (Chapter 3), we investigated the relationship between expression of the CB_2 receptor and other elements of the endocannabinoid system, to neuroinflammation and neurodegeneration in a genetic model of Parkinson's disease using AAV delivery of human α-synuclein with an A53T mutation into the substantia nigra. In the second results chapter (Chapter 4), we endeavoured to develop a more consistent and more rapidly developing model of Parkinson's disease in which to study

alterations in the endocannabinoid system by combining AAV-mediated α-synuclein overexpression with the small molecule FN075 which aggregates α-synuclein. In the third results chapter (Chapter 5), we investigated the potential of targeting the CB_2 receptor for preventing the microglial priming effect in a Parkinsonian animal model combining viral priming with α-synuclein aggregation. In the fourth and final results chapter (Chapter 6), we examined the capability of a CB_2-selective agonist to provide anti-inflammatory and neuroprotective effects in an inflammatory LPS model of Parkinson's disease.

This chapter will detail the techniques and methodologies employed in order to conduct this research. Specific experimental designs will be provided in subsequent results chapters.

2.3 ANIMAL HUSBANDRY

A total of 200 Sprague-Dawley rats (168 female and 32 male) were used in this research. Animals were sourced from Charles River, U.K. unless otherwise indicated. Experiments were initiated when the rats were ~ 8 weeks old. All animals were housed two per cage in plastic bottom cages with the following dimensions: (50.5 x 13 x 24 cm) with a wire grid lid and wood shavings or 3Rs bedding. Each cage contained sizzle-nest and hollow plastic tunnels as environmental enrichment. Animals were allowed to free-feed with access to food and water *ad libitum* unless undergoing food restriction as part of behavioural assessment, in which case animals were provided with sufficient food to maintain 85-90% of their free-feeding body weight, as determined by a weight curve obtained from Charles River. Animals were housed in a 12:12 hour light:dark cycle, under a regulated temperature of 19-23ºC and a regulated humidity of 40-70%. All behavioural testing and *post mortem* analyses were performed blind to the treatment of the animals.

2.4 SURGERY

2.4.1 STEREOTAXIC SURGERY

Animals received bunerorphine (0.01-0.05 mg/kg s.c.) prior to surgery and every 8-12 hours for 24-48 hours following surgery. All surgery was performed under aseptic conditions under isoflurane anaesthesia (5% in O_2 for induction, ~2% in O_2 for

maintenance) in a stereotaxic frame with the nose bar set at -2.3 mm. Before surgery, the head of the rat was shaved at the site of surgery and the animal was secured in the stereotaxic frame using the ear bars and nose bar. EMLA® cream (lidocaine 2.5% w/w & prilocaine 2.5% w/w) was applied to the ears pre-operatively to reduce potential pain caused by the ear bars, and Visidic® Eye Gel (aqueous gel containing carbomer 0.2% w/w) was applied to the eyes to prevent potential discomfort caused by dry eyes. The breathing rate and the temperature of the animal were recorded every five to seven minutes throughout the entire duration of surgery and isoflurane anaesthesia was adjusted accordingly. To initiate surgery, an incision was made on the head to expose the skull using a scalpel. Lidocaine (0.5% w/v) with adrenaline (5 mg/ml) and bupivacaine (0.25% w/v) was administered topically to the incision site to reduce potential pain. The coordinates for bregma were identified and the site of injection was measured relative to this. In each case, the substantia nigra was targeted using coordinates that have been utilised successfully by our research group previously (Mulcahy *et al.*, 2013; Concannon, 2016). An electric drill was used to make a hole in the skull to expose the dura mater. A 30 gauge injection cannula was connected to a 10 µl Hamilton syringe using polyethene tubing of 0.28 mm inner diameter filled with either saline or water, depending on the toxin being administered. An air bubble was deliberately introduced before loading the toxin to allow monitoring of the solution through the plastic tubing. Infusion of the toxins was achieved by slow consistent depression of the plunger on the Hamilton syringe using an automated pump (Harvard apparatus, U.S.A.). The rate was 0.5 µl/min for AAV surgeries and 1 µl/min for other surgeries. Once the delivery was finished and some time was allowed for diffusion, the cannula was removed and the incision was sutured closed. The animal was placed in a recovery cage where it was monitored until it regained full consciousness before being placed back into its home cage.

2.4.2 INTRA-NIGRAL AAV SURGERIES

The viral vectors used in this thesis are AAV6-GFP (green fluorescent protein) and AAV6-α-synuclein viruses, kindly synthesised and provided by Prof. Emmanuel Brouillet and Dr. Alexis Bemelmans of the François Jacob Institute of Biology, CEA, France. Transgenes (GFP, wild-type human α-synuclein and human α-synuclein with the

A53T mutation) under the phosphoglycerate kinase (PGK) promoter were packaged into a pseudotyped AAV6 viral vector.

For the AAV vector surgeries, AAV viral vectors were diluted in sterile phosphate-buffered saline (PBS) with Pluronic F-68 (0.01%) on the day of surgery to the appropriate titre ($1.33x10^{10}$ vector genomes (vg)/µl for surgeries involving the α-synuclein vector with the A53T mutation, $1.67x10^{10}$ vg/µl for surgeries involving the wild-type α-synuclein vector). All surgeries were performed as described in the previous section (section 2.4.1). For these surgeries, the polyethene tubing was filled with sterile water. The substantia nigra was infused unilaterally with a dual injection at the following coordinates from bregma: AP -4.8 & -5.8, ML ± 2.0 and DV -7.2 based on the rat brain axis (Paxinos and Watson, 2006). Infusions were carried out at a rate of 0.5 µl/min with a total volume of 3 µl per site, and a further 5 min was then allowed for diffusion.

2.4.3 INTRA-NIGRAL POLY I:C SURGERIES

For the poly I:C (HMW, tlrl-pic, InvivoGen, France) surgeries, the inflammagen was diluted in sterile endotoxin-free saline (NaCl 0.9%; supplied in kit) to obtain a final concentration of 7.5 µg/µl. All poly I:C surgeries were carried out as described in section 2.4.1. For these surgeries, the polyethene tubing was filled with sterile saline. The nigra was infused unilaterally with poly I:C (30 µg) at the following coordinates from bregma: AP -5.3, ML ± 2 and DV -7.2. Infusions were carried out at a rate of 1 µl/min with a total volume of 4 µl, and a further 3 minutes was then allowed for diffusion.

2.4.4 INTRA-NIGRAL FN075 SURGERIES

FN075 was kindly synthesised and provided by Dr. Andrew G. Cairns, Dr. Jörgen Ådén and Prof. Fredrik Almqvist of the Department of Chemistry, Umeå University, Sweden (Horvath *et al.*, 2012).

For the FN075 surgeries, FN075 was diluted in PBS to a final concentration of 1 mM with dimethyl sulfoxide (DMSO, 0.5%; Sigma, Ireland) and imidazole (100 µM; Sigma, Ireland). For these surgeries, the polyethene tubing was filled with sterile saline. All FN075 surgeries were carried out as described in section 2.4.1. The nigra was infused unilaterally with FN075 (1.9 µg total) or vehicle at the following coordinates from

bregma: AP -5.3, ML ± 2 and DV -7.2. Infusions were carried out at a rate of 1 µl/min with a total volume of 4 µl, and a further 2 minutes was then allowed for diffusion.

2.4.5 INTRA-NIGRAL LPS SURGERIES

For the LPS surgeries (lipopolysaccharides from Escherichia coli O111:B4; purified by phenol extraction, L2630, Sigma-Aldrich, Ireland), the inflammagen was diluted in sterile 0.9% saline to obtain a final concentration of 5 µg/µl. All LPS surgeries were carried out as described in section 2.4.1. For these surgeries, the polyethene tubing was filled with sterile saline. The nigra was infused unilaterally with LPS (10 µg) at the following coordinates from bregma: AP -5.3, ML +2.0, DV -7.2. Infusions were carried out at a rate of 1 µl/min with a total volume of 2 µl, and a further 2 minutes was then allowed for diffusion.

2.5 BEHAVIOURAL TESTS OF MOTOR IMPAIRMENT

2.5.1 CORRIDOR TEST

The Corridor test measures contralateral sensorimotor neglect and was completed as previously described (Dowd *et al.*, 2005; Fitzsimmons *et al.*, 2006). As part of this procedure, rats were food restricted to 85-90% of their free-feeding body weight for the duration of the testing period. The corridor apparatus consisted of two long parallel chambers (length = 150 cm, height = 24.5 cm and width = 7 cm). During the habituation period, rats were allowed to explore the first corridor freely which had CocoPops® scattered along the floor. During the testing period, the rats were placed one by one in an empty corridor for 5 minutes before being transferred to the second corridor where there were 10 adjacently placed pairs of pots, each containing 3-5 CocoPops®. The animal was then allowed to freely explore and retrieve the CocoPops® from the pots on either side (Figure 2.1). The trial was deemed complete when the animal had made a total of 20 retrievals or after 5 minutes had elapsed. The number of retrievals made by each rat from the ipsilateral and contralateral sides (relative to the lesion site) was recorded and expressed as a percentage of the total number of retrievals made. A ‘retrieval’ was defined as the rat poking its nose into the container, whether or not it ate or retrieved any of the food in the container. This test is based on the premise that animals with a unilateral lesion

will tend to ignore food on their contralateral side and will retrieve the CocoPops® predominantly from their ipsilateral side.

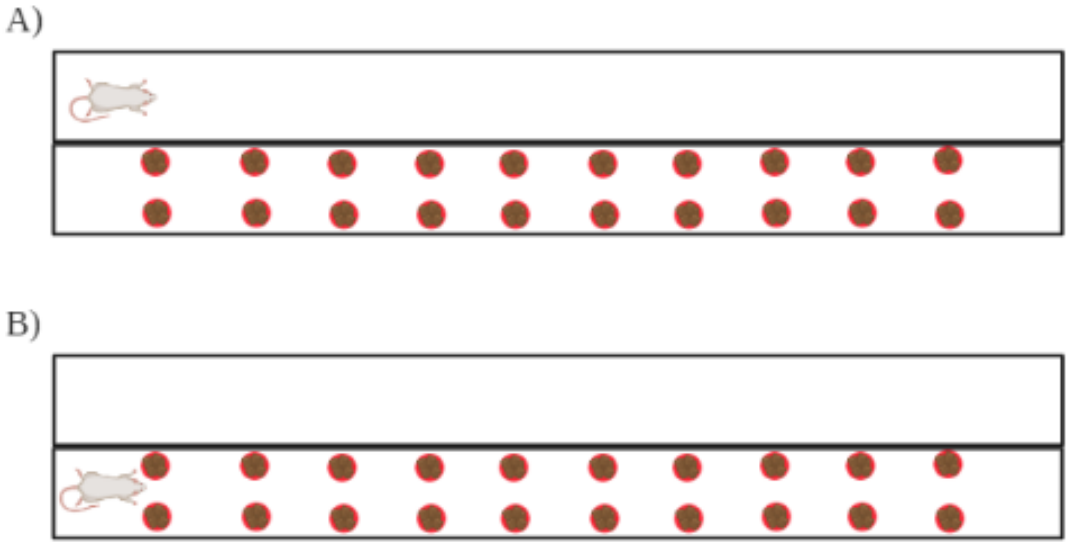

Figure 2.1 Corridor test of sensorimotor neglect. On the day of testing, after 5 minutes of habituation in the empty corridor (A) rats were transferred to the adjacent corridor with the containers with CocoPops® (B). The number of retrievals made by the rat on each side was recorded. Trials were deemed complete after 5 minutes or after 20 retrievals had been made.

2.5.2 STEPPING TEST

The Stepping test measures forelimb akinesia and was carried out as previously described (Olsson *et al.*, 1995). The animals were initially habituated to this test by restraining them in such a manner so that both hindlimbs were resting in the hands of the experimenter and both forelimbs were resting on the edge of a table. Once the animal was habituated to this handling, one forelimb was restrained and the rat was guided horizontally across a table surface at a steady pace (90 cm in ~5 sec). The number of adjusting steps made by the 'free' paw in the forehand and backhand directions was recorded. This was performed for both the ipsilateral and contralateral sides of the rat. Unilaterally lesioned animals have a reduced number of adjusting steps on the contralateral side. Data were represented as the average number of adjusting steps (combining forehand and backhand data) made by either the ipsilateral or the contralateral limb.

2.5.3 WHISKER TEST

The Whisker test is also known as the vibrissae-elicited forelimb placement test and it is a measure of sensorimotor integration (Schallert *et al.*, 2000). This test is based on the sensorimotor reflex underlying vibrissae-elicited movement whereby when the animal's whiskers are brushed against a surface, this will elicit a reflex that causes the animal's ipsilateral limb to move, placing its paw on the surface. This response is impaired in animals with a dopaminergic lesion. The rats were initially habituated to the task by restraining the animal's hindlimbs and one forelimb in such a manner that the animal was comfortable, before gently brushing their whiskers against the corner of the table. During testing, the number of vibrissae-elicited forelimb placings made by the unrestrained forepaw when the animal's whiskers were brushed against the corner of the table 10 times was recorded, and this was performed on both the contralateral and ipsilateral sides of the body. Data were represented as the number of forelimb placings made by either the ipsilateral or the contralateral limb.

2.6 IMMUNOHISTOCHEMISTRY

2.6.1 TISSUE PROCESSING

Rats were sacrificed by terminal anaesthesia (50 mg/kg intraperitoneal (i.p.) pentobarbital) and then transcardial perfusion was performed with heparinised saline (5000 units/litre) followed by formalin solution (Serosep, Ireland) using an automatic pump. Brains were rapidly removed from the skull and were placed in formalin solution overnight before being cryoprotected in 30% sucrose with 0.1% sodium azide solution for long-term storage. Serial brain sections (30 µm) were cut using a freezing sledge microtome (Bright, U.K.) and collected in a series of 12.

2.6.2 IMMUNOHISTOCHEMISTRY

Free-floating immunohistochemistry (IHC) was performed using the streptavidin-biotin-peroxidase method as previously described (Walsh *et al.*, 2011; Hoban *et al.*, 2013). In brief, sections were quenched in a solution containing 3% hydrogen peroxide and 10% methanol in distilled water to eliminate endogenous peroxidase activity. Non-specific antibody binding was blocked by incubation in a solution containing 3% normal horse

serum or normal goat serum (depending on the host species of the secondary antibody) in tris-buffered saline (TBS) with 0.2% Triton X-100 at room temperature for 1 hour. The primary antibody (Table 2.1) was diluted in 1% serum in TBS with 0.2% Triton X-100 and allowed to incubate with the sections overnight at room temperature. Sections were then incubated with the appropriate biotinylated secondary antibody with 1% serum for 3 hours. A streptavidin-biotin-horseradish peroxidase solution (Vector, U.K.) was subsequently added to sections and allowed to incubate for 2 hours. Development of the staining was performed using a 0.5% diaminobenzidine tetrahydrochloride (DAB) (Sigma, Ireland) solution in TBS containing 0.3 µl/ml of hydrogen peroxide. Sections were then mounted onto gelatin-coated slides, dehydrated in an ascending series of alcohols, cleared in xylene and finally coverslipped using DPX mountant (Sigma, Ireland).

Ab	Protein target	Description	Source	Host	Dilution
1º	Tyrosine hydroxylase (TH) (rat)	Catecholaminergic neurons	Millipore	Mouse	1:1000
1º	OX-42 (rat)	Microglia	Millipore	Mouse	1:400
1º	Glial fibrillary acidic protein (GFAP) (rat)	Astrocytes	DAKO	Rabbit	1:2000
1º	α-synuclein (human)	Wild-type α-synuclein protein	Millipore	Mouse	1:10000
1º	α-synuclein filament (rat/human)	α-synuclein filaments – conformation specific	abcam	Rabbit	1:4000
1º	Phospho-α-synuclein (rat/human)	α-synuclein – phosphorylated at serine 129	abcam	Rabbit	1:5000
2º	Biotinylated horse anti-mouse	Targets mouse 1º IgG	Vector	Horse	1:200
2º	Biotinylated goat anti-rabbit	Targets rabbit 1º IgG	Jackson	Goat	1:200

Table 2.1 Antibodies used for immunohistochemistry. Ab, antibody; 1º, primary, 2º, secondary.

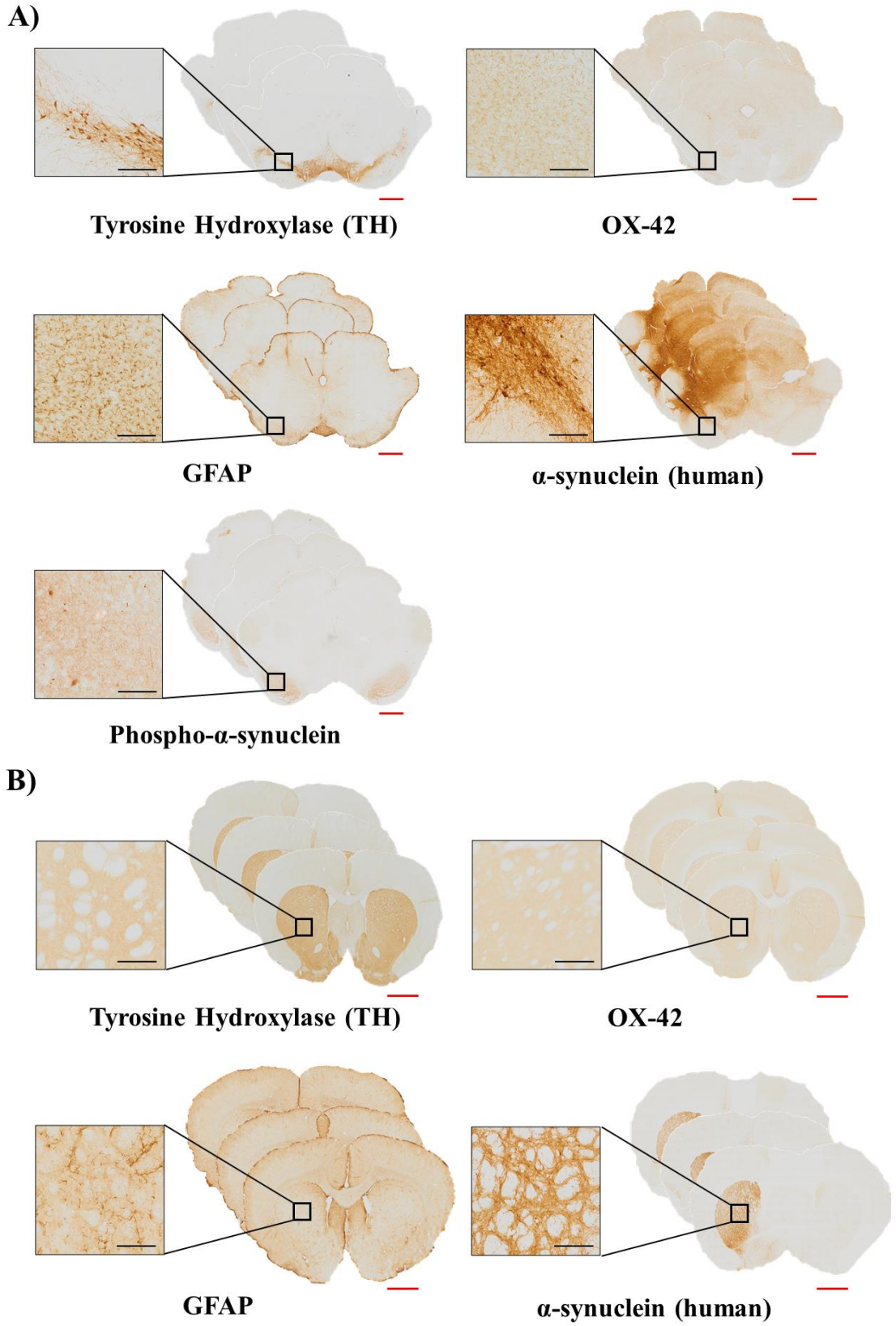

Figure 2.2 Representative images of immunohistochemical stains in the A) substantia nigra and B) striatum. Black scale bar = 200 µm for high magnification images in all cases, red scale bar for slice section images = 1 mm in substantia nigra and = 2 mm in striatum.

2.6.3 HISTOLOGICAL QUANTIFICATION

Slides were imaged using a VS120 Virtual Slide Microscope (Olympus U.K., United Kingdom) with a computer running OlyVIA software (Version 2.9.1). TIFF images were saved from this software for analyses. All histological quantification was carried out using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, U.S.A.). Nigral dopaminergic cells were assessed by counting the number of tyrosine hydroxylase-positive cell bodies in the substantia nigra. Striatal dopaminergic fibres were assessed by measuring the optical density of tyrosine hydroxylase-positive striatal terminals. Similarly, microglial, astrocytic, and α-synuclein staining were measured in both the substantia nigra and the striatum using optical density analyses. In each case, three images were selected along the rostrocaudal axis of the striatum or nigra in an unbiased manner based on their distance from bregma (striatal AP coordinates: +0.7, +1.0, +1.2 mm; nigral AP coordinates: −5.6, −5.8, −6.04 mm).

2.6.3.1 Quantification of nigral tyrosine hydroxylase-positive cell bodies

The number of tyrosine hydroxylase-positive cell bodies in the substantia nigra were counted manually using the cell counter plugin tool in the ImageJ programme (Figure 2.3). For these analyses, the magnification of the images was 4x. The number of positively stained cell bodies were counted on both the ipsilateral and contralateral sides of the brain (relative to the surgery site) and according to boundaries previously delineated (Kirik *et al.*, 1998). The same size and colour criteria of what was counted as a cell was applied across all images. In brief, immune positive cells in the substantia nigra *pars reticulata*, *pars lateralis* and *pars compacta* were counted and immune-positive cells in the ventral tegmental area (VTA) were excluded (Paxinos and Watson, 2006). This was carried out on both the ipsilateral and contralateral sides of the brain for three representative images for each animal and data were expressed as a percentage of the contralateral (intact) side.

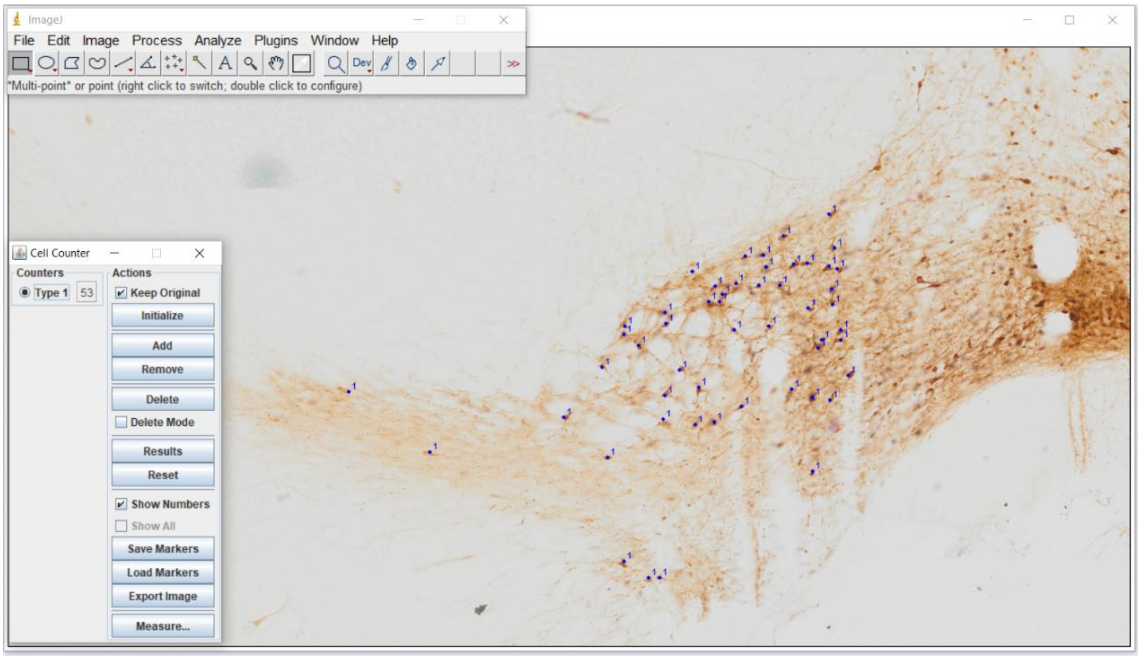

Figure 2.3 ImageJ analysis of nigral cell bodies. Screenshot of ImageJ software and counter tool used to measure tyrosine hydroxylase-positive cell bodies in the substantia nigra. Blue counter dots indicate a cell body.

2.6.3.2 Quantification of nigral density

Optical density measurements were used to analyse microgliosis, astrocytosis and α-synuclein expression in the substantia nigra in ImageJ. Photographs of the immunostained sections were taken using a VS120 Virtual Slide microscope, and three representative images were chosen for each animal. A circular shape was drawn on the substantia nigra on the lesioned side and the mean grey value of the selected region was measured (Figure 2.4). This was then repeated on the intact (contralateral) side. Mean grey values were then converted to optical density values using a logarithmic function applied to the image pixel intensity (optical density = $\log_{10}$(255/mean grey value). Data were then represented as a percentage of the intact side.

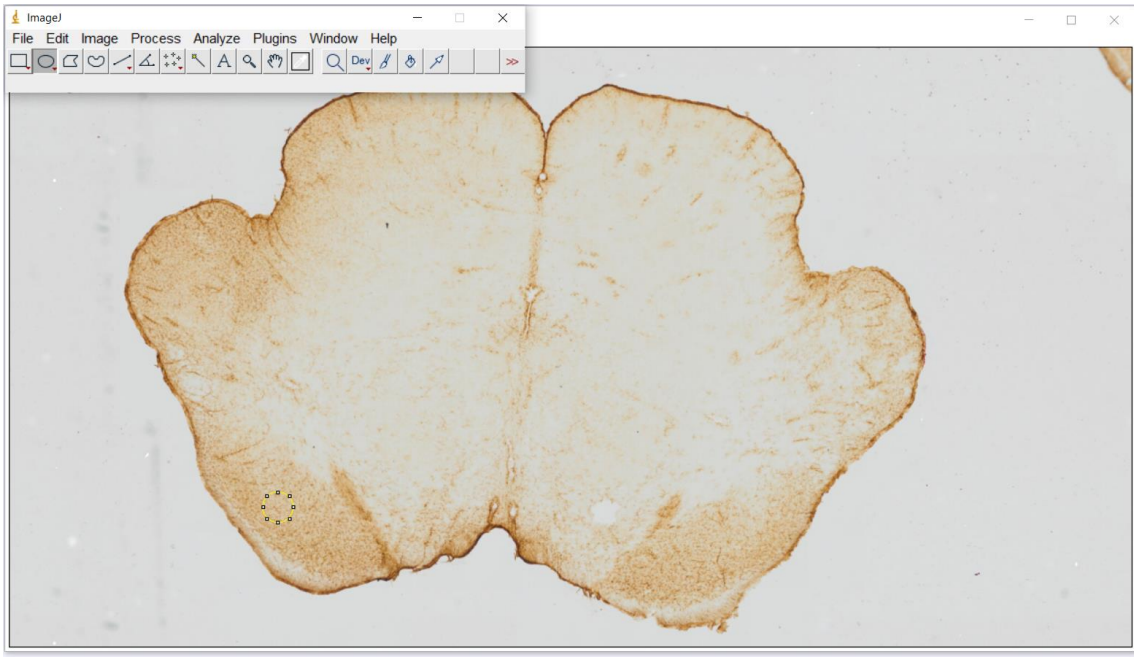

Figure 2.4 ImageJ analysis of nigral density. Screenshot of ImageJ software used to measure nigral density. The area to be measured is delineated by the yellow border in the image above. Mean grey value was measured on both the ipsilateral and contralateral sides.

2.6.3.3 Quantification of striatal density

Optical density measurements were used to analyse tyrosine hydroxylase-positive fibres, microgliosis, astrocytosis and α-synuclein expression in the striatum in ImageJ (Figure 2.5). Photographs of the immunostained sections were taken using a VS120 Virtual Slide microscope, and three representative images were chosen for each animal. A large circular shape was drawn on the striatum on the lesioned side and the mean grey value of the selected region was measured. This was then repeated on the intact (contralateral) side. Mean grey values were then converted to optical density values using a logarithmic function applied to the image pixel intensity (optical density = $\log_{10}$(255/mean grey value). Data were then represented as a percentage of the intact side.

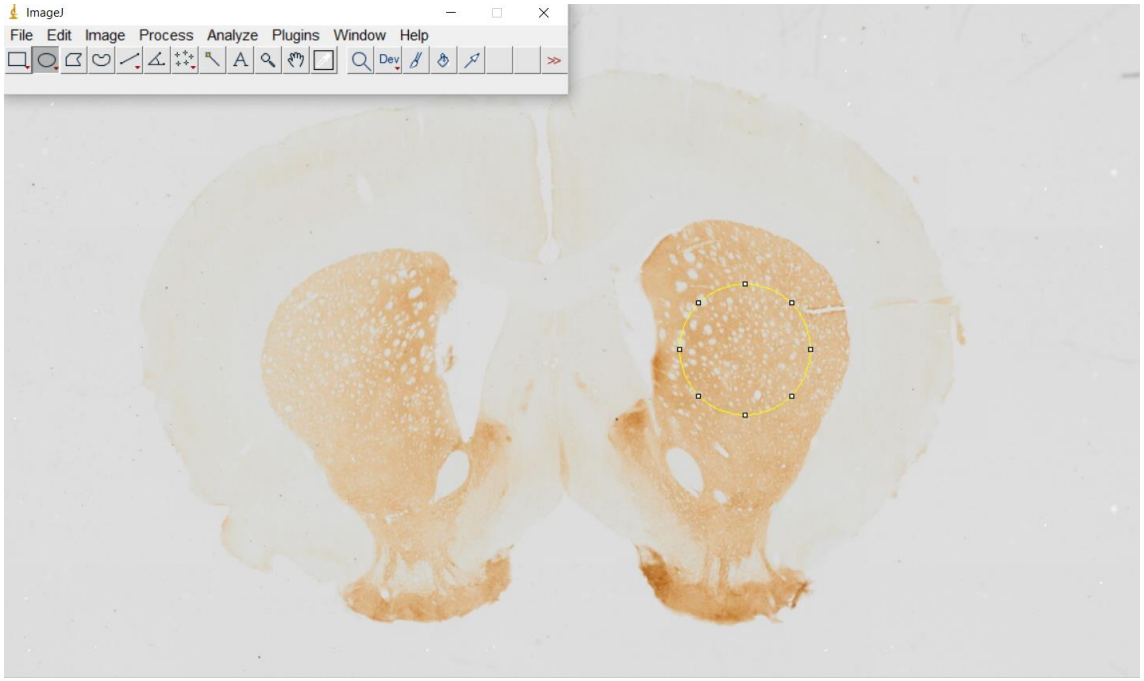

Figure 2.5 ImageJ analysis of striatal density. Screenshot of ImageJ software used to measure striatal density. The area to be measured is delineated by the yellow border in the image above. Mean grey value was measured on both the ipsilateral and contralateral sides.

2.6.3.4 Quantification of tyrosine hydroxylase-positive dystrophic neurites

The number of TH-positive dystrophic neurites in the striatum were counted using ImageJ (Figure 2.6). Dystrophic neurites are swollen, disformed axon terminals, and have previously been reported in Parkinson's disease patients and models (Spillantini *et al.*, 1997; Braak *et al.*, 1999; Kirik *et al.*, 2002). For each animal, photomicrographs were taken from three representative striatal sections at the same level spanning the rostrocaudal axis of the striatum using a VS120 Virtual Slide Microscope. The number of dystrophic neurites in the striatum ipsilateral to the lesion was counted. Data were expressed as the average number of dystrophic neurites.

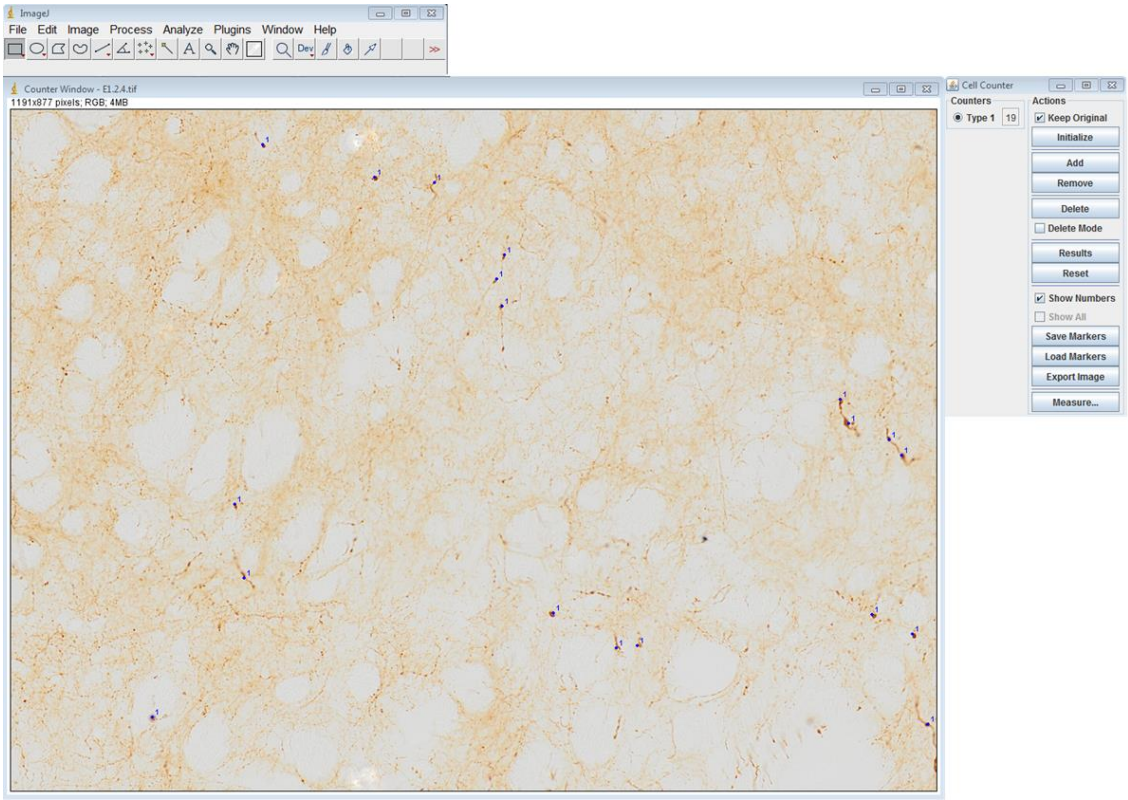

Figure 2.6 ImageJ analysis of TH-positive dystrophic neurites. Screenshot of ImageJ software and counter tool used to measure TH-positive dystrophic neurites in the striatum. Blue counter dots indicate a dystrophic neurite.

2.6.3.5 Quantification of pS129-α-synuclein-positive accumulations

The number of pS129-α-synuclein (α-synuclein phosphorylated at the serine 129 residue) positive accumulations in the substantia nigra were counted in ImageJ (Figure 2.7). Three representative images were used for quantification. The number of positively stained accumulations were counted on the side of the brain ipsilateral to the side of the lesion surgery. The data were expressed as the average number of pS129-α-synuclein positive accumulations.

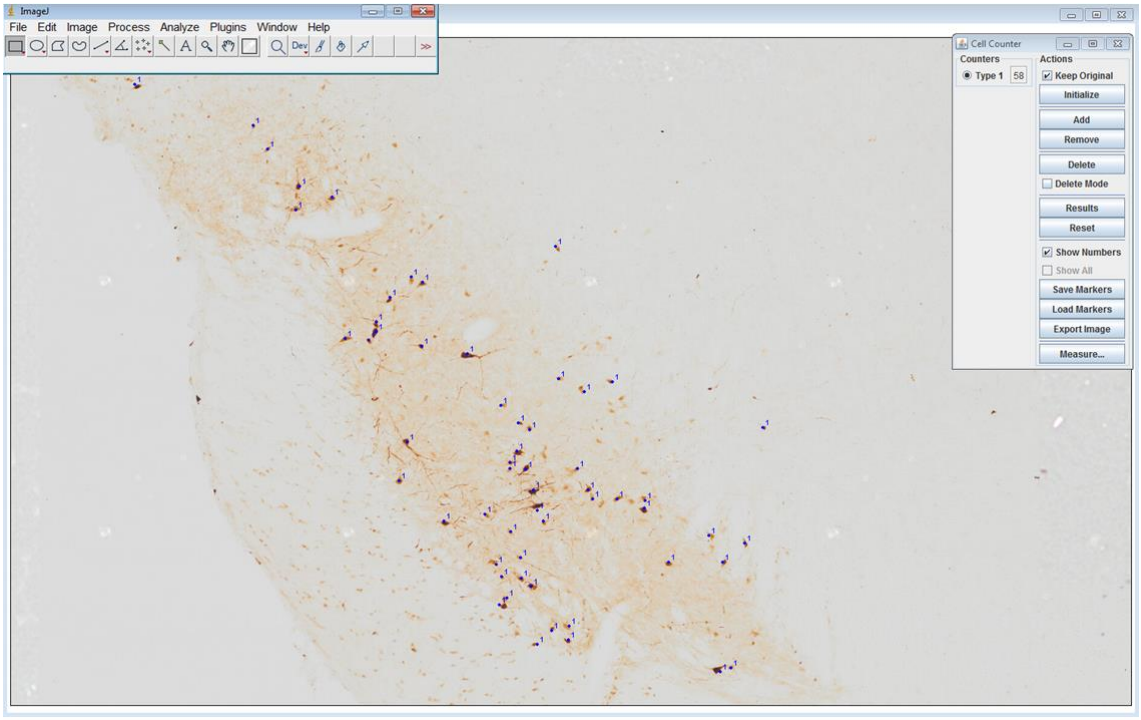

Figure 2.7 ImageJ analysis of pS129-α-synuclein-positive accumulations. Screenshot of ImageJ software and counter tool used to measure pS129-α-synuclein-positive accumulations in the substantia nigra. Blue counter dots indicate an accumulation.

2.7 GENE EXPRESSION ANALYSES USING QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QRT-PCR)

2.7.1 TISSUE PROCESSING

Animals were sacrificed via decapitation under isoflurane anaesthesia (in 5% O_2) and brains were rapidly removed and snap-frozen on dry ice. Brains were stored at -80ºC for processing. In order to obtain nigral and striatal tissue, a cryosectioning and micro-punching technique was used. Brains were mounted on a chuck in Tissue-Tek® O.C.T. mounting medium (Sigma, Ireland) and brain sections were cut at -20ºC on a cryostat (Leica CM3050 S, Germany). The sections were cut at a thickness of 300 µm and collected on Superfrost™ microscope slides (Fisher Scientific, U.K.). For the nigra, tissue was obtained by taking punches from an area encompassing the substantia nigra *pars reticulata*, *pars lateralis* and *pars compacta*, but not the ventral tegmental area. For the striatum, tissue was obtained from serial sections, and additional punches were obtained for mass spectrometry analyses of endogenous cannabinoids (section 2.8). Using a 2 mm internal diameter cylindrical puncher (Harvard Apparatus, U.K.) on a 1 ml syringe, tissue

was excised and quickly displaced into RNase-free tubes and snap-frozen on dry ice. 4-5 punches from the nigra and from the striatum were obtained for RNA isolation and qRT-PCR analyses (4 additional punches from the striatum were obtained for LC-MS/MS analyses). The eppendorfs were weighed before and after the punches were inserted and the difference was calculated to determine the weights of the punches. In all cases, both the lesioned and intact sides of the nigra and the striatum were punched.

2.7.2 RNA ISOLATION

Total RNA was extracted from nigral and striatal samples using a NucleoSpin™ RNA isolation kit (12373368, Macherey-Nagel™, Fisher Scientific, Ireland) according to the manufacturer's instructions. In brief, 354 µl of RA1 lysis buffer containing 1% β-mercaptoethanol (Sigma-Aldrich, Ireland) was added to approximately 10 mg of excised tissue before homogenisation with an IKA T10 basic Ultra-Turrax tissue disrupter (Fisher Scientific, Ireland). Homogenates were then transferred to a NucleoSpin filter column before centrifugation at 11,000 x *g* for 1 min. 350 µl of 70% molecular grade ethanol (Sigma-Aldrich, Ireland) was added to the lysates and mixed by pipetting up and down 8 times. The samples were then transferred to a set of NucleoSpin RNA II columns and centrifuged at 11,000 x *g* for 30 seconds to allow RNA to bind to the column. The columns were placed in a new set of collection tubes and 350 µl of membrane salting buffer was added prior to centrifugation at 11,000 x *g* for 1 min. Genomic DNA was then digested using a 10% v/v rDNase solution in DNase reaction buffer by pipetting 95 µl of this solution directly into the centre of each column and allowing it to stand for 15 min at room temperature. Following this incubation, 200 µl of RAW2 buffer was added to each column before centrifugation at 11,000 x *g* for 30 seconds. The columns were then placed in new collection tubes and 600 µl of RA3 wash buffer was added prior to centrifugation at 11,000 x *g* for 30 seconds. The flow-through was discarded and 250 µl of RA3 buffer was added followed by centrifugation at 11,000 x *g* for 2 minutes. The columns were then placed in RNAse-free collection tubes and the RNA was eluted by the addition of 50 µl of RNase-free water (Sigma-Aldrich, Ireland) followed by centrifugation at 11,000 x *g* for 1 minute. The eluted RNA was then quantified before storage at -80ºC until reverse transcription.

2.7.3 ASSESSMENT OF RNA QUALITY AND CONCENTRATION

The quality and quantity of RNA were assessed using a DeNovix DS-11 spectrophotometer (DeNovix, U.S.A.). RNA quantity was determined by measuring optical density (OD) at 260 nm (1 OD unit at 260 nm corresponds to 40 µg/ml RNA). RNA quality was determined by measuring the OD_{260}/OD_{280} ratio where a value of 1.6-2.2 was deemed indicative of pure RNA. Where possible, samples were equalised to the same concentration of RNA (13 ng/µl for the substantia nigra and 21.5 ng/µl for the striatum) by the addition of RNase free water, although in some cases low RNA yield did not allow for this.

2.7.4 cDNA SYNTHESIS

Complementary DNA (cDNA) synthesis was performed using an Invitrogen Superscript™ reverse transcriptase custom kit (Biosciences, Ireland). Initially, a master mix consisting of 1 µl random primers and 1 µl dNTP mix (10 mM) was added to the sample containing 10 µl of normalised RNA to make up a reaction volume of 12 µl. Samples were then incubated for 5 min at 65ºC in a PTC-200 Peltier MJ Research Research Thermocycler (Bio-Rad, Ireland). A second master mix consisting of 4 µl of First Strand buffer, 2 µl dithiothreitol (DTT) and 1 µl RNaseOUT (Biosciences, Ireland) was added to the tubes and further incubated at 37ºC for 2 min. Finally, 1 µl Superscript™ reverse transcriptase (Biosciences, Ireland) was then added to the sample before a final incubation at room temperature for 10 minutes, at 50ºC for 50 minutes and 70ºC for 15 minutes before being cooled to 4ºC. Samples were then diluted 1:4 with RNase-free water before being stored at -20ºC.

2.7.5 QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QRT-PCR)

Gene expression of the genes of interest was determined using commercially available Taqman gene expression assays (Biosciences, Ireland) containing specific target primers and fluorescein amidite (FAM)-labelled minor groove binder (MGB) probes. β-actin (4352340E, ThermoFisher, Ireland) was used as an endogenous control to normalize gene expression. In all samples, a multiplex reaction was performed simultaneously measuring β-actin using a VIC-labelled MGB probe in addition to the target gene (Table 2.2). A reaction master mix was first prepared containing 5 µl Taqman master mix, 1.5 µl RNase-

free water, 0.5 µl β-actin control primer and 0.5 µl target gene primer per well. 2.5 µl of each sample was pipetted in duplicate into an optical 96 well plate. 7.5 µl of the appropriate master mix was then added to each well, giving a total reaction volume of 10 µl. RNase-free water was used as a non-template control (NTC), controlling for the possibility of genomic DNA contamination. Plates were then sealed with optical adhesive covers and spun briefly at 1600 x g to ensure all liquid was at the bottom of the wells and to eliminate bubbles. The PCR reaction took place in an Applied Biosystems StepOne Plus™ Real-Time PCR system (Applied Biosystems, U.S.A.) to a preset run with the following relative quantification cycling programme; Step 1: 50ºC for 2 min, 95ºC for 10 min, and Step 2: 40 cycles of 95ºC for 15 sec/ 60ºC for 1 min. The level of gene expression was measured by fluorescence amplification and measured during the annealing and extension phase at 60ºC during the programme.

Target Gene	Assay ID
SNCA	Hs00240906_m1
CB_1	Rn00562880_m1
CB_2	Rn03993699_s1
FAAH	Rn00577086_m1
MAGL	Rn00593297_m1
CD11b	Rn00709342_m1
GFAP	Rn00566603_m1

Table 2.2 TaqMan® gene expression assays.

2.7.6 ANALYSIS OF QRT-PCR DATA

Amplification plots and cycle threshold (Ct) values were examined using Applied Biosystems StepOne Software v2.3 (Figure 2.8). The threshold was detected automatically by the software programme at the linear exponential phase of the amplification curve and then manually adjusted if necessary. Ct values were then exported to Microsoft Excel for analyses. Relative gene expression was calculated using the formula $2^{-\Delta Ct}$. ΔCt was determined using the formula $Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$, where the average of the Ct values was used, normalising to the endogenous control. The fold change in gene expression is then given by the formula $2^{-\Delta Ct}$. This method was applied to

both treated and control samples and all samples were then expressed as a percentage of the mean control value. As Chapter 3 was a timecourse study, each treated and control sample was represented as a percentage of the mean control value for that time-point. This method allowed us to determine the difference in expression of the target gene in treated animals compared to control animals.

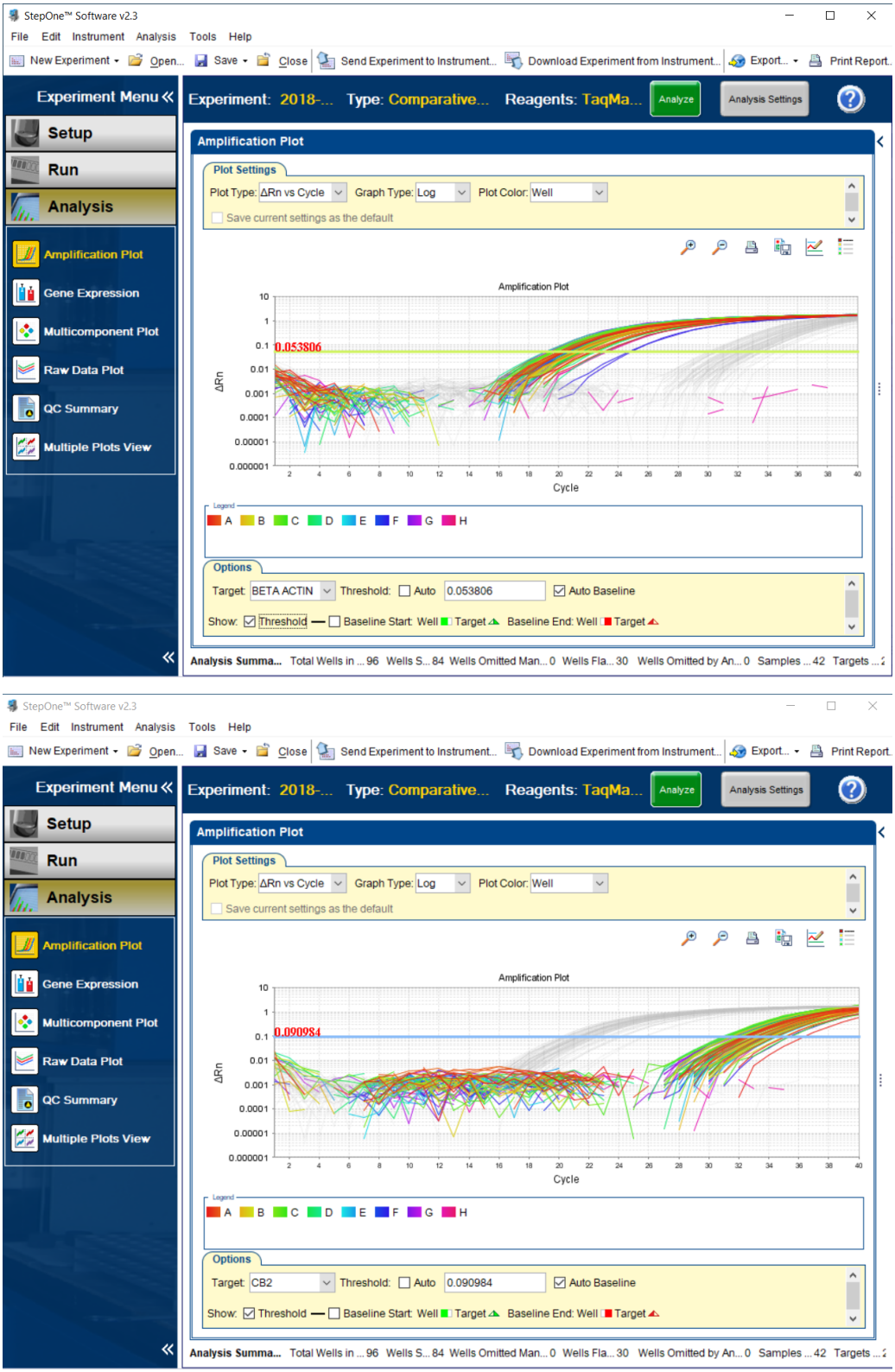

Figure 2.8 Sample amplification plots for A) β-actin and B) CB_2

2.8 QUANTIFICATION OF ENDOCANNABINOID AND RELATED *N*-ACYLETHANOLAMINE LEVELS USING LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LC-MS/MS)

2.8.1 TISSUE PROCESSING

Striatal tissue was obtained using the protocol described in section 2.7.1. Tissue punches taken from serial sections were used to measure levels of endocannabinoids and related *N*-acylethantolamines in the striata of animals that had been injected in the substantia nigra with AAV-GFP or AAV-α-synuclein.

2.8.2 PREPARATION OF STANDARDS

Samples and standards were prepared essentially as previously described (Corcoran *et al.*, 2020; Boullon *et al.*, 2021). Non-deuterated stock solutions were prepared in 100% acetonitrile for *N*-arachidonyl ethanolamide (AEA), 2-arachidonylglycerol (2-AG), *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA) (Cayman chemicals, Cambridge Biosciences, U.K.) at concentrations of 2.5 mg/ml for AEA, PEA, OEA and 0.5 mg/ml for 2-AG. A single stock solution of all the above standards was then prepared to contain 2-AG at a concentration of 5 µg/ml and AEA, PEA and OEA at a concentration of 0.5 µg/ml, in 100% acetonitrile.

Stock solutions of the deuterated form of AEA-d8, 2-AG-d8, PEA-d4 and OEA-d4 (Cayman Chemicals, Cambridge Biosciences, U.K.) were prepared in acetonitrile, each at a concentration of 100 µg/ml. From these stocks, a deuterated homogenising buffer was prepared to contain 2-AG-d8 at 50 ng/200 µl and AEA-d8, PEA-d4 and OEA-d4 at 2.5 ng/200 µl. The deuterated homogenizing buffer was then dispensed into 20 ml aliquots and stored with non-deuterated standards at -80ºC.

2.8.3 PREPARATION OF STANDARD CURVE AND SAMPLES

A 10-point standard curve was prepared by a 1:4 serial dilution. The highest standard (Standard 10) was made up by adding 25 µl of 100% acetonitrile containing a known fixed amount of non-deuterated internal standard (12.5 ng AEA, PEA and OEA and 125 ng 2-AG) to 75 µl of pure acetonitrile solution. Once the serial dilution was complete,

200 µl of 100% acetonitrile containing a known fixed amount of deuterated internal standard was added to each standard.

2.8.4 QUANTITATION OF ENDOCANNABINOIDS AND *N*-ACYLETHANOLAMINE LEVELS

Quantitation of the concentration of endocannabinoids and related *N*-acylethanolamines was carried out in the NUIG Biological Mass Spectrometry Core Facility according to a standardised protocol (unpublished). Pre-weighed frozen tissue (~10 mg) in 75 µl of 100% acetonitrile and 200 µl deuterated internal standard was carefully homogenised for ~3 sec using a sonicator (Branson Sonifier 150, Branson, U.K.). Following this, homogenates were centrifuged at 14,000 x g for 15 min at 4ºC (Hettich® centrifuge Mikro 220R, Hettich, Germany). The supernatant from each sample was transferred to a new Eppendorf. Forty microliters of the supernatant of each sample and standard curve point were added to high-performance liquid chromatography (HPLC) vials.

Mobile phases consisted of solution A (HPLC grade water with 0.1% (v/v) formic acid) and solution B (100% acetonitrile with 0.1% (v/v) formic acid), maintained at a flow rate of 0.3 ml/min, injected onto a Zorbax® SB C18 column (Agilent Santa Clara, U.S.A.) having length, internal diameter, and particle size dimensions of 50 mm, 2.1 mm, and 1.8 µm respectively. Reversed-phase gradient elution was used, comprising of 45% Solution B for the first minute, then linearly increased to 100% until 5 minutes into the run, which was then maintained until the assay run finished at 15 min. A further 5 min was used to re-equilibrate the column at 45% Solution B before the next injection.

AEA, 2-AG, PEA and OEA eluted at the following retention times: 6.7 min, 7.0 min, 7.1 min and 7.3 min, respectively. Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1260 infinity 2 HPLC system coupled to a SCIEX QTRAP 4500 mass spectrometer operated in triple quadrupole mode (SCIEX Ltd., U.K.). Instrument conditions were optimised for each analyte by infusing standards separately.

2.8.5 QUANTIFICATION OF ANALYTES AND ANALYSIS OF ANALYTE CONCENTRATIONS

Quantitation of target endocannabinoids was achieved by positive ion electrospray ionization and multiple reaction monitoring (MRM) mode, allowing simultaneous detection of the protonated precursor and product molecular ions [M+ H+] of the analytes of interest and the deuterated forms of the internal standards.

Ratiometric analysis was performed using Skyline Quantitative Analysis Software, version 21.1 (MacCoss Lab Software, University of Washington, U.S.A.). The amount of analyte in unknown samples was calculated from the analyte/internal standard peak area response ratio using a 10-point calibration curve constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard (Figure 2.9 & Figure 2.10). The values obtained were initially expressed in ng per mg of tissue by dividing by the weight of the punched tissue. To express values as nmol or pmol per mg the corresponding values were then divided by the molar mass of each analyte and expressed as ng/nmol or pg/pmol.

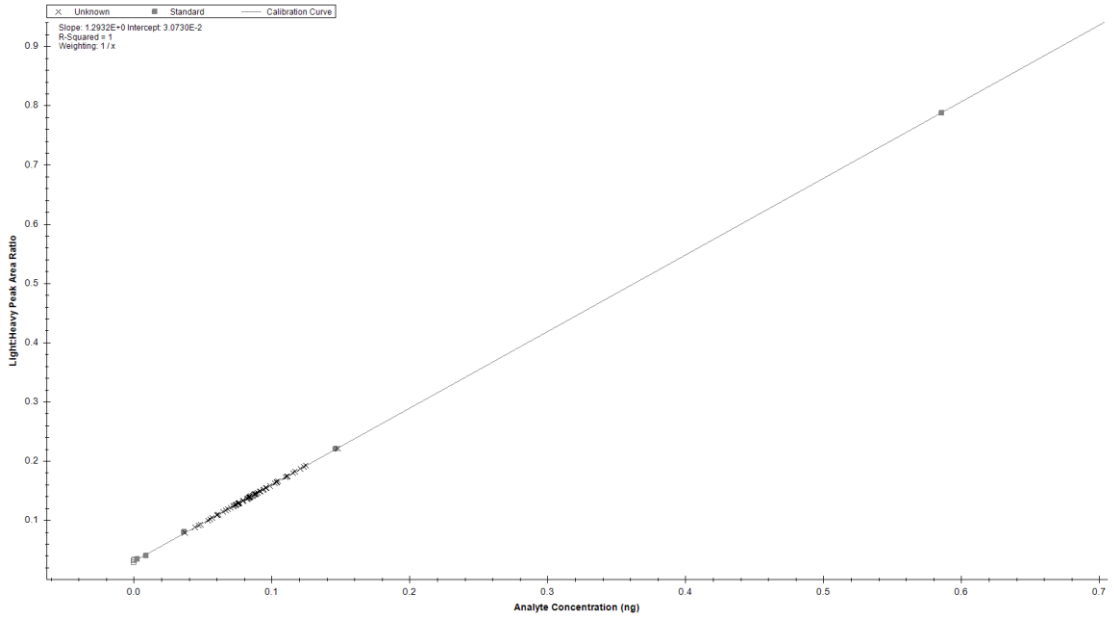

Figure 2.9 Plot of relative response vs. relative concentration for 2-AG.

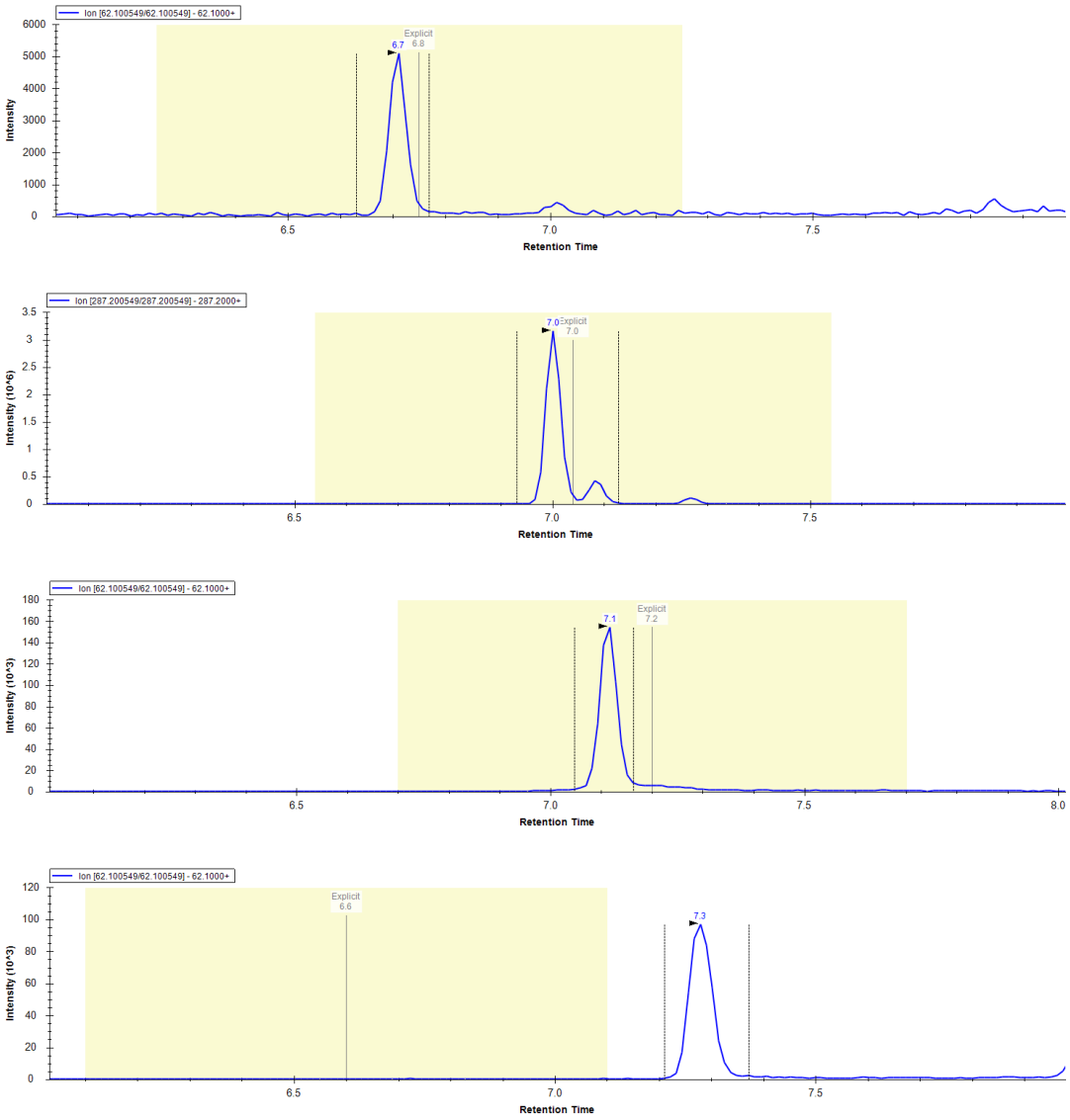

Figure 2.10 Representative chromatographs of each analyte – AEA, 2-AG, PEA and OEA.

2.9 STATISTICAL ANALYSIS

Statistical analyses were completed using GraphPad Prism software (Version 9.1.2.). Prior to analysis, data were tested for normality using Shapiro-Wilk's test and homogeneity of variance by Brown-Forsythe's test to determine if the data were parametric or non-parametric. All parametric data were expressed as mean ± standard error of the mean (SEM). As the vast majority of the data were parametric, one-way ANOVA (Analysis of Variance) was used in all instances to compare the mean of more

than two groups on one factor, with *post hoc* Tukey analysis used to determine where the difference lay between groups. Two-way ANOVA was used to compare the means of two or more groups on two factors simultaneously, with *post hoc* Tukey analysis. Behavioural data were analysed using a two-way ANOVA repeated-measures (with within subject-subject factor of time and between subject-factor of group) with *post hoc* Tukey analysis. Any other test used is mentioned at the relevant point in the text. Results were deemed significant if $P<0.05$. Throughout the result chapters, the main outcomes from the ANOVA analyses are given in the text while the outcome of any *post hoc* analyses are shown in the relevant figure and explained in the corresponding legend.

Chapter 3: Characterisation of the endocannabinoid system in an α-synuclein overexpression model of Parkinson's disease

3.1 INTRODUCTION

Parkinson's disease is a chronic, progressive neurodegenerative disease. Clinically, it manifests primarily as a motor disorder, with patients presenting with bradykinesia, resting tremor, rigidity and postural instability (Sveinbjornsdottir, 2016). Pathologically, Parkinson's disease is characterised by the death of the nigrostriatal dopaminergic neurons and the formation of abnormal intracellular protein aggregates termed Lewy bodies, the principal component of which is α-synuclein protein (Spillantini *et al.*, 1997). Further to this, it has emerged in recent decades that neuroinflammation is also a crucial component of Parkinson's disease pathophysiology. In the neurodegenerative disease state, microglia, the resident immune cells of the brain, become chronically activated and release an excess of cytotoxic factors which induce further neuronal death, thus initiating a self-sustaining cycle of neuroinflammation and neurodegeneration (Frank-Cannon *et al.*, 2009; Tansey and Goldberg, 2010). Therefore, the concept of anti-inflammatory therapy for Parkinson's disease emerged, to potentially interrupt this cycle and halt the progression of the disease.

In recent years, the immunomodulatory cannabinoid CB_2 receptor has garnered considerable interest as a potential target for anti-inflammatory disease modulation. This is by virtue of its presence on microglial cells, and the pronounced increase in CB_2 expression that has been observed in neurodegenerative disease patients, in addition to in numerous animal models of these disorders (reviewed in Kelly *et al.*, 2020). In 2003, Benito and colleagues noted an increased expression of the CB_2 receptor on the microglia surrounding neuritic plaques in Alzheimer's disease patients (Benito *et al.*, 2003). In the years since, upregulation of the receptor on glial cells in brain regions crucial to Parkinson's disease pathology has also been observed (Gómez-Gálvez *et al.*, 2016; Navarrete *et al.*, 2018). Furthermore, increases in CB_2 receptor expression have been detected in assorted Parkinsonian models, including neurotoxin models such as the 6-OHDA and rotenone models, in addition to inflammatory models such as the LPS and poly I:C models (García *et al.*, 2011; Concannon *et al.*, 2015b; Concannon *et al.*, 2016).

Interestingly, a more pronounced upregulation was observed in the inflammatory models relative to the neurotoxic models. Clearly, the relationship between the CB_2 receptor and the pathology of Parkinson's disease is complex, and thus it is imperative that we investigate the links between the alterations in CB_2 receptor expression and the pathological events of Parkinson's disease, in order to more comprehensively understand the feasibility of this receptor as a target for anti-inflammatory disease modification.

However, as of yet, little is known about the alterations in CB_2 receptor expression in the highly relevant AAV-α-synuclein animal model. This model involves the intracerebral administration of a viral vector that causes the overexpression of human α-synuclein, a principal constituent of the pathological Lewy body aggregates (Spillantini *et al.*, 1997). Deniz Kirik and co-workers (Kirik *et al.*, 2002) were amongst the first to attempt this viral vector-mediated approach to develop an α-synuclein model. They injected an adeno-associated viral (AAV) vector that expressed either wild-type or A53T mutated human α-synuclein into the substantia nigra of rats. They observed a loss of dopaminergic cell bodies in the substantia nigra in addition to a reduction in striatal dopamine, which was accompanied by motor dysfunction in animals in which a critical threshold of 50-60% loss of neurons was reached. They also noted the presence of intracellular aggregates that stained positive for α-synuclein, in addition to dystrophic neurites similar to those seen in Parkinson's disease patients. Subsequently, AAV vectors have been used to successfully produce both mouse (St Martin *et al.*, 2007; Ip *et al.*, 2017) and primate α-synuclein models (Kirik *et al.*, 2003; Eslamboli *et al.*, 2007). Lentiviral vectors have also been utilised but to a lesser extent, as they are not as effective at inducing transgene expression and causing dopaminergic degeneration (Lo Bianco *et al.*, 2002; Lauwers *et al.*, 2003).

This viral vector-mediated α-synuclein overexpression model is one of the best currently available with regard to recapitulating the pathophysiology of Parkinson's disease. The gradual progression of this model more accurately represents the normal disease course compared to other neurotoxic models, which typically induce rapid and extensive degeneration of the nigrostriatal dopamine neurons. Furthermore, many neurotoxic models do not incorporate α-synuclein pathology and do not present with intracellular aggregates. Despite the validity of this model, our comprehension regarding the relationship between the levels of endocannabinoid system components and the

progression of this model is scant. It is imperative to understand the temporal alterations in the expression of the CB_2 receptor and other constituents of the cannabinoid system in order to fully discern the link between the receptor and the disease state, and accordingly to determine the feasibility of targeting of the CB_2 receptor for anti-inflammatory disease modification.

Taking the aforementioned evidence into consideration, we hypothesised that CB_2 receptor expression would be increased in the AAV-α-synuclein animal model of Parkinson's disease. Therefore, the aim of this chapter was to examine the temporal alterations in the expression of the cannabinoid CB_2 receptor, as well as other components of the cannabinoid system, in the highly relevant viral-mediated α-synuclein overexpression rat model.

3.2 METHODS AND MATERIALS

All methods are described in detail in Chapter 2.

3.2.1 EXPERIMENTAL DESIGN

The CB_2 receptor is recognised to be intricately associated with the neuroinflammatory aspect of Parkinson's disease pathology, but decidedly little is known about the alterations in the expression of this receptor in the viral-mediated α-synuclein overexpression model. The work presented in this chapter assessed the temporal levels of components of the cannabinoid system in the AAV-α-synuclein rat model of Parkinson's disease. We used qRT-PCR analyses to assess the expression of these constituents as the validity of the currently available CB_2 receptor antibodies remains contentious due to their non-specificity (Baek *et al.*, 2013; Marchalant *et al.*, 2014).

To investigate this, 56 female rats (born in-house) received dual injections unilaterally into the substantia nigra (at the stereotaxic coordinates: AP -4.8 & -5.8, ML -2.0, DV -7.2) of an AAV vector expressing either human α-synuclein with the A53T mutation or green fluorescent protein (GFP) driven by the PGK promoter (3.98×10^{10} vg per site, with two sites per rat (7.96×10^{10} vg total)). In order to assess lateralised motor function, animals underwent Corridor, Stepping and Whisker behavioural tests throughout the study. Rats were subsequently sacrificed for qRT-PCR analyses of the expression levels of the CB_2 receptor and other cannabinoid system genes in the substantia nigra and the striatum, as well as for α-synuclein expression and expression of microglial and astrocytic markers at 4, 8 and 12 weeks post-surgery (AAV-GFP: n=5 per time-point; AAV-α-synuclein: n=8-9 per time-point). Striatal punches from these rats were also used to assess alterations in endocannabinoid levels. In addition, 2 rats (AAV-GFP) or 3 rats (AAV-α-synuclein) were sacrificed by transcardial perfusion per time-point for histological visualisation of α-synuclein expression, neurodegeneration and neuroinflammation in the substantia nigra and the striatum.

3.3 RESULTS

3.3.1 UNILATERAL, INTRA-NIGRAL AAV-ALPHA-SYNUCLEIN DID NOT INDUCE CONTRALATERAL MOTOR IMPAIRMENT

Before embarking on qRT-PCR analyses, which was the primary focus of this research, we first sought to determine if the administration of AAV-α-synuclein induced motor impairments in the animals. The surgeries were performed unilaterally so any motor dysfunction would be restricted to one side of the body (contralateral to the side of the brain injected) and thus the other side could serve as an internal control.

As expected, there were no differences in ipsilateral motor function between groups in the Corridor test of sensorimotor neglect (Figure 3.1A; Group x Time, $F_{(3,51)}$=0.40, P>0.05), the Stepping test of forelimb akinesia (Figure 3.1B; Group x Time, $F_{(3,51)}$=0.99, P>0.05), or the Whisker test of sensorimotor integration (Figure 3.1C; Group x Time, $F_{(3,51)}$=1.98, P>0.05). In the Stepping test, there was a significant effect of time with the average number of steps decreasing over time in all groups, suggesting a training effect as the animals became more accustomed to the handling (Figure 3.1B; Time, $F_{(3,51)}$=54.84, P<0.05).

Importantly, in this study, the AAV-α-synuclein vector did not induce contralateral motor impairments in the Corridor (Figure 3.2A; Group x Time, $F_{(3,51)}$=0.40, P>0.05), Stepping (Figure 3.2B; Group x Time, $F_{(3,51)}$=0.69, P>0.05) or Whisker (Figure 3.2C; Group x Time, $F_{(3,51)}$=0.43, P>0.05) behavioural tests at any time-point. A significant effect of time was also observed in the contralateral Stepping test (Figure 3.2B; Time, $F_{(3,51)}$=14.77, P<0.05), which reinforces that there may be a training effect.

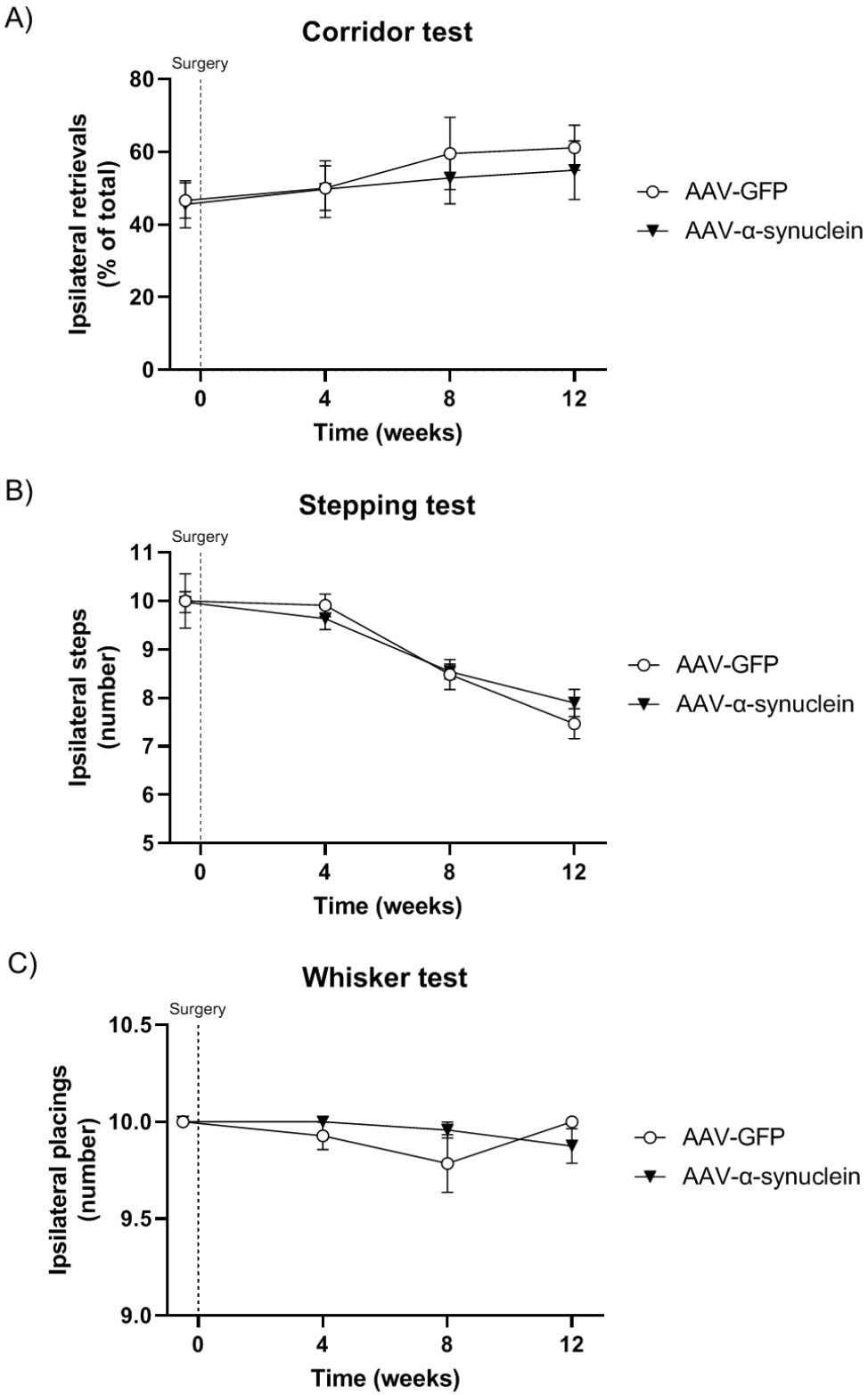

Figure 3.1 Impact of AAV-α-synuclein on ipsilateral motor function. Unilateral administration of AAV-α-synuclein did not induce ipsilateral motor dysfunction in the Corridor (A), Stepping (B), or Whisker (C) behavioural tests. Data are represented as mean ± SEM with n=7-12 per group and were analysed by two-way repeated-measures ANOVA. Dashed lines represent the day of infusion surgeries.

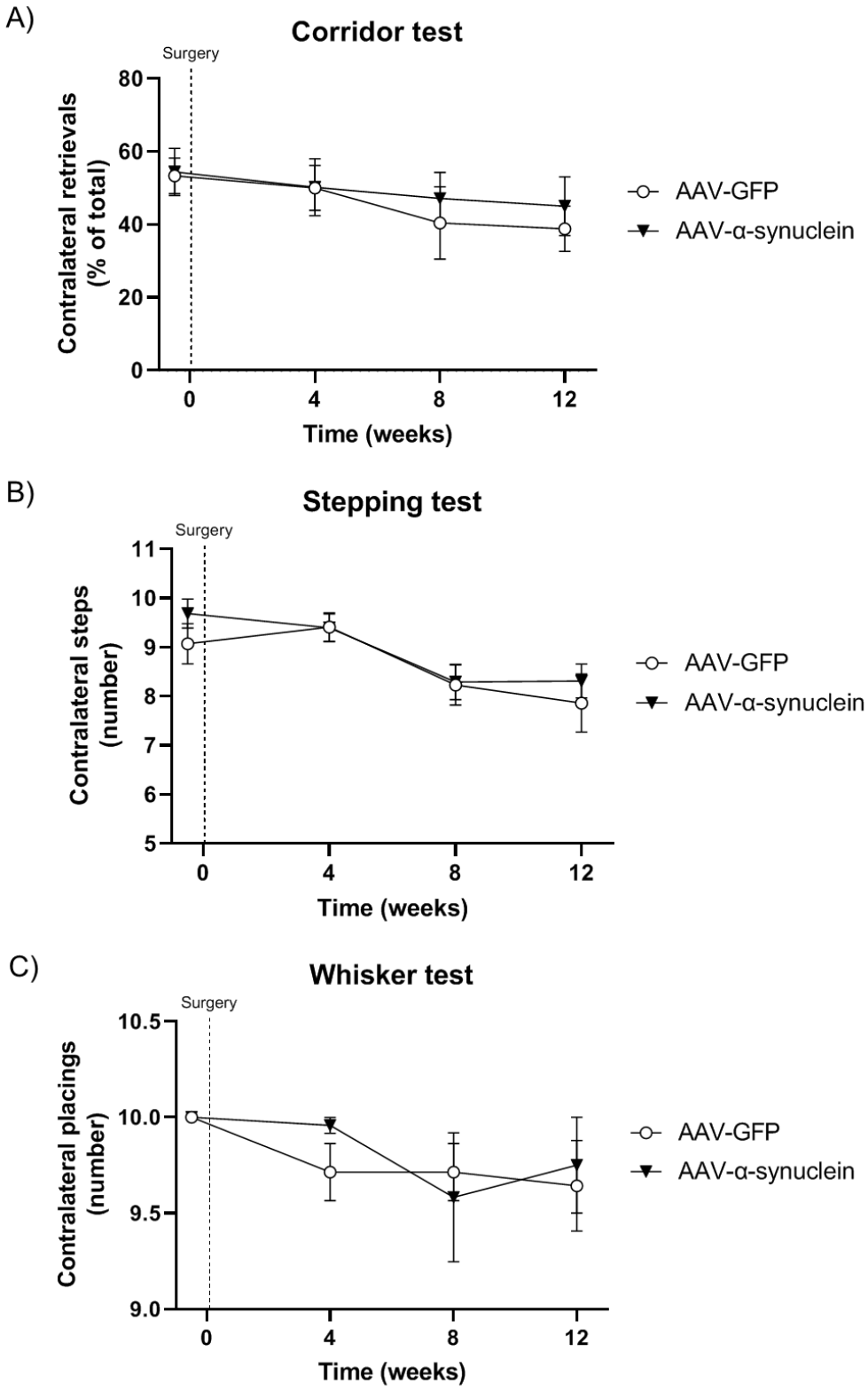

Figure 3.2 Impact of AAV-α-synuclein on contralateral motor function. Unilateral administration of AAV-α-synuclein did not induce contralateral motor dysfunction in the Corridor (A), Stepping (B) or Whisker (C) behavioural tests. Data are represented as mean ± SEM with n=7-12 per group and were analysed by two-way repeated-measures ANOVA. Dashed lines represent the day of infusion surgeries.

3.3.2 SEMI-QUANTITATIVE ANALYSES OF ALPHA-SYNUCLEIN EXPRESSION IN THE NIGROSTRIATAL PATHWAY

Once behavioural analyses were complete and the animals were sacrificed, *post mortem* we firstly wished to visually confirm the presence of human α-synuclein in the nigrostriatal pathway. Immunohistochemical staining for human α-synuclein revealed that, as anticipated, intra-nigral administration of AAV-α-synuclein, but not AAV-GFP, induced the widespread expression of human α-synuclein protein in the substantia nigra and across the midbrain (Figure 3.3; Group, $F_{(1,9)}$=14.49, P<0.05). We also sought to investigate if there were differences in the expression of this protein in the nerve terminals of the striatum. Similarly to the substantia nigra, α-synuclein staining is clearly evident in the striatum on the side of the brain ipsilateral to the lesion in the rats which received AAV-α-synuclein but not in the animals which received AAV-GFP (Figure 3.4; Group, $F_{(1,8)}$=18.90, P<0.05). In both brain regions while an overall group effect was detected, *post hoc* tests did not detect any significant differences between groups.

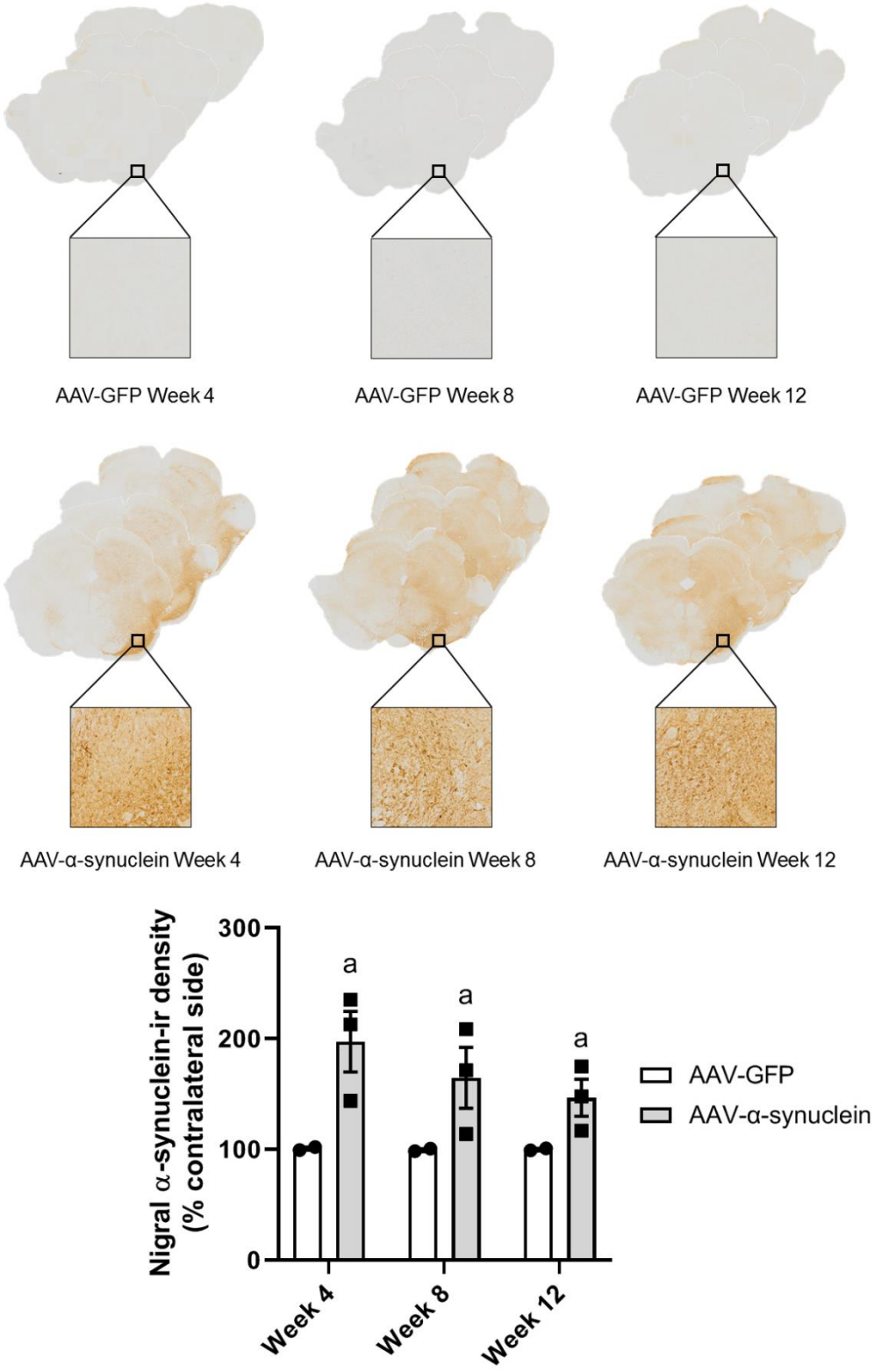

Figure 3.3 Semi-quantitative analyses of the impact of AAV-α-synuclein on the expression of human α-synuclein in the substantia nigra. Animals that received AAV-α-synuclein expressed human α-synuclein protein in the substantia nigra, but animals that received AAV-GFP did not. Data are represented as mean ± SEM with 2-3 animals per group and were analysed by two-way ANOVA with post hoc Tukey. 'a' indicates an overall group effect..

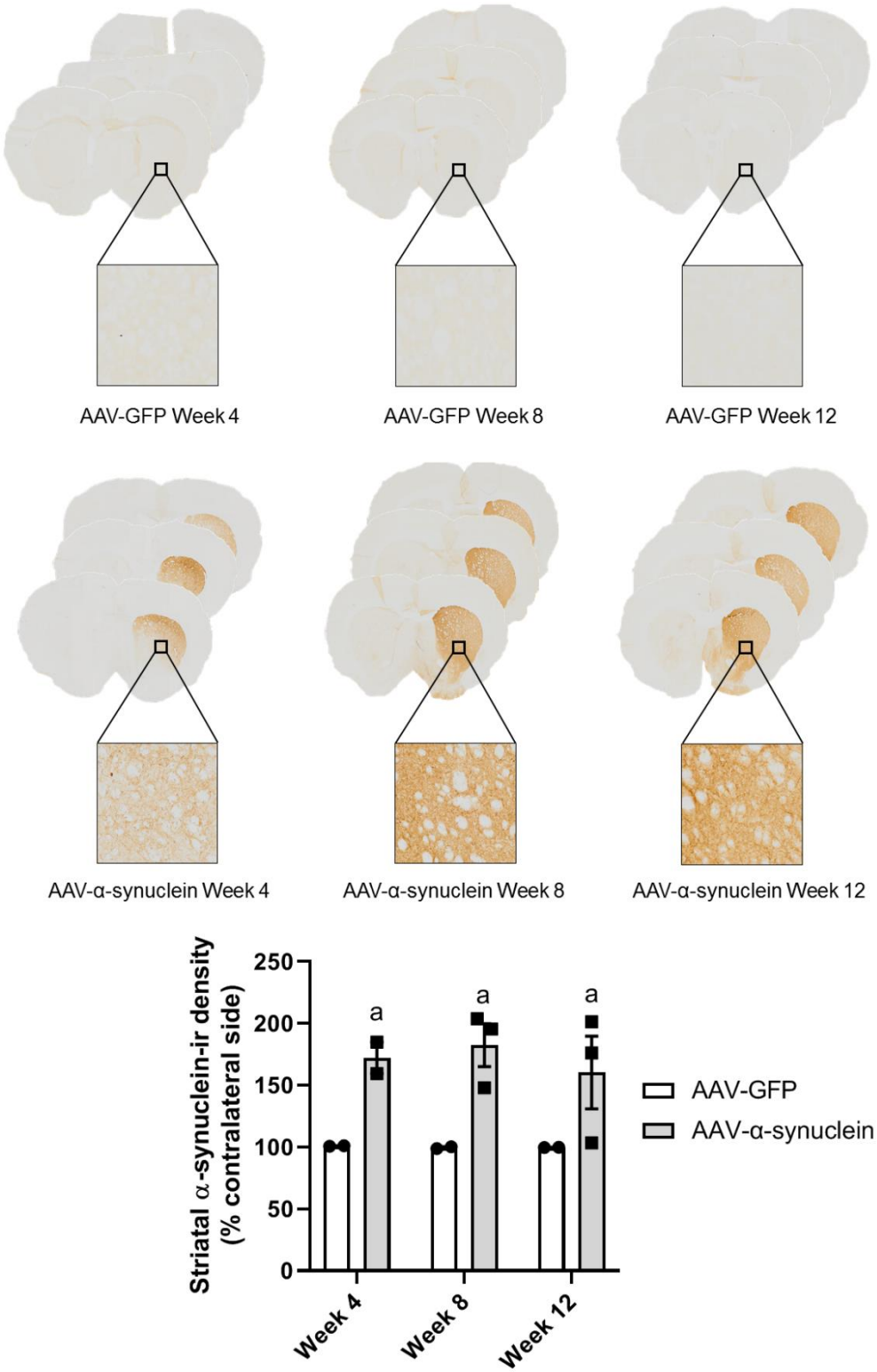

Figure 3.4 Semi-quantitative analyses of the impact of AAV-α-synuclein on the expression of human α-synuclein in the striatum. Animals that received AAV-α-synuclein expressed human α-synuclein protein in the striatum, whereas animals that received AAV-GFP did not. Data are represented as mean ± SEM with 2-3 animals per group were analysed by two-way ANOVA with post hoc Tukey. 'a' indicates an overall group effect..

3.3.3 SEMI-QUANTITATIVE ANALYSES OF THE INTEGRITY OF NIGROSTRIATAL DOPAMINE NEURONS

Following our visual confirmation that human α-synuclein was overexpressed in the nigrostriatal pathway, we continued with our immunohistological analyses. We carried out tyrosine hydroxylase (TH) staining to determine if administration of AAV-α-synuclein induced conspicuous degeneration of dopaminergic cell bodies in the substantia nigra, or the loss of the dopaminergic nerve terminals in the striatum. Despite the α-synuclein overexpression, there was no overt differences between groups at any time-point with regard to the staining in the counts of TH-immunoreactive cell bodies in the substantia nigra (Figure 3.5; Group, $F_{(1,9)}$=1.29, P>0.05) or the optical density of TH staining in the striatum (Figure 3.6; Group, $F_{(1,8)}$=3.28, P>0.05).

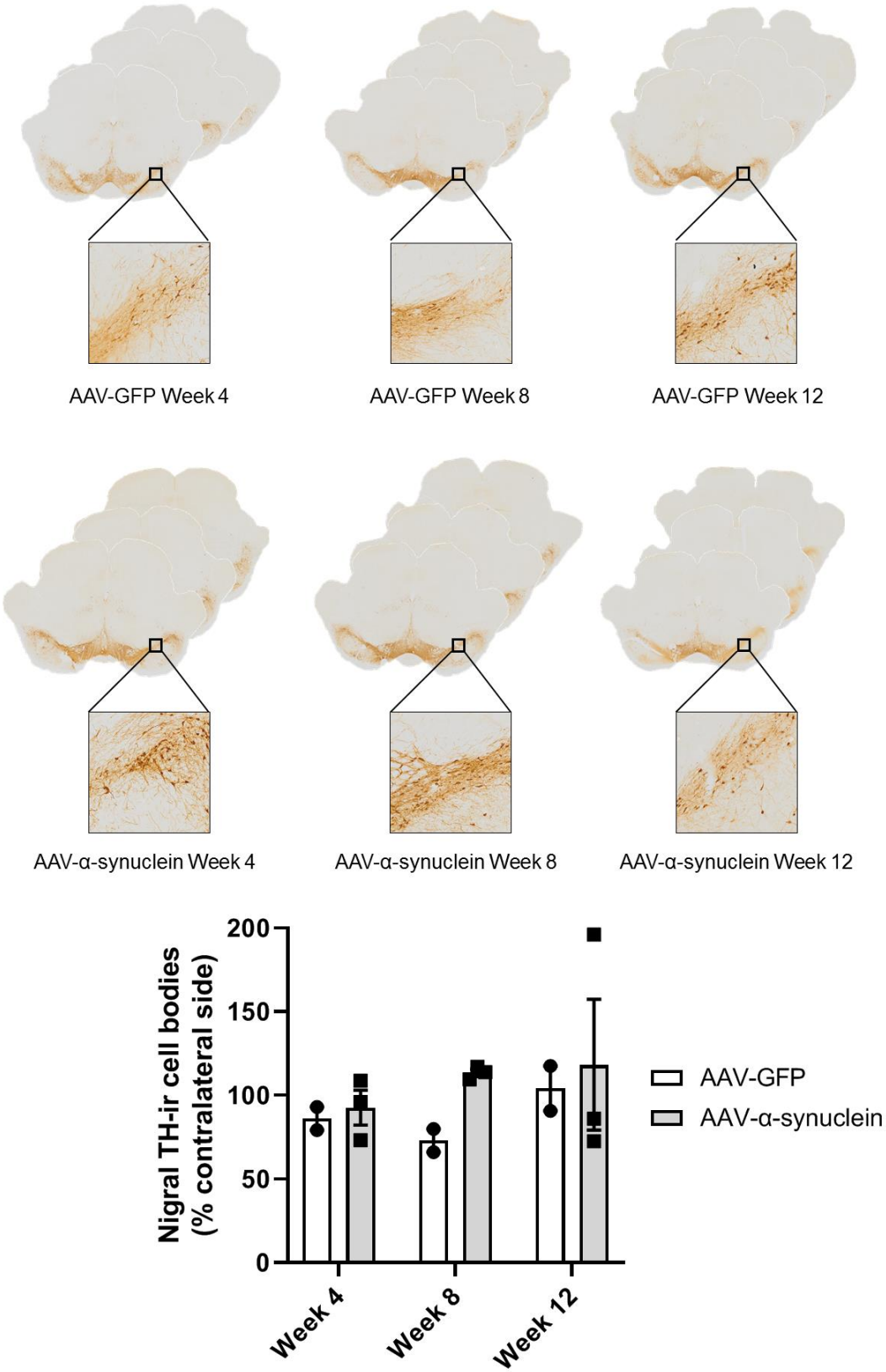

Figure 3.5 Semi-quantitative analyses of the impact of AAV-α-synuclein on nigral cell counts. AAV-α-synuclein did not lead to any overt changes in the number of TH-immunoreactive cells in the substantia nigra. Data are represented as mean ± SEM with 2-3 animals per group and were analysed by two-way ANOVA.

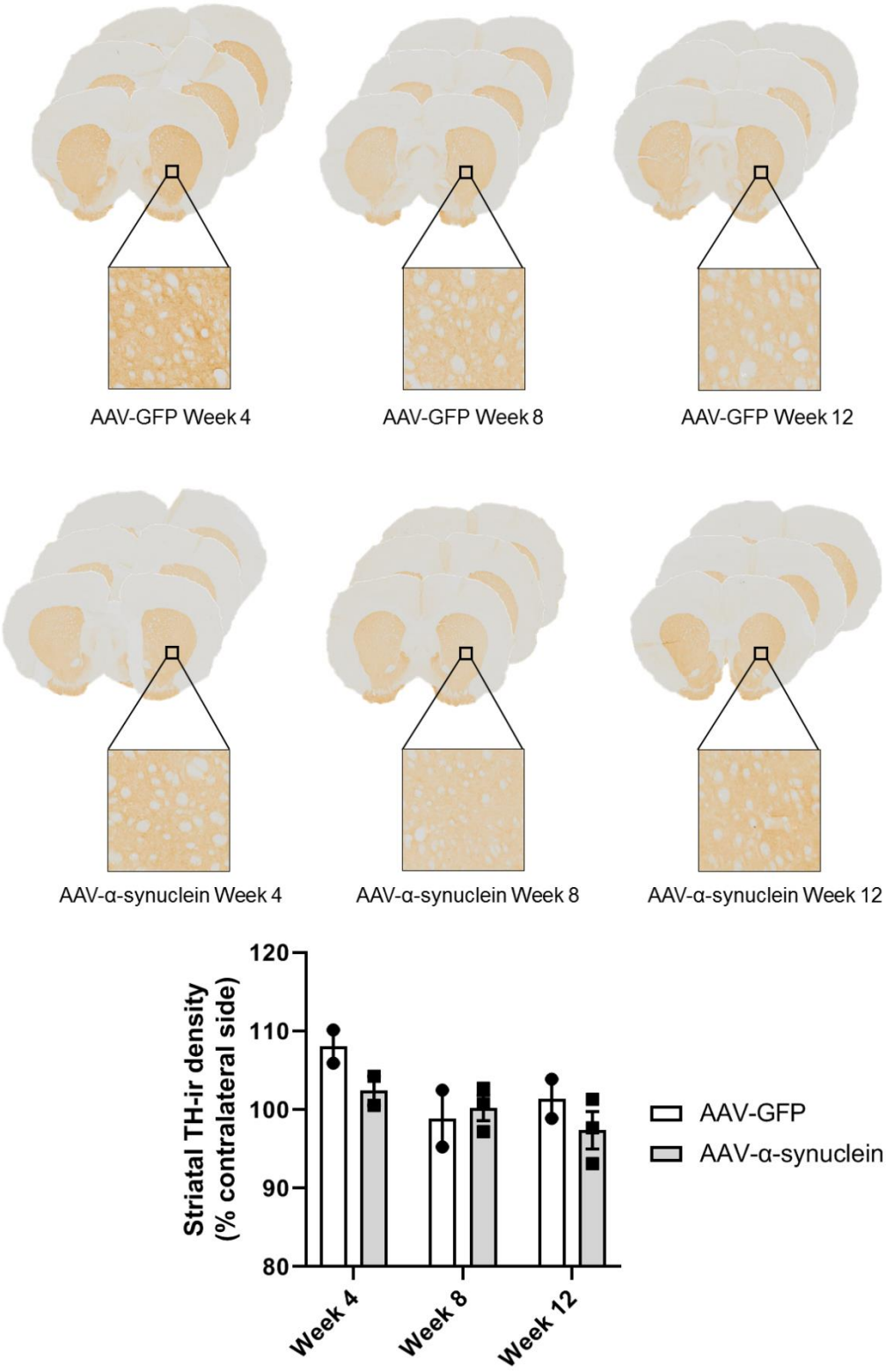

Figure 3.6 Semi-quantitative analyses of the impact of AAV-α-synuclein on striatal terminals. AAV-α-synuclein did not induce alterations in the density of TH staining in the striatum. Data are represented as mean ± SEM with 2-3 animals per group and were analysed by two-way ANOVA.

3.3.4 SEMI-QUANTITATIVE ANALYSES OF MICROGLIOSIS AND ASTROCYTOSIS IN THE NIGROSTRIATAL PATHWAY

Following our visualisation of α-synuclein expression and dopaminergic neuronal integrity in the nigrostriatal pathway, we also carried out immunostaining for microglial and astrocytic cells to determine if AAV-α-synuclein induced a neuroinflammatory response in the substantia nigra or the striatum. However, despite the pronounced overexpression of α-synuclein in the nigrostriatal pathway, there was no overt microgliosis in either the substantia nigra (Figure 3.7A; Group, $F_{(1,9)}$=3.40, P>0.05) or the striatum (Figure 3.7B; Group, $F_{(1,8)}$=2.79, P>0.05) at any time-point. Similarly, there were no obvious differences in astrocytic density in the substantia nigra (Figure 3.8A; Group, $F_{(1,9)}$=3.536, P>0.05) or the striatum (Figure 3.8B; Group, $F_{(1,8)}$=0.2176, P>0.05).

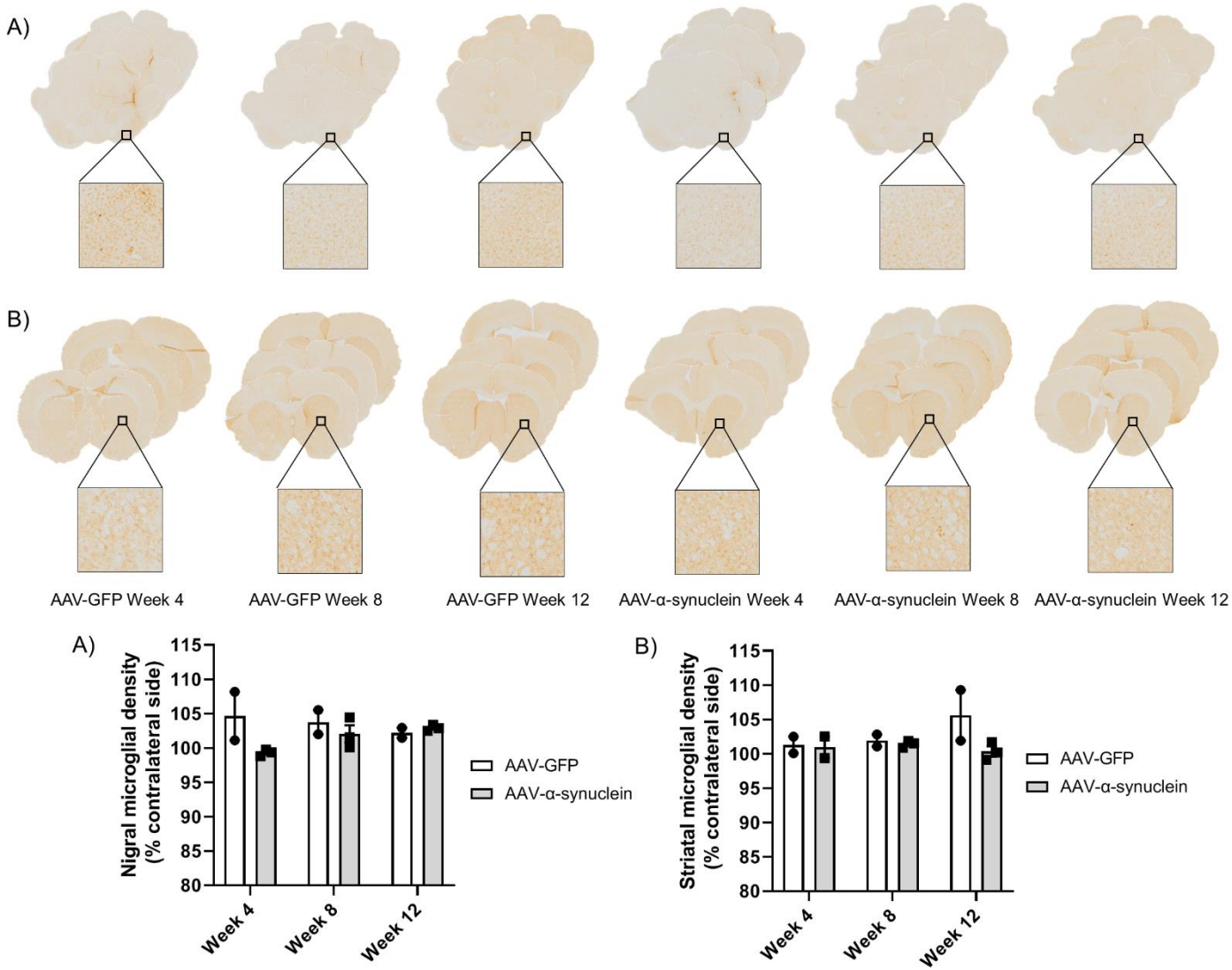

Figure 3.7 Semi-quantitative analyses of the impact of AAV-α-synuclein on microglial density in the nigrostriatal pathway. AAV-α-synuclein did not affect the optical density of OX-42 staining in the substantia nigra (A) or in the striatum (B). Data are represented as mean ± SEM with n=2-3 animals per group and were analysed by two-way ANOVA.

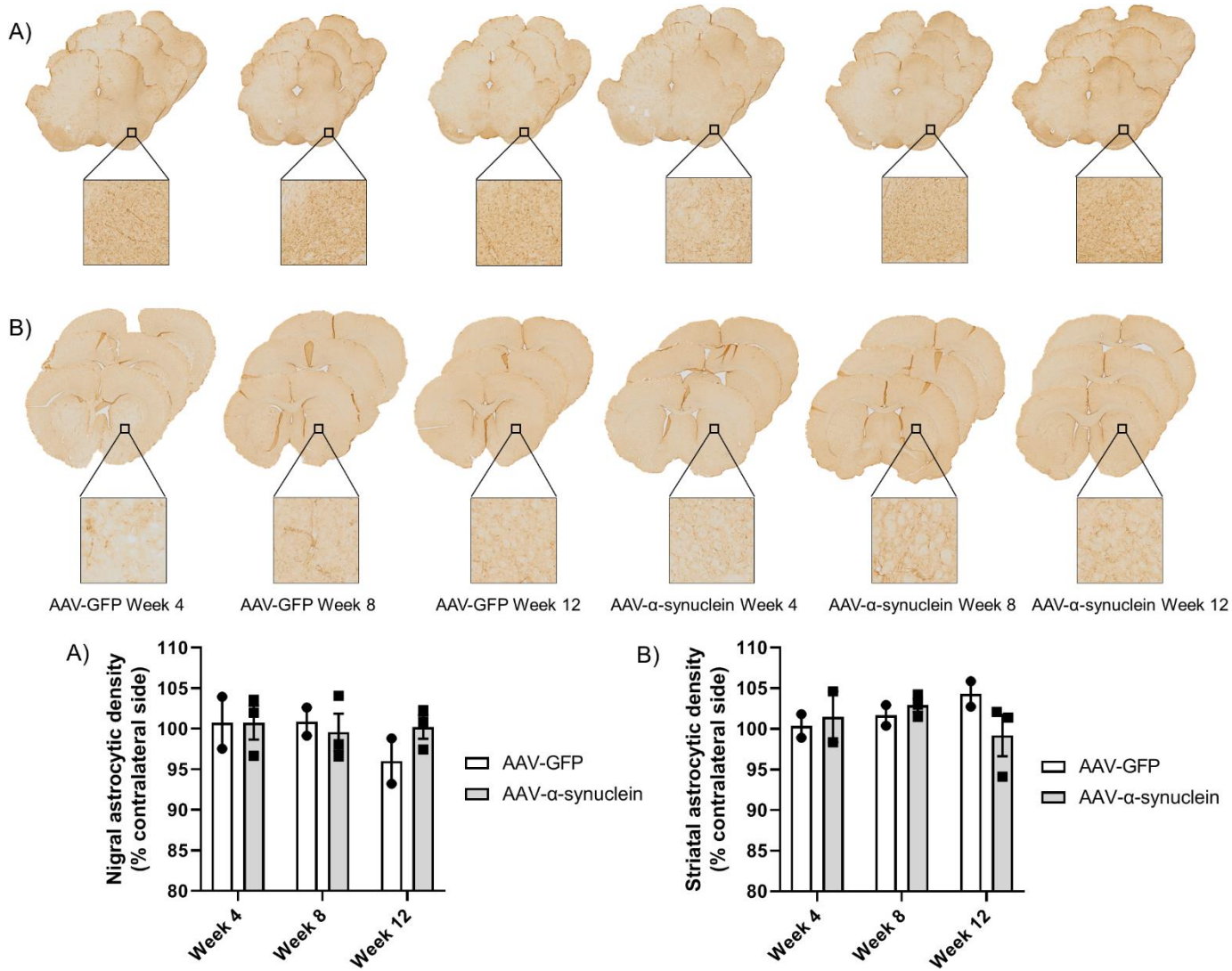

Figure 3.8 Semi-quantitative analyses of the impact of AAV-α-synuclein on astrocytic density in the nigrostriatal pathway. AAV-α-synuclein did not affect the optical density of GFAP staining in the substantia nigra (A) or in the striatum (B). Data are represented as mean ± SEM with n=2-3 animals per group and were analysed by two-way ANOVA.

3.3.5 AAV-ALPHA-SYNUCLEIN ADMINISTRATION LED TO INCREASED EXPRESSION OF HUMAN ALPHA-SYNUCLEIN

Following the behavioural analyses and the immunohistological visualisation of some of the alterations in the nigrostriatal pathway, we began our qRT-PCR analyses by quantification of the alterations of human α-synuclein (SNCA) expression at the gene level in the nigrostriatal pathway. The expression of this gene was normalised to the expression of β-actin, our housekeeping gene. Because there were large differences in the expression levels between animals, we decided to log this data to make it simpler to visualise and for ease of analysis. Administration of AAV-α-synuclein resulted in a significant overexpression of human α-synuclein in the substantia nigra compared to the rats which received GFP (Figure 3.9A; Group, $F_{(1,32)}$=166.80, P<0.05). In the striatum, α-synuclein was similarly overexpressed (Figure 3.9B; Group, $F_{(1,29)}$=77.82, P<0.05).

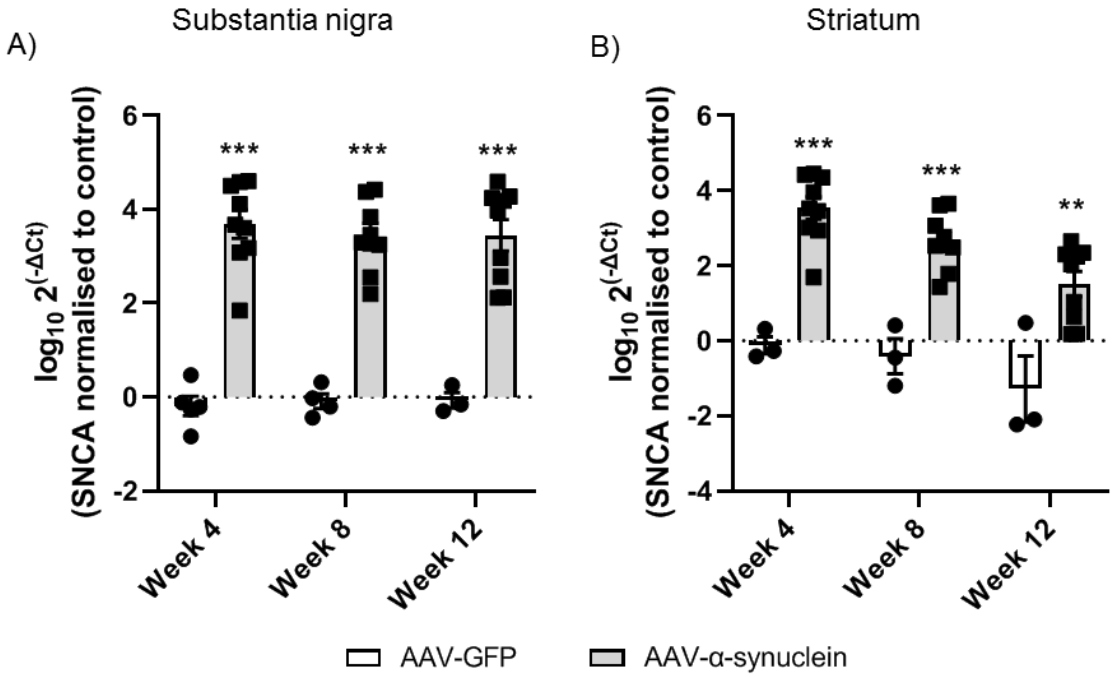

Figure 3.9 qRT-PCR analyses of the human α-synuclein gene in the nigrostriatal pathway. The administration of AAV-α-synuclein induced a significant upregulation in the expression of human α-synuclein in the substantia nigra (A), and in the striatum (B) across all time-points. Data are represented as mean ± SEM with 3-9 animals per group. **P<0.01, ***P<0.001 vs. corresponding AAV-GFP group by two-way ANOVA with *post hoc* Tukey.

3.3.6 AAV-ALPHA-SYNUCLEIN INDUCED A REDUCTION IN MICROGLIAL AND ASTROCYTIC MARKER GENE EXPRESSION IN THE SUBSTANTIA NIGRA

Continuing with our analyses of gene expression, we proceeded to assess and compare temporal changes in the expression of neuroinflammatory markers in the nigrostriatal pathway, as neuroinflammation has been acknowledged to be intricately linked to the pathophysiology of Parkinson's disease.

We appraised temporal changes in the expression of the microglial marker, CD11b, and of the astrocytic marker, GFAP, in nigral and striatal tissue by qRT-PCR. Surprisingly, an overall reduction in mRNA levels of CD11b was identified in the substantia nigra of AAV-α-synuclein animals compared to control animals (Figure 3.10A; Group, $F_{(1,35)}$=4.69, P<0.05), although this was not found to be significant at any particular time-point in the *post hoc* test. Contrastingly, no differences in CD11b expression were identified in the striatum (Figure 3.10B; Group, $F_{(1,33)}$=0.29, P>0.05).

Similar results were seen with regard to the expression of the astrocytic marker GFAP. AAV-α-synuclein induced an overall decrease in GFAP expression in the substantia nigra (Figure 3.10A; Group, $F_{(1,35)}$=4.16, P<0.05), but this did not reach significance at any particular time-point in the *post hoc* Tukey test. No alterations were detected in the expression levels of GFAP in the striatum (Figure 3.10B; Group, $F_{(1,31)}$=0.26, P>0.05).

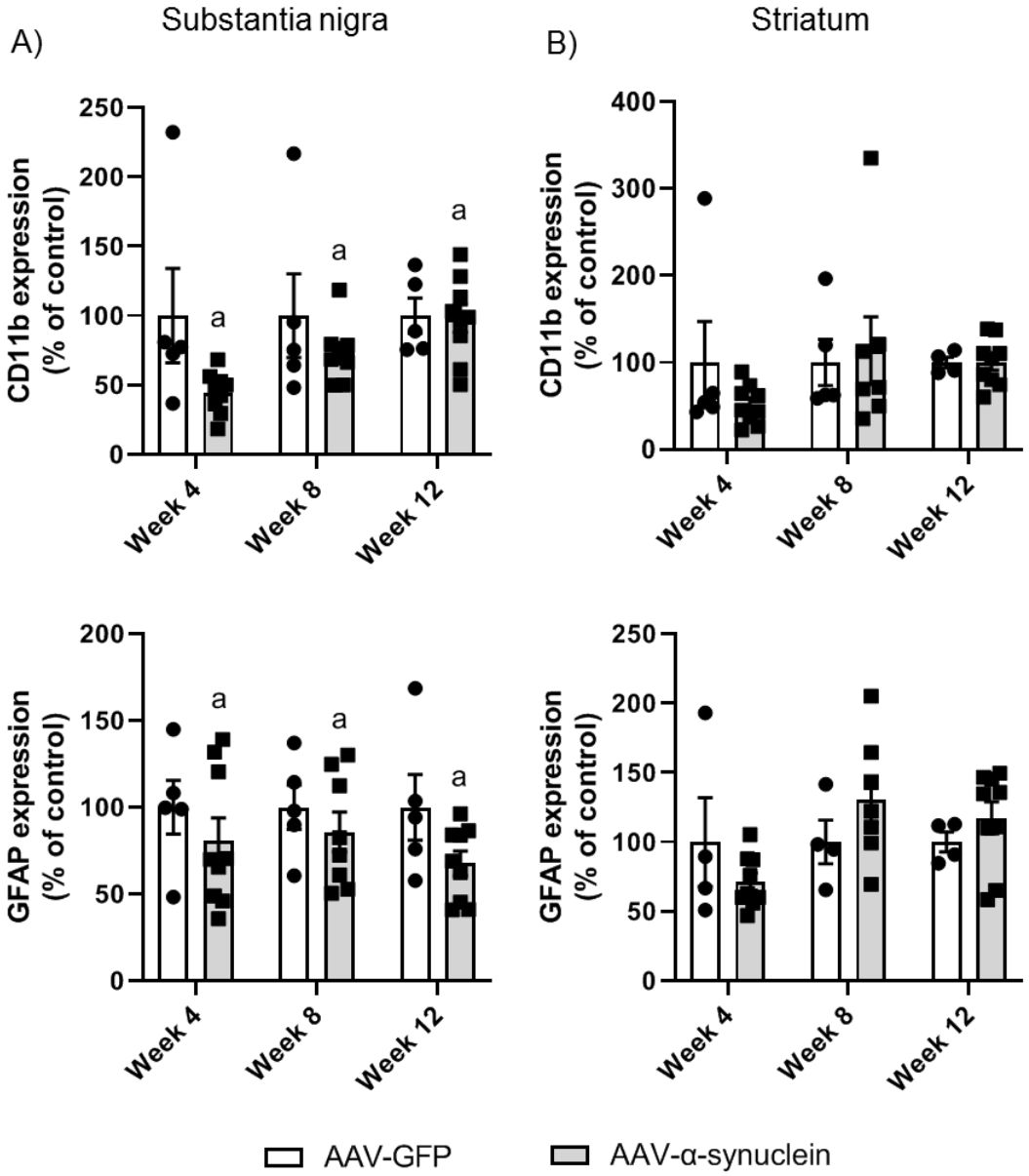

Figure 3.10 qRT-PCR analyses of astrocytic and microglial markers in the nigrostriatal pathway. In the substantia nigra (A), AAV-α-synuclein administration led to an overall reduction in the expression of the microglial marker CD11b and the astrocytic marker GFAP. There were no differences between groups in the striatum (B) with regard to either gene. Data are represented as mean ± SEM with 4-9 animals per group and were analysed by two-way ANOVA with *post hoc* Tukey. 'a' indicates an overall group effect.

3.3.7 AAV-ALPHA-SYNUCLEIN DID NOT ALTER CB_1 OR CB_2 RECEPTOR EXPRESSION IN THE NIGROSTRIATAL PATHWAY

Following our analyses of the mRNA levels of the microglial and astrocytic markers, we proceeded to determine if AAV-α-synuclein induced alterations in the expression of the cannabinoid receptors CB_1 and CB_2, which was the primary research query of this study. The expression levels of these cannabinoid receptors, particularly the CB_2 receptor, have been demonstrated to be altered in Parkinson's disease patients and in other Parkinsonian models, so we wished to determine if there were alterations in this genetic Parkinson's disease model.

In the substantia nigra, there were no differences in the levels of CB_1 expression between the two groups at any time-point (Figure 3.11A; Group, $F_{(1,35)}$=1.46, P>0.05) and similarly, the expression of the CB_2 receptor was not altered (Figure 3.11A; Group, $F_{(1,32)}$=0.61, P>0.05). In the striatum, there were no differences in the mRNA levels of either CB_1 (Figure 3.11B; Group, $F_{(1,35)}$=3.79, P>0.05) or CB_2 (Figure 3.11B; Group, $F_{(1,34)}$=2.13, P>0.05) at any time-point.

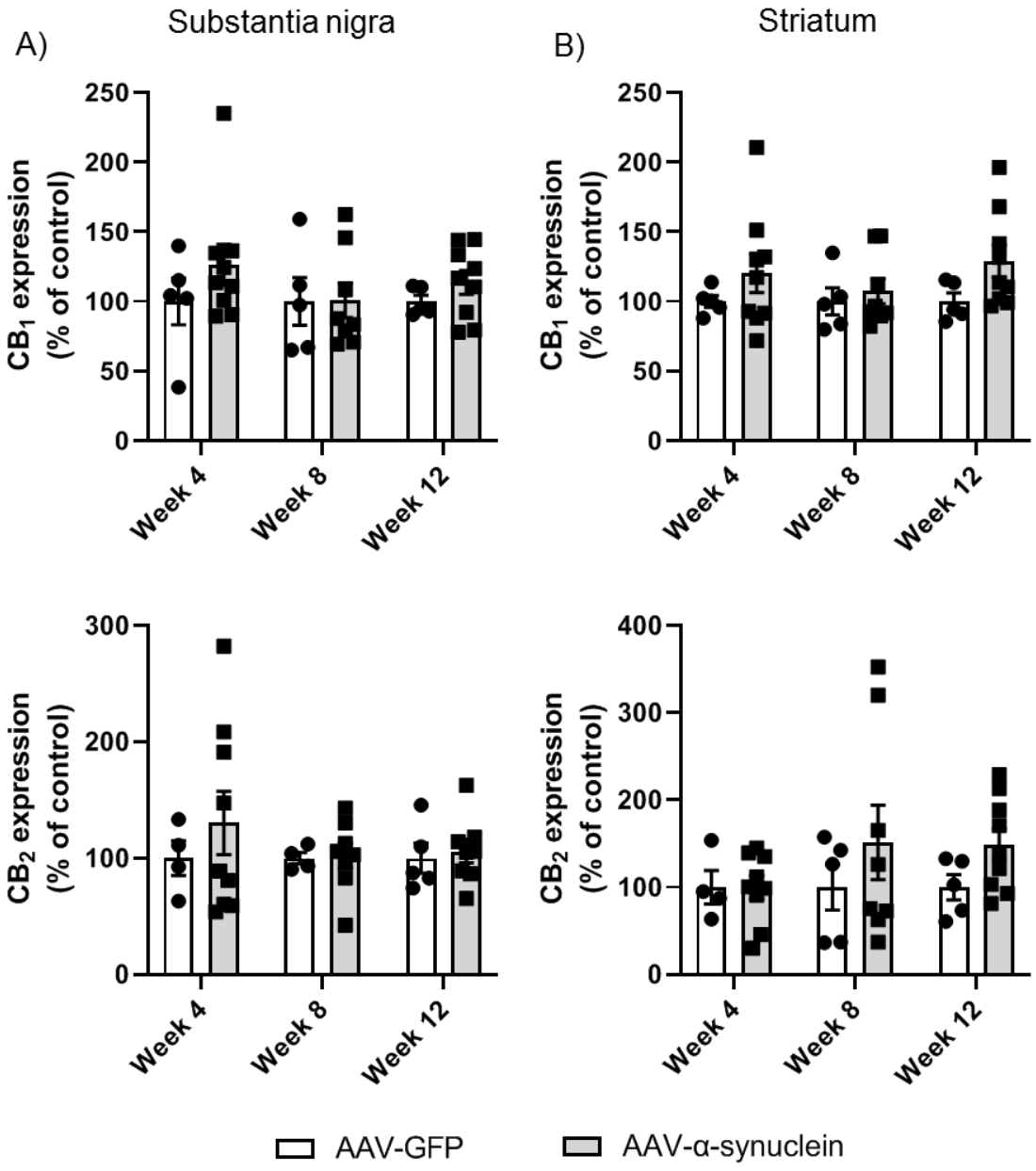

Figure 3.11 qRT-PCR analyses of cannabinoid receptor expression in the nigrostriatal pathway. There was no effect of AAV-α-synuclein expression on CB_1 or CB_2 expression in the substantia nigra (A), or of either gene in the striatum (B). Data are represented as mean ± SEM with 4-9 animals per group and were analysed by two-way ANOVA.

3.3.8 AAV-ALPHA-SYNUCLEIN DID NOT ALTER CANNABINOID ENZYME EXPRESSION IN THE NIGROSTRIATAL PATHWAY

In addition to investigating the changes in the expression of the endocannabinoid receptors, we also wished to assess the expression of endocannabinoid enzymes to determine if the levels of these enzymes involved in degradation were altered in this genetic model.

This analysis revealed that administration of AAV-α-synuclein did not alter mRNA levels of MAGL, the 2-AG degrading enzyme, or FAAH, the anandamide degrading enzyme, in either the substantia nigra (Figure 3.12A; MAGL: Group, $F_{(1,34)}$=0.44, P>0.05; FAAH: Group, $F_{(1,35)}$=0.60, P>0.05) or the striatum (Figure 3.12B; MAGL: Group, $F_{(1,35)}$=0.17, P>0.05; FAAH: Group, $F_{(1,35)}$=2.69, P>0.05).

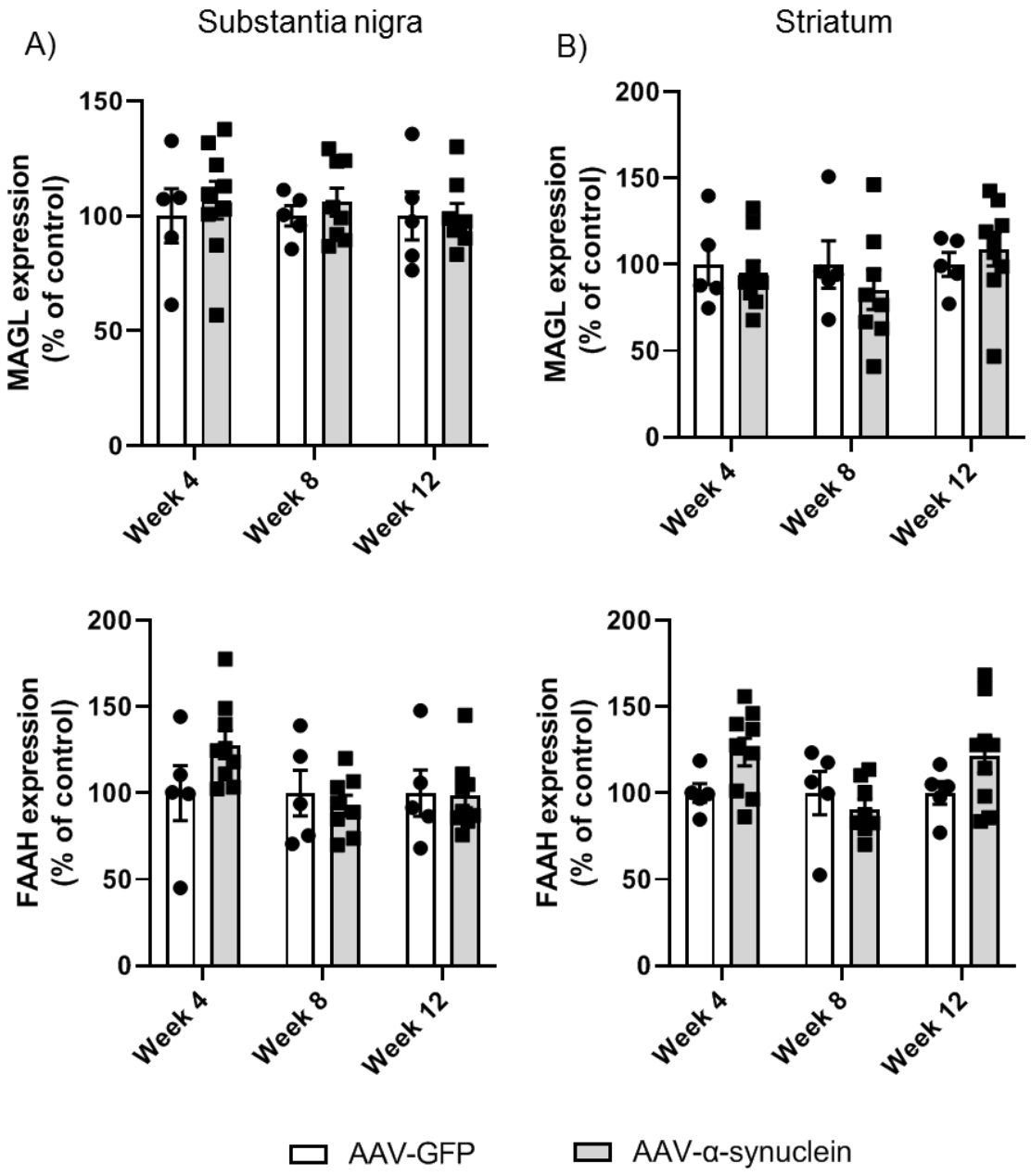

Figure 3.12 qRT-PCR analyses of the expression of endocannabinoid degrading enzymes in the nigrostriatal pathway. Administration of AAV-α-synuclein did not induce alterations in the expression of MAGL or FAAH in the substantia nigra (A) or the striatum (B). Data are represented as mean ± SEM with 5-9 animals per group and were analysed by two-way ANOVA.

3.3.9 AAV-ALPHA-SYNUCLEIN ADMINISTRATION REDUCED THE STRIATAL LEVELS OF THE ENDOCANNABINOID 2-AG

Further to our analyses of the expression of components of the cannabinoid system at the genomic level, we sought to investigate if the intra-nigral administration of AAV-α-synuclein altered striatal endocannabinoid levels. To do so, we measured striatal levels of the endocannabinoids, anandamide and 2-AG, as well as the related lipid mediators, PEA and OEA, by LC-MS/MS. Striatal levels of AEA (Figure 3.13; Group, $F_{(1,35)}$=0.83, P>0.05) and PEA (Figure 3.13; Group, $F_{(1,34)}$=3.69, P>0.05) were not altered by AAV-α-synuclein administration. While there was no overall group effect of AAV-α-synuclein observed with regard to any of the endocannabinoids, at Week 12 the levels of 2-AG (Figure 3.13; Group, $F_{(1,34)}$=5.17, P<0.05) and OEA (Figure 3.13; Group, $F_{(1,34)}$=6.17, P<0.05) were significantly lower in the AAV-α-synuclein group compared to the AAV-GFP group at that time point.

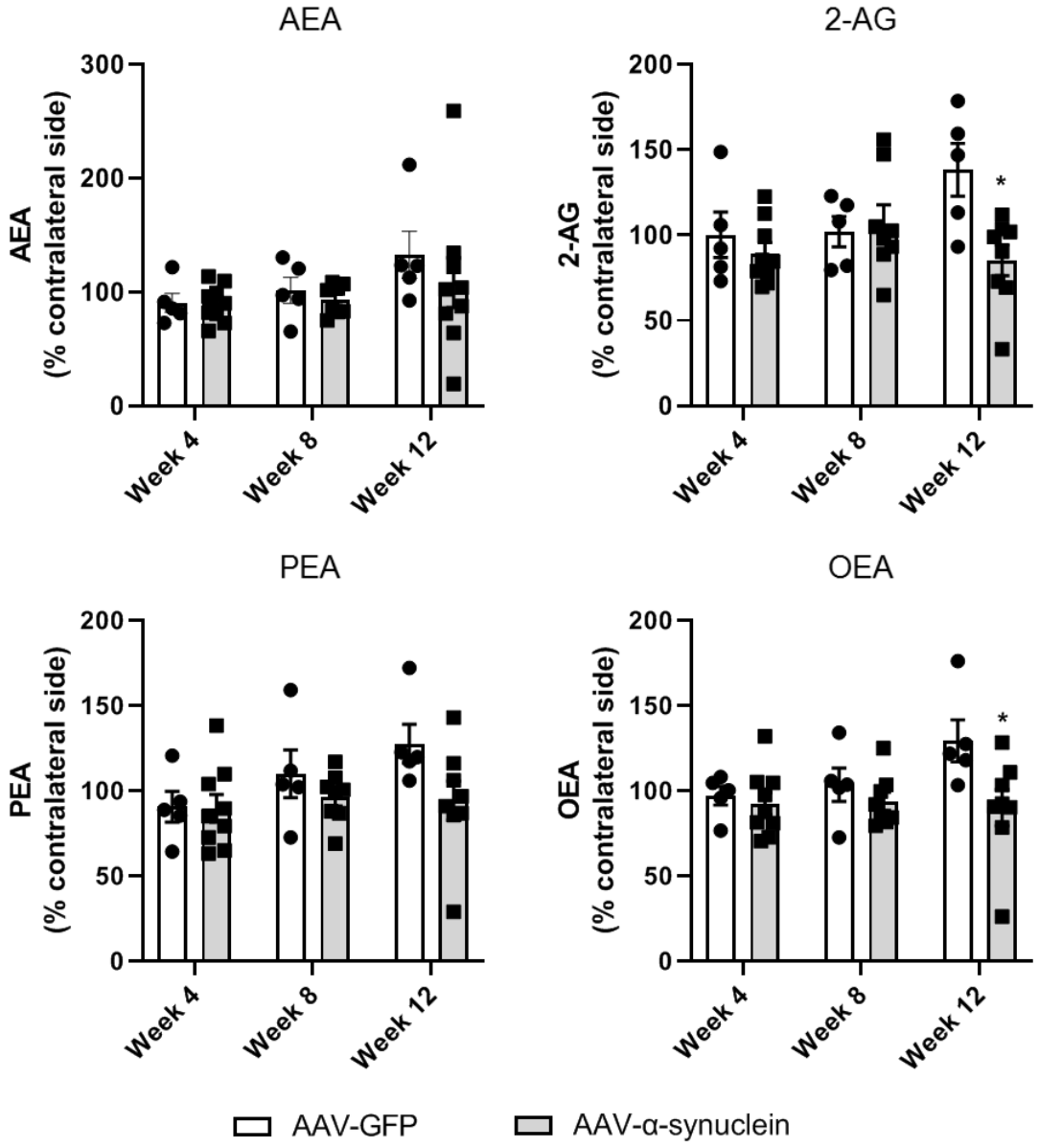

Figure 3.13 Mass spectrometry measurements of striatal endocannabinoid levels. Unilateral intra-nigral AAV-α-synuclein did not alter striatal AEA or PEA levels but induced a reduction in the levels of 2-AG and OEA. Data are analysed as mean ± SEM with 5-9 animals per group. **P*<0.05 vs. corresponding AAV-GFP group by two-way ANOVA with *post hoc* Tukey.

3.4 DISCUSSION

In recent years, it has become increasingly evident that microglia and neuroinflammation play a crucial role in the progression of Parkinson's disease (Frank-Cannon *et al.*, 2009; Tansey and Goldberg, 2010). The immunomodulatory cannabinoid CB_2 receptor is present on microglial cells in the brain (reviewed in Kelly *et al.*, 2020), and massive alterations in the levels of this receptor have been reported in patients suffering from Parkinson's disease (García *et al.*, 2015; Gómez-Gálvez *et al.*, 2016; Navarrete *et al.*, 2018), as well as in animal models of the disease (García *et al.*, 2011; Concannon *et al.*, 2015b; Concannon *et al.*, 2016). Notwithstanding, very little is known about whether CB_2 receptor expression is altered in viral-mediated α-synuclein overexpression Parkinson's disease models. These models are considered some of the most relevant models presently available, due to their gradual degeneration of dopamine neurons and their incorporation of α-synuclein pathology, a feature frequently absent in many Parkinsonian models (Volpicelli-Daley *et al.*, 2016). A comprehensive understanding of the alterations of components of the cannabinoid system in these models is necessary in order to fully assess the potential of the CB_2 receptor as a target for anti-inflammatory disease modulation.

Therefore, this first results chapter sought to investigate the temporal alterations in the expression of the immunomodulatory cannabinoid CB_2 receptor in the highly valid AAV-α-synuclein genetic model of Parkinson's disease. We assessed lateralised motor performance, and the expression of α-synuclein, of immune activation markers and of cannabinoid genes for a period of up to 12 weeks post-surgery. We also carried out mass spectrometry to assess striatal endocannabinoid levels, and immunohistochemistry to visualise α-synuclein, the nigrostriatal dopamine neurons and immune cells.

As anticipated, we found that intra-nigral delivery of AAV-α-synuclein induced widespread overexpression of human α-synuclein in the nigrostriatal pathway, both at the mRNA level and the protein level; however, this was not associated with a conspicuous loss of dopaminergic neurons or an impairment in motor function. Furthermore, α-synuclein overexpression did not cause any cellular neuroinflammation that was discernible by immunostaining in the substantia nigra, but was surprisingly associated

with an overall reduction in the mRNA levels of microglial and astrocytic markers in this brain region.

The main purpose of this study was to assess alterations in the cannabinoid system, particularly of the CB_2 receptor, in this viral-induced α-synuclein overexpression model. Despite the profound overexpression of α-synuclein and the neuroinflammatory dysregulation seen in the substantia nigra, we detected no differences in CB_2 receptor expression in the nigrostriatal pathway, nor in the gene expression of other endocannabinoid system components.

Further to our PCR analyses, we also carried out LC-MS/MS measurements of endocannabinoids in the striatum, to determine if the lesion surgery induced alterations in the levels of these lipid neurotransmitters at the nerve terminals. At Week 12 post-surgery, we found that AAV-α-synuclein induced a significant reduction in the levels of the lipid immune mediators 2-AG and OEA compared to the control group, indicating dysregulation of the endocannabinoid system in this model. However, it is unclear if this is a true decrease in endocannabinoid levels, or rather a prevention of an increase, as the levels of all four endocannabinoids seem to be increasing slightly over time in the control group. Whether this is perhaps age-mediated or is induced by the overexpression of GFP is unclear, and further studies may require a sham group to assist in elucidating this link.

Clinically, an increase in the levels of anandamide has been reported in the cerebrospinal fluid of Parkinson's disease patients (Pisani *et al.*, 2005; Pisani *et al.*, 2010), and similar elevations have been reported in animal models of Parkinson's disease (Gubellini *et al.*, 2002; Maccarrone *et al.*, 2003; van der Stelt *et al.*, 2005). Increases in 2-AG have been described in the brains of several animal models of the disease (Di Marzo *et al.*, 2000; van der Stelt *et al.*, 2005; Concannon *et al.*, 2015b; Mounsey *et al.*, 2015), but levels were unaffected in other models (Gubellini *et al.*, 2002; Maccarrone *et al.*, 2003; Concannon *et al.*, 2016). Contrastingly, a decrease in 2-AG in the plasma and cerebrospinal fluid of Parkinson's disease patients has been reported (Marchioni *et al.*, 2020). Furthermore, treatment with dopaminergic therapy has been noted to reduce the abnormally elevated levels of these cannabinoids, indicating that the increases in endocannabinoids observed may be a compensatory mechanism, with an aim to normalise dopamine depletion (Di Marzo *et al.*, 2000; Maccarrone *et al.*, 2003; Pisani *et al.*, 2010). Clearly, the relationship

between endocannabinoids and the pathophysiology of Parkinson's disease is complex, and further research is required to illuminate this connection.

The AAV-α-synuclein model, despite its abundant advantages, has various limitations which are evident in this study. While this model has been reported to induce dopaminergic degeneration and motor dysfunction, this can be highly variable (Kirik *et al.*, 2002; Gorbatyuk *et al.*, 2008; Lindgren *et al.*, 2012), and this unpredictability limits its usefulness as a pre-clinical model to researchers. In this study, despite the profound overexpression of human α-synuclein, no neurodegeneration was evident and hence there was no impairment in the motor behavioural tests. Without the death of these dopamine neurons, which is the key neuropathological feature of Parkinson's disease, it is unsurprising that there are no alterations in CB_2 receptor expression as has been seen in other Parkinsonian models (Concannon *et al.*, 2015b; Concannon *et al.*, 2016; Gómez-Gálvez *et al.*, 2016). However, the results from this study are still interesting with regard to investigating the prodromal or subclinical condition, and the altered levels of endocannabinoids indicate that dysregulation of the cannabinoid system may proceed cell death in Parkinson's disease. It is unclear why neurodegeneration was not induced in this instance, as there may be a number of contributing factors. The promotor used was a PGK1 promotor encoding a phosphoglycerate kinase that is required in every cell for glycolysis. In the future, the use of a neuron-specific promotor, such as the human synapsin-1 gene (Kügler *et al.*, 2003), could be beneficial by providing more targeted α-synuclein expression. A higher titre could also be used, but GFP-induced toxicity has been reported at higher titres (Klein *et al.*, 2006; Khabou *et al.*, 2018). Pilot studies may need to be carried out in the future to ensure the validity of the vectors before further studies are executed.

Relative to other neurotoxic models, the neuroinflammatory response to AAV-α-synuclein expression is very mild, with subtle alterations in glial morphology rather than overt gliosis (Sanchez-Guajardo *et al.*, 2010). Therefore, it is not too surprising that in our immunostained sections for microglial and astrocytic markers, there was no obvious increase in glial density. The overall reduction in astrocytic and microglial marker gene expression in the substantia nigra was unanticipated, but whether this reflects a decrease in the glial cell numbers in this region cannot be concluded without proper quantitative immunohistochemical analyses with larger *n* numbers, which was outside the scope of

this study. The link between α-synuclein expression and glial cells is very complex and is not yet fully elucidated. The CD11b protein is part of the CR3 complex, which is involved in phagocytic activity (Ross and Větvička, 1993). Alpha-synuclein has been shown previously to modulate the phagocytic function of microglia (Park *et al.*, 2008; Roodveldt *et al.*, 2010), and the reduction of CD11b expression observed here may indicate that α-synuclein may be interfering with the phagocytic functionality of microglia. In addition, a triplication of the SNCA gene was seen to compromise phagocytosis in iPSC-derived macrophages (Haenseler *et al.*, 2017). Furthermore, studies have demonstrated that the activation of microglia by α-synuclein may be dependent both on the degree of aggregation of α-synuclein as well as the protein isoform (Roodveldt *et al.*, 2010; Hoenen *et al.*, 2016; Hoffmann *et al.*, 2016). With regard to astrocytes, the reports on astrocytic activation in Parkinson's disease patients are highly variable (Damier *et al.*, 1993; Mirza *et al.*, 2000; Vila *et al.*, 2001; Brück *et al.*, 2016). It is not clear whether astrocytes facilitate the removal of α-synuclein by phagocytosis, or contribute to its propagation (reviewed in Sorrentino *et al.*, 2019). Undoubtedly, much remains to be learnt concerning the link between α-synuclein and the neuroinflammatory aspect of Parkinson's disease.

In summary, the results presented in this chapter depict that in this AAV-α-synuclein model of Parkinson's disease, there were changes induced in the neuroinflammatory system, in addition to a dysregulation of the endocannabinoid system. Although there was no overt neurodegeneration of the nigrostriatal pathway in this study, nevertheless the results from this chapter provide interesting insights into the prodromal condition, before cell death and thus motor dysfunction are induced. However, at present, this model is not reliable enough to faithfully investigate changes in the endocannabinoid system. Therefore in the next chapter, we attempted to enhance the AAV-α-synuclein model by sequential administration with a small molecule α-synuclein aggregator.

Chapter 4: Enhancing the AAV-α-synuclein Parkinson's disease model using a small molecule α-synuclein aggregator

4.1 INTRODUCTION

Many of the animal models of Parkinson's disease that have been developed to date involve the administration of a toxin either systemically or cerebrally, such as the direct toxins 6-OHDA and MPTP; the pesticide, rotenone, and the herbicide, paraquat; and inflammagens such as LPS and poly I:C (Duty and Jenner, 2011). However, while undoubtedly useful, these toxin-based models are problematic in that they are usually not progressive, and therefore generally not optimal for testing potential neuroprotective therapeutic interventions. Moreover, they do not replicate all the neuropathological changes occurring in the disease in a molecular manner, often just inducing nigrostriatal degeneration in a molecular manner that is not necessarily relevant to the disease, and rarely incorporating α-synuclein pathophysiology. Therefore there was a clear need for a model which exhibited a gradual progression and which more closely resembled the pathological changes that are occurring in the brains of Parkinson's disease patients.

Viral vector-mediated overexpression of α-synuclein was first introduced as a potential model of Parkinson's disease just a few years after the implication of α-synuclein in the pathogenesis of the disease as the major component of Lewy bodies (Spillantini *et al.*, 1997). Deniz Kirik and colleagues employed adeno-associated viral vectors that expressed either wild-type or A53T mutated human α-synuclein and injected them into the substantia nigra of rats (Kirik *et al.*, 2002). They observed a substantial loss of nigral dopamine neurons and a subsequent loss of striatal dopamine, which was accompanied by motor dysfunction in animals in which the dopamine neuron loss exceeded a critical threshold of 50-60%. They also noted the presence of α-synuclein-positive inclusions and dystrophic neurites similar to those seen in Parkinson's disease patients. Subsequently, AAV vectors have been used with similar success in mice (St Martin *et al.*, 2007; Ip *et al.*, 2017) and primates (Kirik *et al.*, 2003; Eslamboli *et al.*, 2007). Lentiviral vectors have also been used, but to a lesser extent as they have a lower transgene expression in

midbrain dopamine neurons and relatively reduced death of neuronal cells (Lo Bianco *et al.*, 2002; Lauwers *et al.*, 2003).

However, despite the advantages of this viral vector-induced α-synuclein overexpression model, it has its limitations: primarily, a very slowly developing pathology and a high degree of variability. Although this variability can partially be attributed to the different capsid serotypes and titres of vectors used by distinct research groups, this does not fully explain the disparities in neuronal loss (Volpicelli-Daley *et al.*, 2016). Even within a singular study, discernible inconsistencies have been noted with regard to the extent of observable neurodegeneration between animals (Kirik *et al.*, 2002). This variability is emphasised in the results from the previous chapter, where the viral vector did not cause dopaminergic degeneration despite inducing profound α-synuclein expression. In addition, the levels of α-synuclein expression required to produce dopaminergic degeneration are much higher (up to 4-5 times) than those present in the human disease (Decressac *et al.*, 2012), rendering it physiologically less relevant. These limitations are substantial disadvantages which indicate an improved α-synuclein model of Parkinson's disease is required.

FN075 is a novel peptidomimetic small molecule that has been shown to inhibit the production of functional curli or bacterial amyloid fibres, but also to stimulate the aggregation of α-synuclein (Cegelski *et al.*, 2009; Horvath *et al.*, 2012). *In vivo*, a single injection of FN075 into the brains of mice caused impairments in motor function and induced a significant reduction of tyrosine hydroxylase-positive neurons at 6 months. In α-synuclein knockout mice, there were no differences in neuronal numbers, indicating that this was an α-synuclein-mediated effect (Chermenina *et al.*, 2015). Previous members of our group studied FN075 in the context of the interaction between viruses and α-synuclein, and found that priming with a viral mimetic significantly exacerbated the α-synuclein aggregate-induced behavioural and neuropathological effects which were mediated by FN075. Specifically, sequential exposure to poly I:C and FN075 caused significant nigrostriatal neurodegeneration and microgliosis, as well as impairments in forelimb kinesis and sensorimotor integration (Olsen *et al.*, 2019).

Given the aforementioned problems with the current AAV-α-synuclein model, and the promising results reported from studies germane to the small molecule FN075, we

hypothesised that we could produce a more consistent, rapidly developing model of Parkinson's disease by the combination of AAV-mediated α-synuclein overexpression with FN075-mediated α-synuclein aggregation. Therefore, the aim of this chapter was to determine if sequential administration of an AAV vector expressing either wild-type α-synuclein or A53T mutant α-synuclein and the α-synuclein aggregating molecule FN075 could induce a more pronounced and rapidly developing Parkinson's disease pathology in rats.

4.2 MATERIALS AND METHODS

All methods are described in detail in Chapter 2.

4.2.1 EXPERIMENTAL DESIGN

Animal models of Parkinson's disease induced by viral-mediated overexpression of α-synuclein are widely considered to be the most valid currently available models of Parkinson's disease, but they are limited by their very slowly developing pathology and their high degree of variability. The studies presented in this chapter combined viral-mediated overexpression of α-synuclein with FN075-mediated aggregation of α-synuclein to determine if this produced a more consistent, rapidly developing model of Parkinson's disease.

4.2.1.1 Combination of AAV-induced wild-type α-synuclein overexpression with the α-synuclein aggregating molecule, FN075

In order to investigate the effect of combining viral-induced human wild-type α-synuclein with the α-synuclein aggregating molecule FN075, 40 female rats (Charles River, U.K.) received dual injections unilaterally into the substantia nigra (at the stereotaxic coordinates: AP -4.8 & -5.8, ML +2.0, DV -7.2) of an AAV vector expressing either wild-type human α-synuclein or GFP, driven by the PGK promoter (5×10^{10} vg per site, with two sites per rat (10×10^{10} vg total)). Four weeks later (i.e. once α-synuclein expression was quite extensive but the model was still developing), the animals were performance-matched into groups (Table 4.1) and received an intra-nigral injection (at the stereotaxic coordinates: AP -5.3, ML +2.0, DV -7.2) of the α-synuclein aggregating molecule, FN075 (1.926 µg), or its vehicle (DMSO/imidazole/PBS). The dose of FN075 was chosen based on previously published *in vivo* studies using this molecule (Chermenina *et al.*, 2015; Olsen *et al.*, 2019). Animals underwent Corridor, Stepping, and Whisker behavioural tests throughout the study to assess if the AAV-α-synuclein vector and/or FN075 induced motor impairment. The animals were sacrificed at Week 20 post-AAV surgery for immunohistological analyses.

Group	1st surgery	2nd surgery	n
Control	AAV-GFP	Vehicle	10
FN075	AAV-GFP	FN075	10
AAV-α-SYN (WT)	AAV-α-synuclein (wild-type)	Vehicle	10
AAV-α-SYN (WT) & FN075	AAV-α-synuclein (wild-type)	FN075	10

Table 4.1 Groups used in AAV-α-synuclein (wild-type) and FN075 study. AAV-α-synuclein viral vectors used in this study expressed wild-type human α-synuclein.

4.2.1.2 Combination of AAV-induced A53T mutant α-synuclein overexpression with the α-synuclein aggregating molecule, FN075

To investigate the effect of the combination of AAV-induced A53T mutant α-synuclein overexpression with FN075-mediated α-synuclein aggregation, 40 female rats (born in-house) received dual injections unilaterally into the substantia nigra (at the stereotaxic coordinates: AP -4.8 & -5.8, ML +2.0, DV -7.2) of an AAV vector expressing either human α-synuclein with the A53T mutation or GFP, driven by the PGK promoter (3.98 x 10^{10} vg per site, with two sites per rat (7.96 x 10^{10} vg total)). Four weeks later, the animals were performance-matched into groups (Table 4.2) and received an intra-nigral injection (at the stereotaxic coordinates: AP -5.3, ML +2.0, DV -7.2) of the α-synuclein aggregating molecule, FN075 (1.926 µg), or vehicle (DMSO/imidazole/PBS). Animals underwent Corridor, Stepping, and Whisker behavioural tests at regular intervals throughout the study to assess if any motor dysfunctions were induced. They were subsequently sacrificed at Week 20 post-AAV surgery for immunohistological analyses.

Group	1st surgery	2nd surgery	n
Control	AAV-GFP	Vehicle	10
FN075	AAV-GFP	FN075	10
AAV-α-SYN (A53T)	AAV-α-synuclein (A53T mutant)	Vehicle	10
AAV-α-SYN (A53T) & FN075	AAV-α-synuclein (A53T mutant)	FN075	10

Table 4.2 Groups used in AAV-α-synuclein (A53T) and FN075 study. AAV-α-synuclein viral vectors used in this study expressed human α-synuclein with the A53T mutation.

4.3 RESULTS

4.3.1 COMBINATION OF AAV-INDUCED WILD-TYPE ALPHA-SYNUCLEIN OVEREXPRESSION WITH THE ALPHA-SYNUCLEIN AGGREGATING MOLECULE, FN075

4.3.1.1 Unilateral, intra-nigral AAV-α-synuclein administration alone or in combination with FN075 did not induce contralateral motor impairment

Before we commenced *post mortem* analyses, which were the main objective of this study, we first sought to investigate if an impairment in motor function was induced in the animals by the administration of AAV-α-synuclein or FN075, alone or in combination. The surgeries were performed unilaterally so any motor dysfunction would be restricted to one side of the body (contralateral to the side of the brain injected) and thus the other side could serve as an internal control.

As expected, there were no differences in ipsilateral motor function between groups in the Corridor test of sensorimotor neglect (Figure 4.1A; Group x Time, $F_{(6,72)}$=1.07, P>0.05), the Stepping test of forelimb akinesia (Figure 4.1B; Group x Time, $F_{(6,72)}$=0.79, P>0.05), or the Whisker test of sensorimotor integration (Figure 4.1C; Group x Time, $F_{(6,72)}$=1.06, P>0.05). In the Stepping test, there was a significant effect of time, with the average number of steps decreasing over time in all groups, suggesting a habituation effect as the animals became more accustomed to the handling (Figure 4.1B; Time, $F_{(1.93,69.30)}$=34.22, P<0.05).

Importantly, in this study, AAV-wild-type-α-synuclein, administered either alone or sequentially with FN075, did not induce any impairment in contralateral motor function in the Corridor test (Figure 4.2A; Group x Time, $F_{(6,72)}$=1.07, P>0.05), the Stepping test (Figure 4.2B; Group x Time, $F_{(6,72)}$=1.09, P>0.05), or the Whisker test (Figure 4.2C; Group x Time, $F_{(6,72)}$=0.83, P>0.05). Akin to the ipsilateral data, there was a significant effect of time in the Stepping test (Figure 4.2B; Time, $F_{(1.71,61.70)}$=30.89, P<0.05), indicating a training effect.

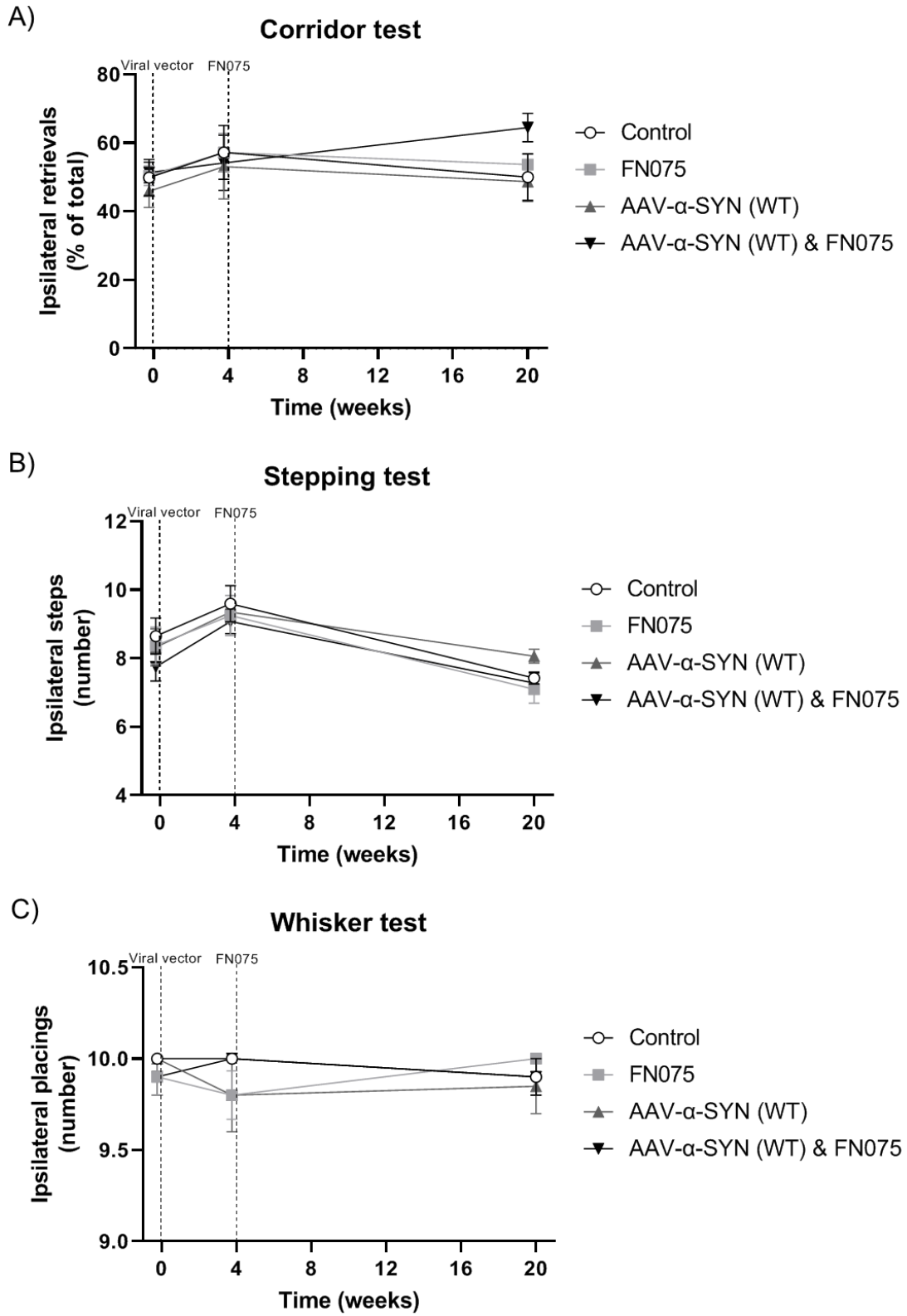

Figure 4.1 Impact of AAV-α-SYN (WT) alone or in combination with FN075 on ipsilateral motor function. There were no differences in ipsilateral motor function between groups in the Corridor (A), Stepping (B) or Whisker (C) behavioural tests. Data are represented as mean ± SEM with n=10 animals per group and were analysed by two-way repeated-measures ANOVA. Dashed lines represent the days of infusion surgeries.

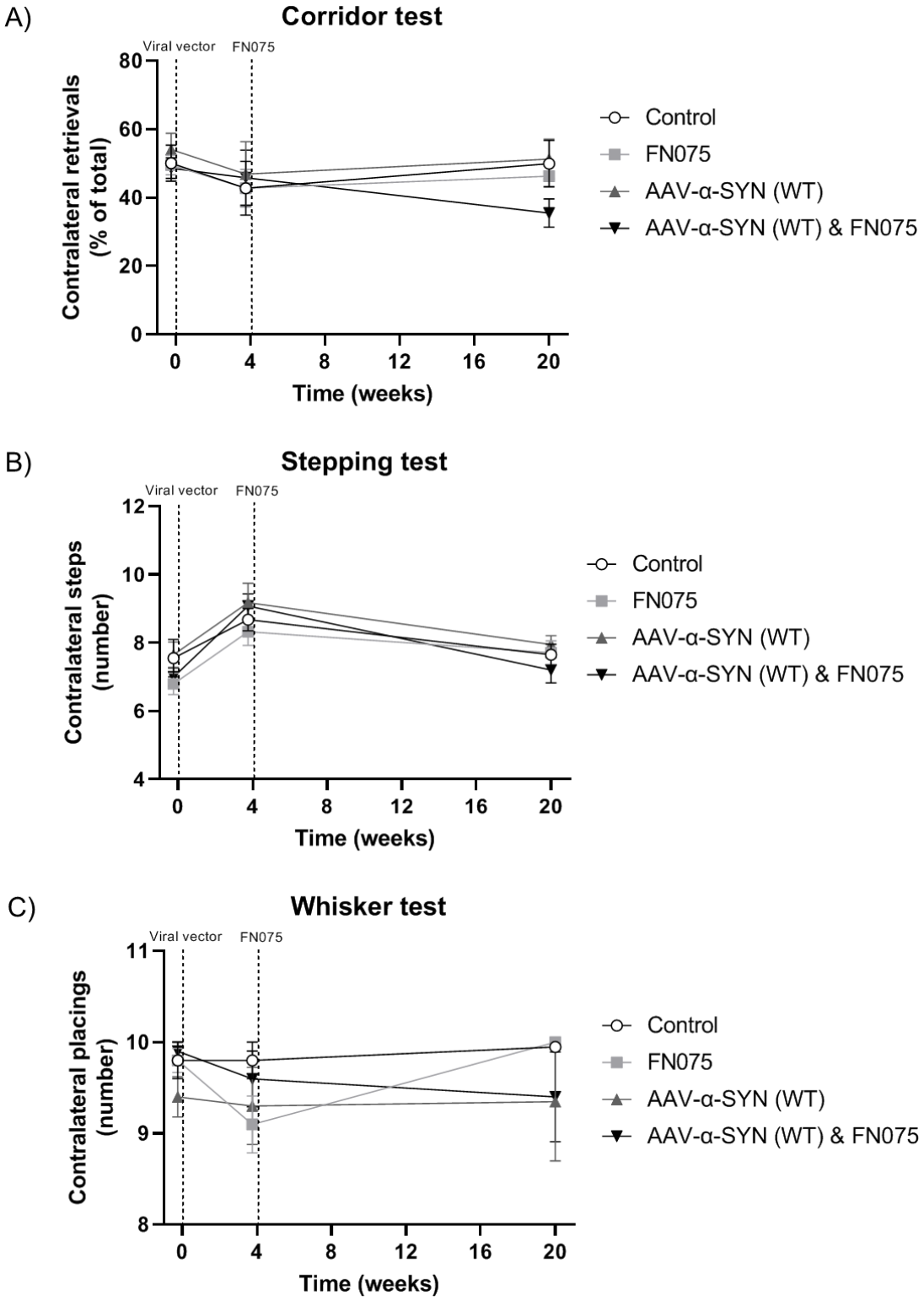

Figure 4.2 Impact of AAV-α-SYN (WT) alone or in combination with FN075 on contralateral motor function. AAV-α-SYN (WT), administered alone or sequentially with FN075, did not induce contralateral motor dysfunction in the Corridor (A), Stepping (B) or Whisker (C) behavioural tests. Data are represented as mean ± SEM with n=10 animals per group and were analysed by two-way repeated-measures ANOVA. Dashed lines represent the days of infusion surgeries.

4.3.1.2 AAV-α-synuclein administration induced significant α-synuclein expression

Once the animals were sacrificed, *post mortem* we first sought to investigate if the administration of AAV-wild-type-α-synuclein induced the overexpression of human α-synuclein in the nigrostriatal pathway. Immunohistochemical staining for human α-synuclein verified that the animals that received the AAV-α-synuclein injection had widespread expression of α-synuclein in the substantia nigra (Figure 4.3; Group, $F_{(3,36)}$=22.11, P<0.05) and across the midbrain. As expected, this effect was restricted to groups that received the AAV-α-synuclein injection, and animals that were administered the AAV-GFP vector exhibited no α-synuclein expression. The α-synuclein aggregating molecule, FN075, did not significantly affect the density of α-synuclein immunohistochemical staining, either alone or in combination with AAV-α-synuclein.

In the striatum, a similar effect was observed. AAV-α-synuclein induced extensive human α-synuclein expression ipsilateral to the side of injection compared to the control group (Figure 4.4; Group, $F_{(3,35)}$=7.76, P<0.05), but FN075 did not have an additive effect on expression.

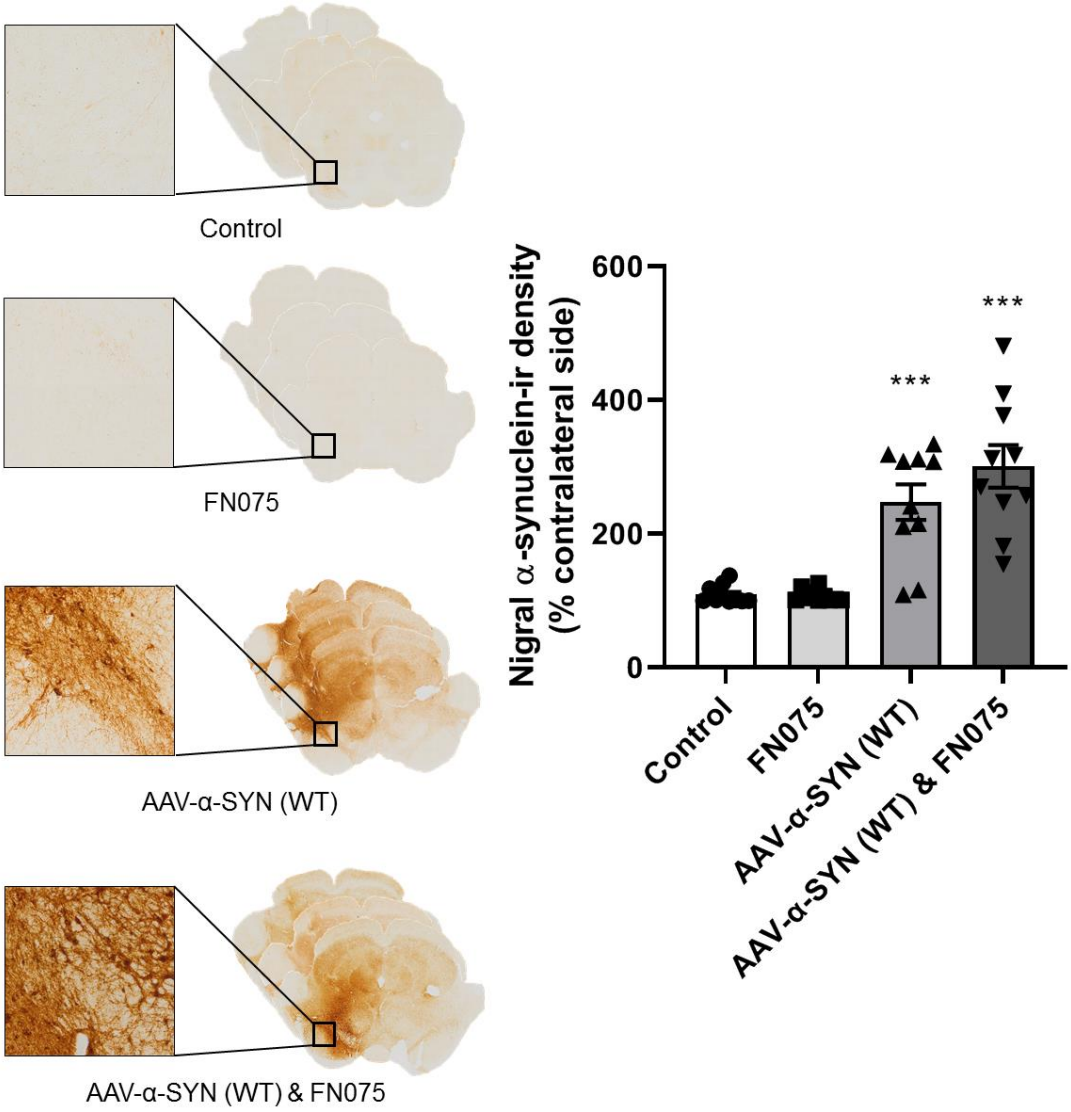

Figure 4.3 Immunohistological assessment of the impact of AAV-α-SYN (WT) alone or in combination with FN075 on the expression of human α-synuclein in the substantia nigra. AAV-α-SYN (WT), alone or with FN075, caused a significant overexpression of human α-synuclein in the substantia nigra. Data are represented as mean ± SEM with n=10 animals per group. ****P*<0.001 vs. Control by one-way ANOVA with *post hoc* Tukey.

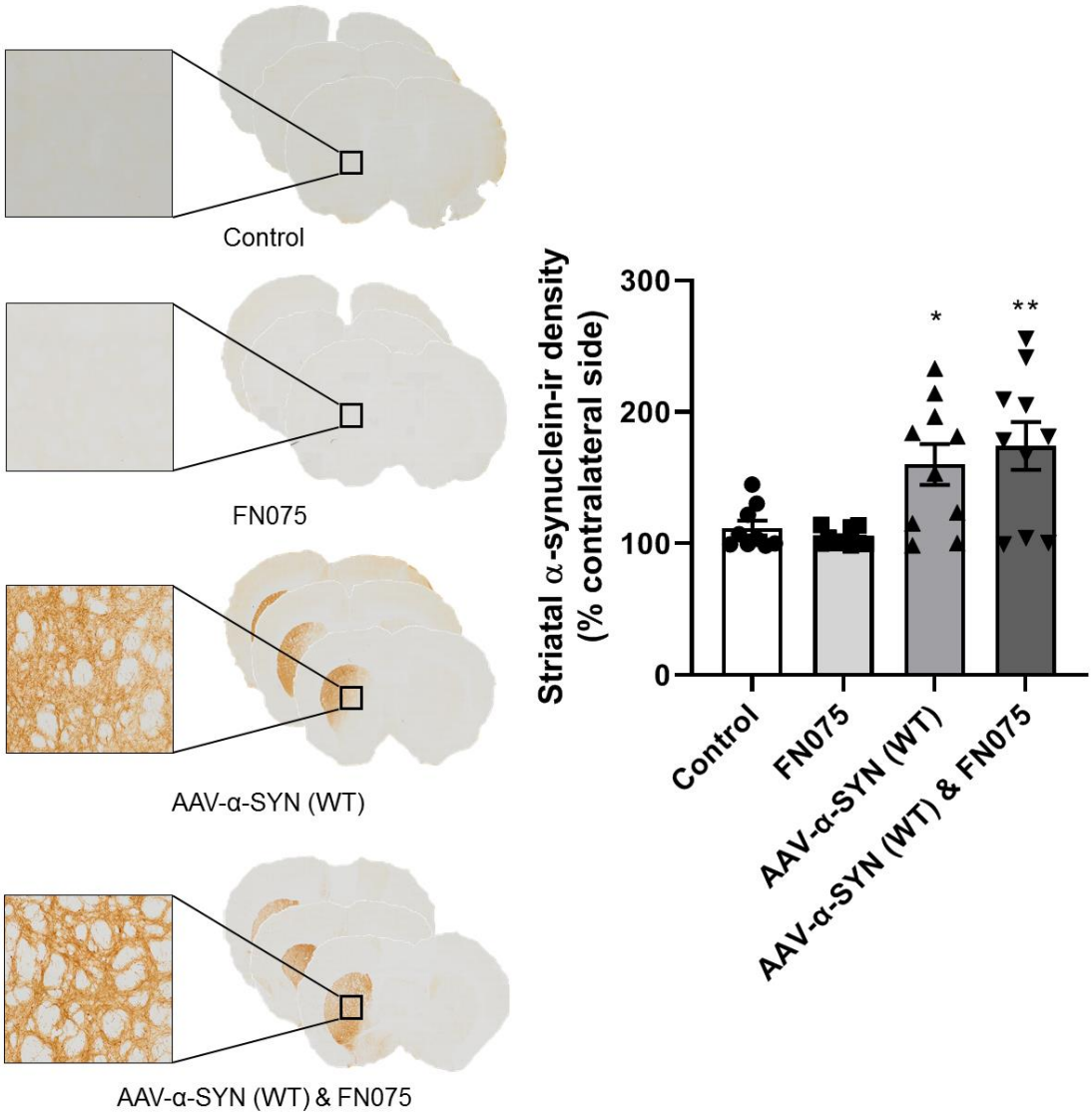

Figure 4.4 Immunohistological assessment of the impact of AAV-α-SYN (WT) alone or in combination with FN075 on the expression of human α-synuclein in the striatum. AAV-α-SYN (WT) caused a significant overexpression of human α-synuclein in the striatum, alone or with FN075. Data are represented as mean ± SEM with n=9-10 animals per group. **P*<0.05, ***P*<0.01 vs. Control by one-way ANOVA with *post hoc* Tukey.

4.3.1.3 FN075 significantly increased α-synuclein phosphorylation at serine 129

Following our verification that the AAV-wild-type-α-synuclein vector induced an overexpression of human α-synuclein in the nigrostriatal pathway, we sought to investigate if the combination of AAV-α-synuclein and the α-synuclein aggregating molecule FN075 affected the structure of or modified the form of the α-synuclein protein. α-synuclein can contribute to Parkinson's disease pathology in numerous ways, and the

formation of aberrant aggregates and the presence of phosphorylated α-synuclein have been implicated in the pathogenesis of Parkinson's disease (Xu *et al.*, 2015; Oueslati, 2016; Mehra *et al.*, 2019). Therefore, we carried out a further immunohistochemical stain for α-synuclein, specific for α-synuclein phosphorylated on serine 129 (pS129).

Upon carrying out the staining for the pS129-α-synuclein, accumulations following a neuronal morphology were immediately evident in some sections under high magnification. Optical density analyses revealed that FN075, in combination with the wild-type AAV-α-synuclein vector, induced a significant increase in the density of the abnormal pS129-α-synuclein protein compared to both the control group and compared to the AAV-α-SYN (WT) group (Figure 4.5; Group, $F_{(3,35)}$=10.70, P<0.05). We also carried out an alternative analysis by counting the number of pS129-α-synuclein-positive accumulations on the side of the brain that was lesioned. Similarly, we found that FN075 in combination with AAV-α-SYN (WT) induced a significant increase in the number of pS129-α-synuclein-positive accumulations compared to the control group and compared to the AAV-α-SYN (WT) group (Figure 4.6; Group, $F_{(3,36)}$=11.92, P<0.05)

Furthermore, we carried out a stain specific for α-synuclein in the filamentous form. Under high magnification, aggregates were visible in some animals suggesting that FN075 in combination with AAV-α-synuclein does induce α-synuclein aggregation on the ipsilesional side (Figure 4.7).

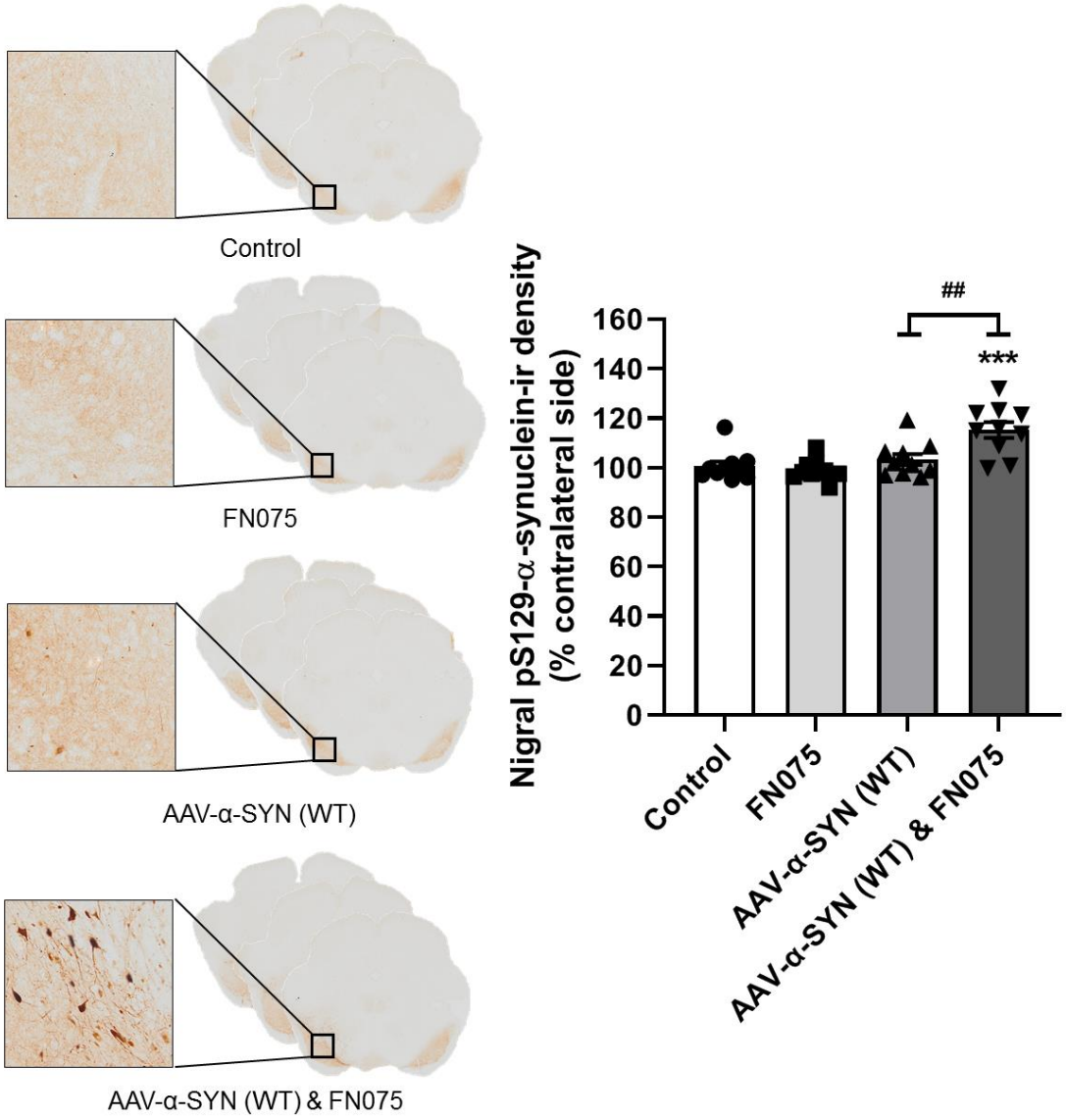

Figure 4.5 Immunohistological assessment of the impact of the small molecule FN075 on the phosphorylation of α-synuclein at serine 129 in the substantia nigra. FN075 in combination with AAV-α-SYN (WT) induced a significant increase in the density of phosphorylated α-synuclein in the substantia nigra. Data are represented as mean ± SEM with n=10 animals per group. ***P<0.001 vs. Control, $^{\#\#}P$<0.01 vs. AAV-α-SYN (WT) by one-way ANOVA with *post hoc* Tukey.

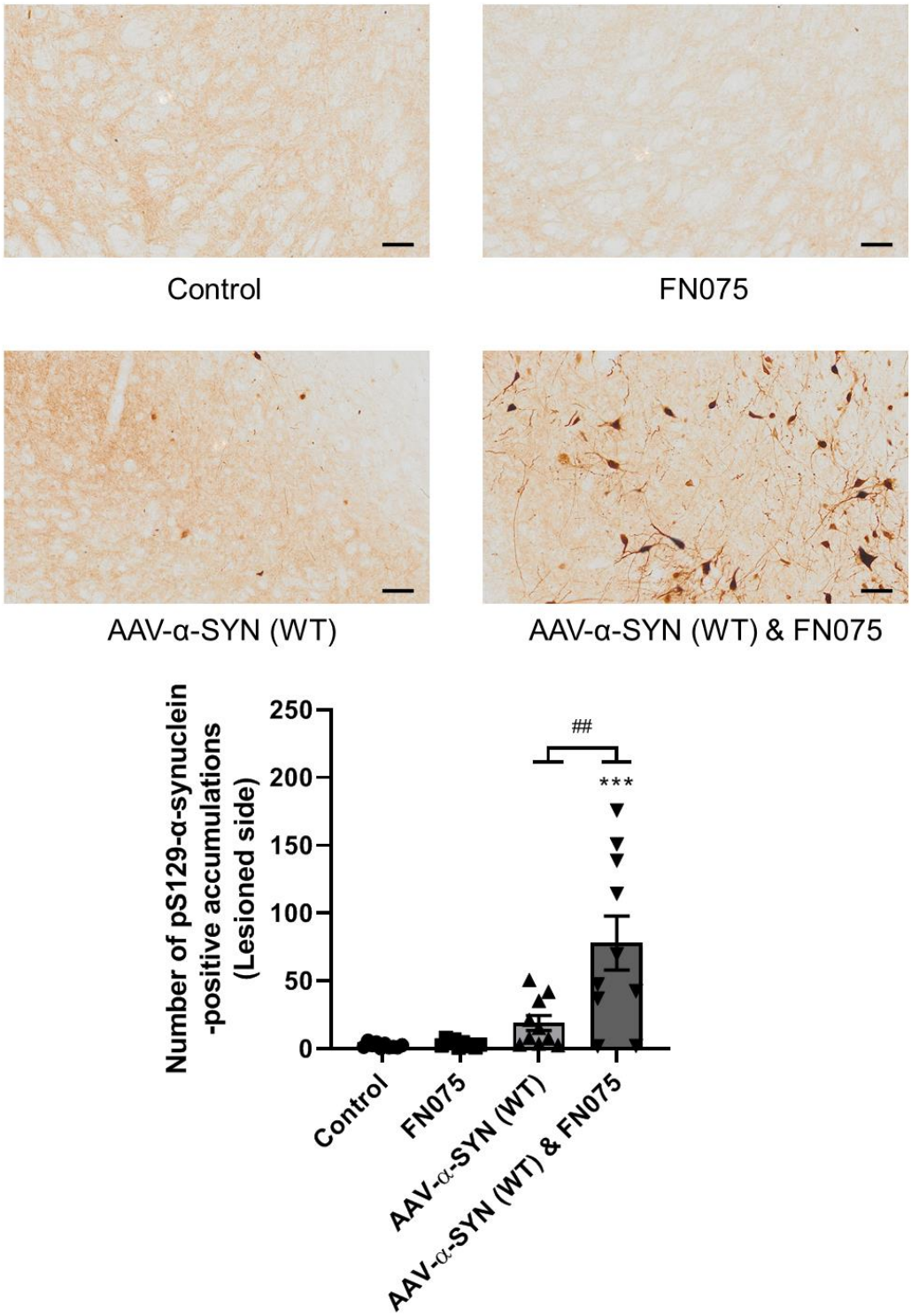

Figure 4.6 Immunohistological assessment of the impact of the small molecule FN075 on the accumulation of phosphorylated α-synuclein in the substantia nigra. FN075 in combination with AAV-α-SYN (WT) induced a significant increase in the number of pS129-α-synuclein-positive accumulations in the substantia nigra. Scale bars represent 50 µm. Data are represented as mean ± SEM with n=10 animals per group. ***P<0.001 vs. Control, $^{\#\#}P$<0.01 vs. AAV-α-SYN (WT) by one-way ANOVA with *post hoc* Tukey.

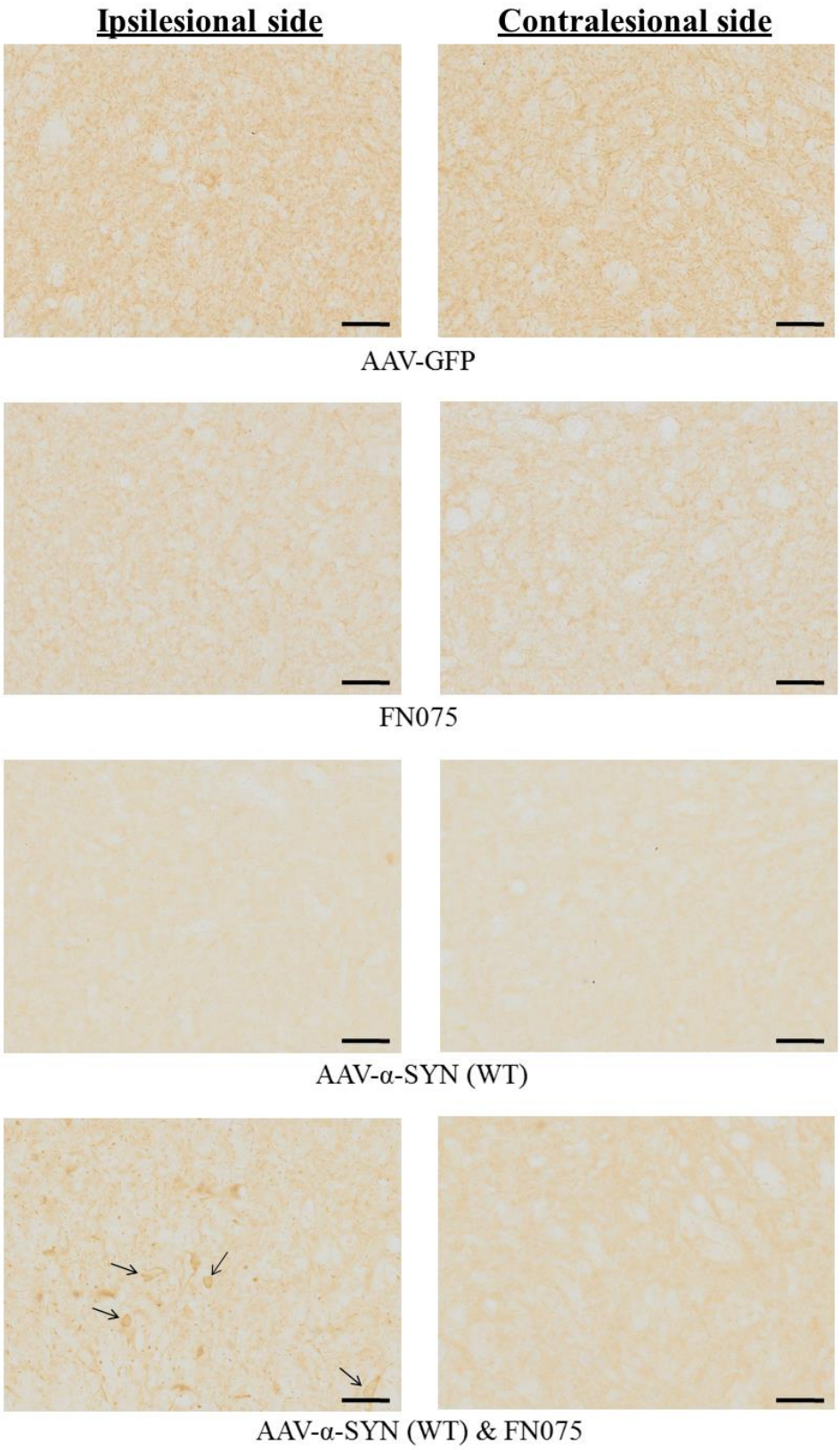

Figure 4.7 Immunohistological visualisation of the impact of the small molecule FN075 on the expression of α-synuclein filaments in the substantia nigra. FN075 in combination with AAV-α-SYN (WT) induced the aggregation of α-synuclein in the substantia nigra on the ipsilesional side. Scale bars represent 50 µm.

4.3.1.4 AAV-α-synuclein administration did not cause degeneration in the nigrostriatal pathway

Following our analyses of α-synuclein expression, we next sought to investigate if the overexpression of α-synuclein we observed in the substantia nigra and the striatum induced degeneration of the dopaminergic neurons in the nigrostriatal pathway. The slow, progressive death of these neurons is the central pathological feature of Parkinson's disease and thus a crucial constituent of a novel animal model. We visualised these dopaminergic neurons by carrying out staining for tyrosine hydroxylase (TH), a dopamine synthesis enzyme. In this study, there were no differences observed between groups with regard to either cell numbers in the substantia nigra (Figure 4.8; Group, $F_{(3,35)}$=0.72, P>0.05), or the density of nerve terminals in the striatum (Figure 4.9; Group, $F_{(3,36)}$=0.50, P>0.05). The tyrosine hydroxylase-stained striatal sections were also analysed for the presence of dystrophic neurites, as α-synuclein-induced dystrophy of the nigrostriatal terminals has been reported previously in the AAV-α-synuclein model (Kirik *et al.*, 2002). While there was a trend for increased dystrophy in the combined AAV-α-SYN (WT) & FN075 group compared to the control group, this did not reach significance (Figure 4.10; Group, $F_{(3,35)}$=3.63, P=0.02; in *post hoc* Tukey test P=0.0588 for Control vs. AAV-α-SYN (WT) & FN075).

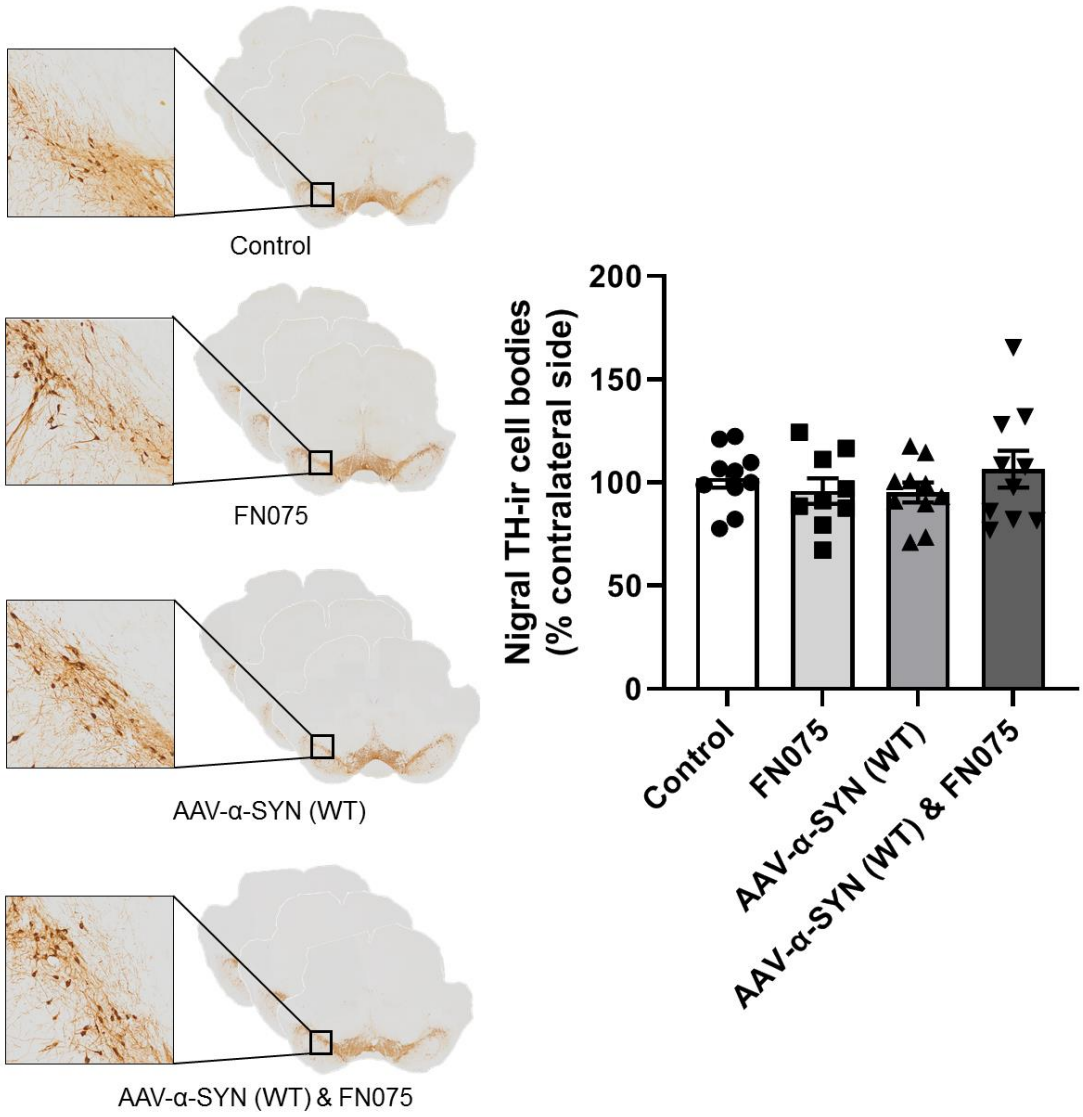

Figure 4.8 Immunohistological assessment of the impact of AAV-α-SYN (WT) alone or in combination with FN075 on nigral cell counts. AAV-α-SYN (WT), administered alone or with FN075, did not significantly affect the number of TH-immunoreactive cell bodies in the substantia nigra. Data are represented as mean ± SEM with n=9-10 animals per group and were analysed by one-way ANOVA.

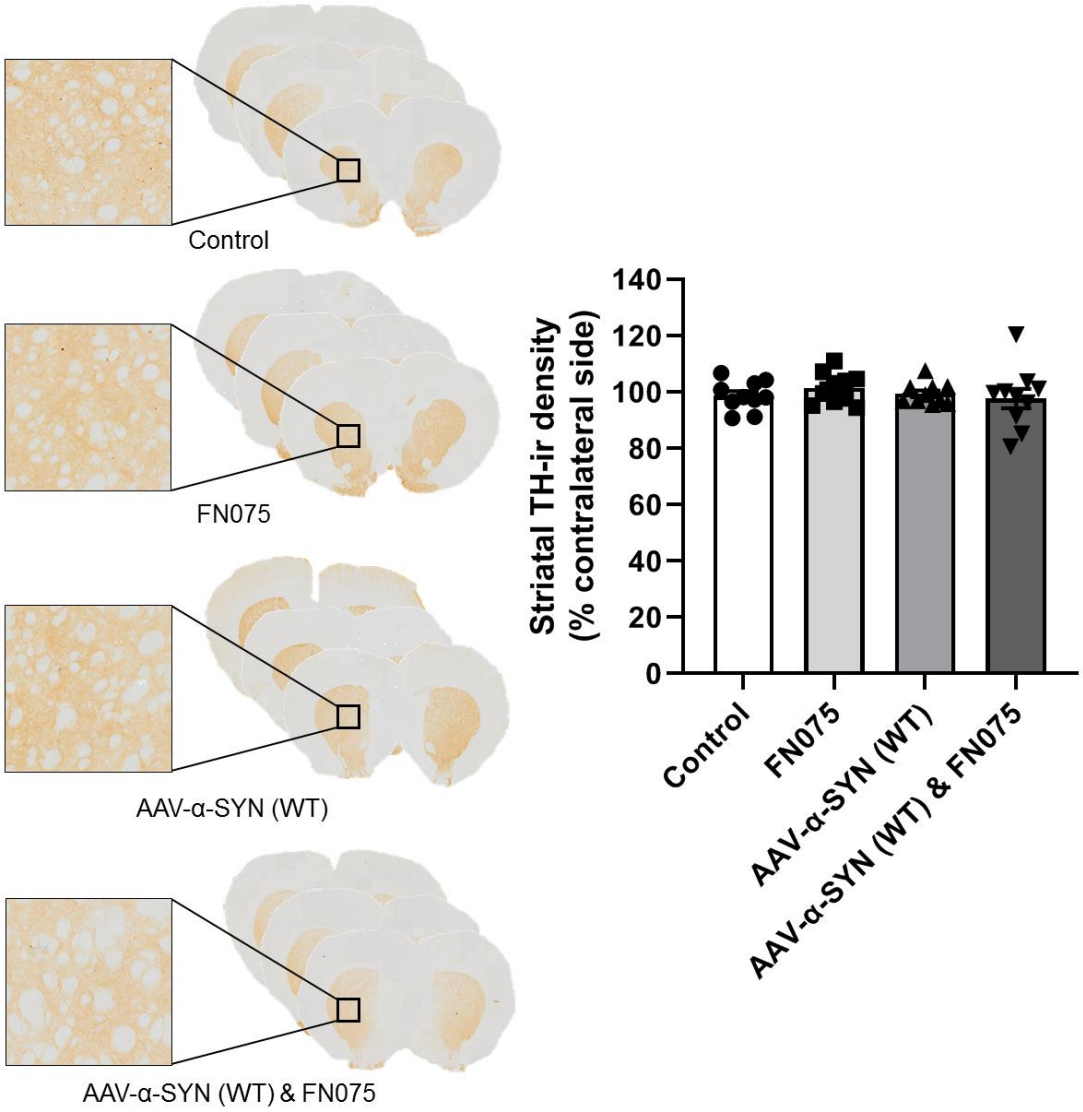

Figure 4.9 Immunohistological assessment of the impact of AAV-α-SYN (WT) alone or in combination with FN075 on the density of dopaminergic nerve terminals in the striatum. AAV-α-SYN (WT), administered alone or with FN075, did not significantly affect the optical density of TH staining in the striatum. Data are represented as mean ± SEM with n=10 animals per group and were analysed by one-way ANOVA.

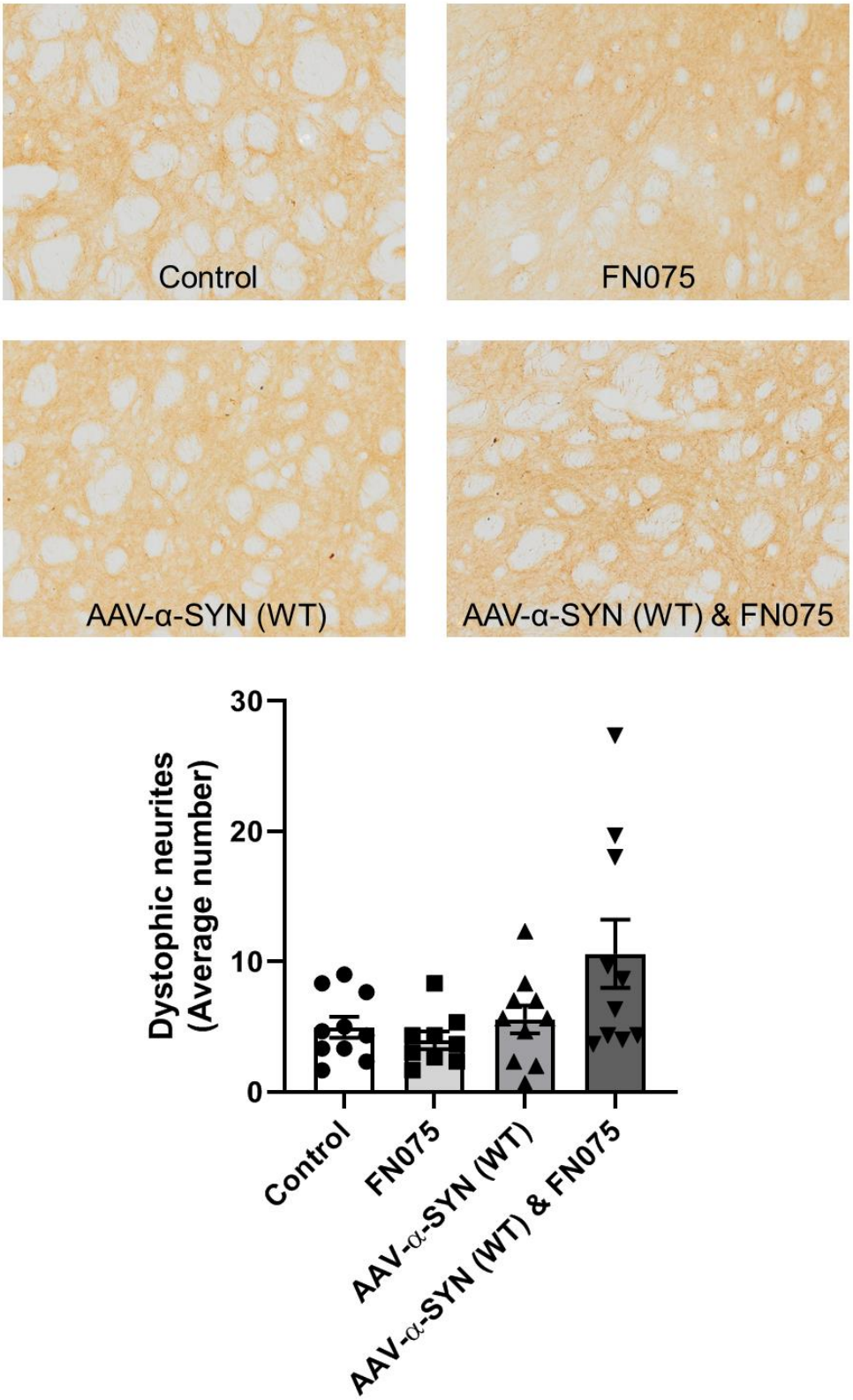

Figure 4.10 Immunohistological assessment of the impact of AAV-α-SYN (WT) alone or in combination with FN075 on striatal dopaminergic dystrophic neurites. AAV-α-SYN (WT), administered alone or sequentially with FN075, did not significantly affect the number of dystrophic neurites in the striatum. Data are represented as mean ± SEM with n=9-10 animals per group and were analysed by one-way ANOVA.

4.3.1.5 AAV-α-synuclein administration did not affect microgliosis

Although we did not detect any alterations in the numbers of dopaminergic cell bodies in the substantia nigra or in the density of the striatal nerve terminals, we still wished to assess if there were any alterations to the microglial response in the nigrostriatal pathway as neuroinflammation is an important component of the pathology of Parkinson's disease. We visualised the microglial cells by carrying out quantitative OX-42 staining, which recognises the CD11b protein that is highly expressed on macrophages and microglia. In this study, although some animals had conspicuous microglial staining along the cannula tract, we did not detect significant differences in microgliosis between groups in either the substantia nigra (Figure 4.11A; Group, $F_{(3,36)}$=1.76, P>0.05) or the striatum (Figure 4.11B; Group, $F_{(3,36)}$=0.37, P>0.05).

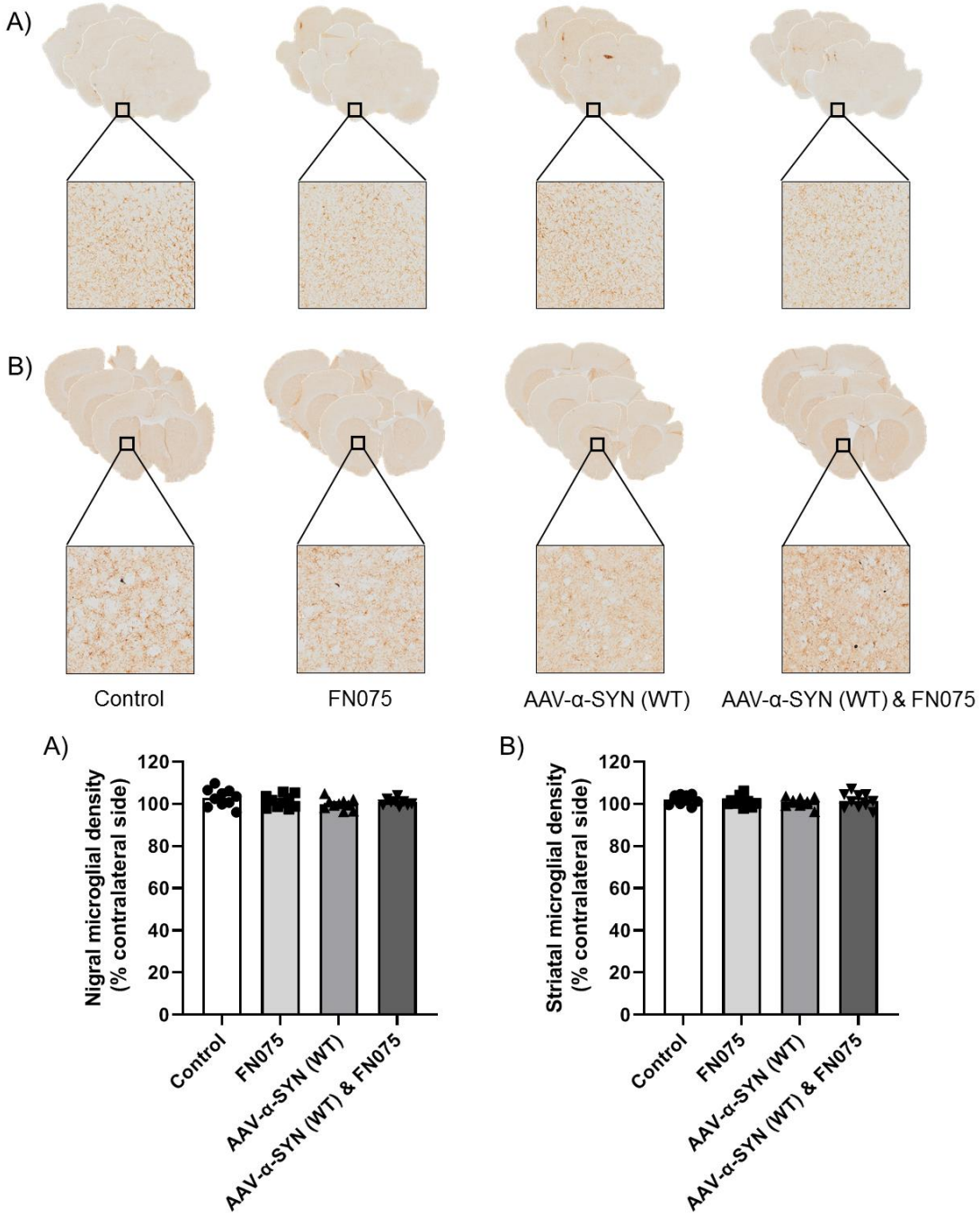

Figure 4.11 Immunohistological assessment of the impact of AAV-α-SYN (WT) alone or in combination with FN075 on the density of microglial cells. AAV-α-SYN (WT), administered alone or with FN075, did not affect the optical density of OX-42 staining in the substantia nigra (A) or the striatum (B). Data are represented as mean ± SEM with n=10 animals per group and were analysed by one-way ANOVA.

4.3.1.6 AAV-α-synuclein administration did not affect astrocytosis

Further to our analyses of microglial cells, we also sought to investigate if either the viral vectors or the α-synuclein aggregating molecule FN075 induced alterations in the densities of astrocytes in the nigrostriatal pathway. Astrocytes were visualised by carrying out quantitative staining for glial fibrillary acidic protein (GFAP), a type III intermediate filament protein expressed by astrocytes. We did not observe any differences in astrocytic density between groups in either the substantia nigra (Figure 4.12A; Group, $F_{(3,36)}$=1.33, P>0.05) or the striatum (Figure 4.12B; Group, $F_{(3,36)}$=2.28, P>0.05).

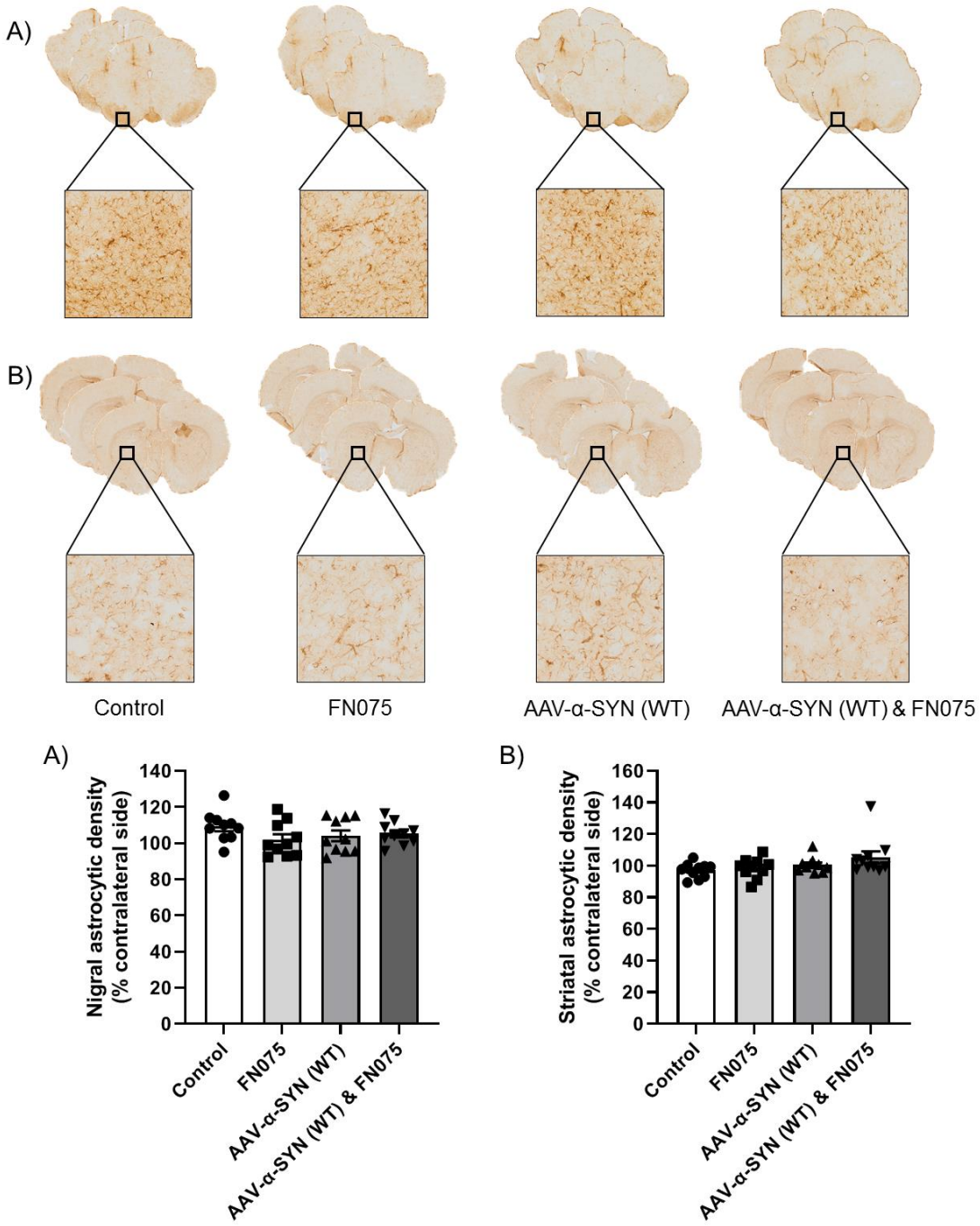

Figure 4.12 Immunohistological assessment of the impact of AAV-α-SYN (WT) alone or in combination with FN075 on the density of astrocytic cells. AAV-α-SYN (WT), either alone or with FN075, did not affect the optical density of GFAP staining in the substantia nigra (A) or in the striatum (B). Data are represented as mean ± SEM with n=10 animals per group and were analysed by one-way ANOVA.

4.3.2 COMBINATION OF AAV-INDUCED A53T MUTANT ALPHA-SYNUCLEIN OVEREXPRESSION WITH THE ALPHA-SYNUCLEIN AGGREGATING MOLECULE, FN075

4.3.2.1 Unilateral, intra-nigral AAV-α-synuclein administration alone or in combination with FN075 did not induce contralateral motor impairment

As we did not detect dopaminergic degeneration using the wild-type vector, we decided to carry out a similar study using a vector that expresses the A53T mutated form of the α-synuclein protein, a mutation that is associated with an autosomal dominant familial form of Parkinson's disease (Polymeropoulos *et al.,* 1997).

Before embarking on *post mortem* immunohistochemical analyses for this study, we first sought to determine if a deterioration in motor function was induced throughout the study by administration of the AAV-α-synuclein A53T mutant vector or FN075, separately or in combination. Motor impairment is one of the major characteristics of Parkinson's disease patients and thus a key element in a Parkinsonian model. We performed the surgeries unilaterally, so any impairment would be restricted to one side of the body (contralateral to the side of the brain injected), thus allowing the other side (ipsilateral) to serve as an internal control.

As expected, in this study there were no differences in ipsilateral motor function in the Corridor test of sensorimotor neglect (Figure 4.13A; Group x Time, $F_{(21,217)}$=0.91, P>0.05), the Stepping test of forelimb akinesia (Figure 4.13B; Group x Time, $F_{(27,279)}$=0.76, P>0.05), or the Whisker test of sensorimotor integration (Figure 4.13C; Group x Time, $F_{(27,279)}$=0.82, P>0.05). In the Stepping test, there was a significant effect of time similar to the previous study, suggesting a habituation effect as the animals became more accustomed to the handling (Figure 4.13B; Time, $F_{(2.98,92.30)}$=89.54, P<0.05).

With regard to contralateral motor function, there were no differences observed after intra-nigral AAV-α-SYN (A53T) administration alone or in combination with FN075 at any time-point in contralateral performance in the Corridor test (Figure 4.14A; Group x Time, $F_{(21,217)}$=0.91, P>0.05), the Stepping test (Figure 4.14B; Group x Time, $F_{(27,279)}$=0.81, P>0.05), or the Whisker test (Figure 4.14C; Group x Time, $F_{(27,279)}$=0.84,

P>0.05). Similar to the ipsilateral data, a time effect was seen in the Stepping test, indicating a training effect (Figure 4.14B; Time, $F_{(3.05,94.45)}$=118.20, *P*<0.05).

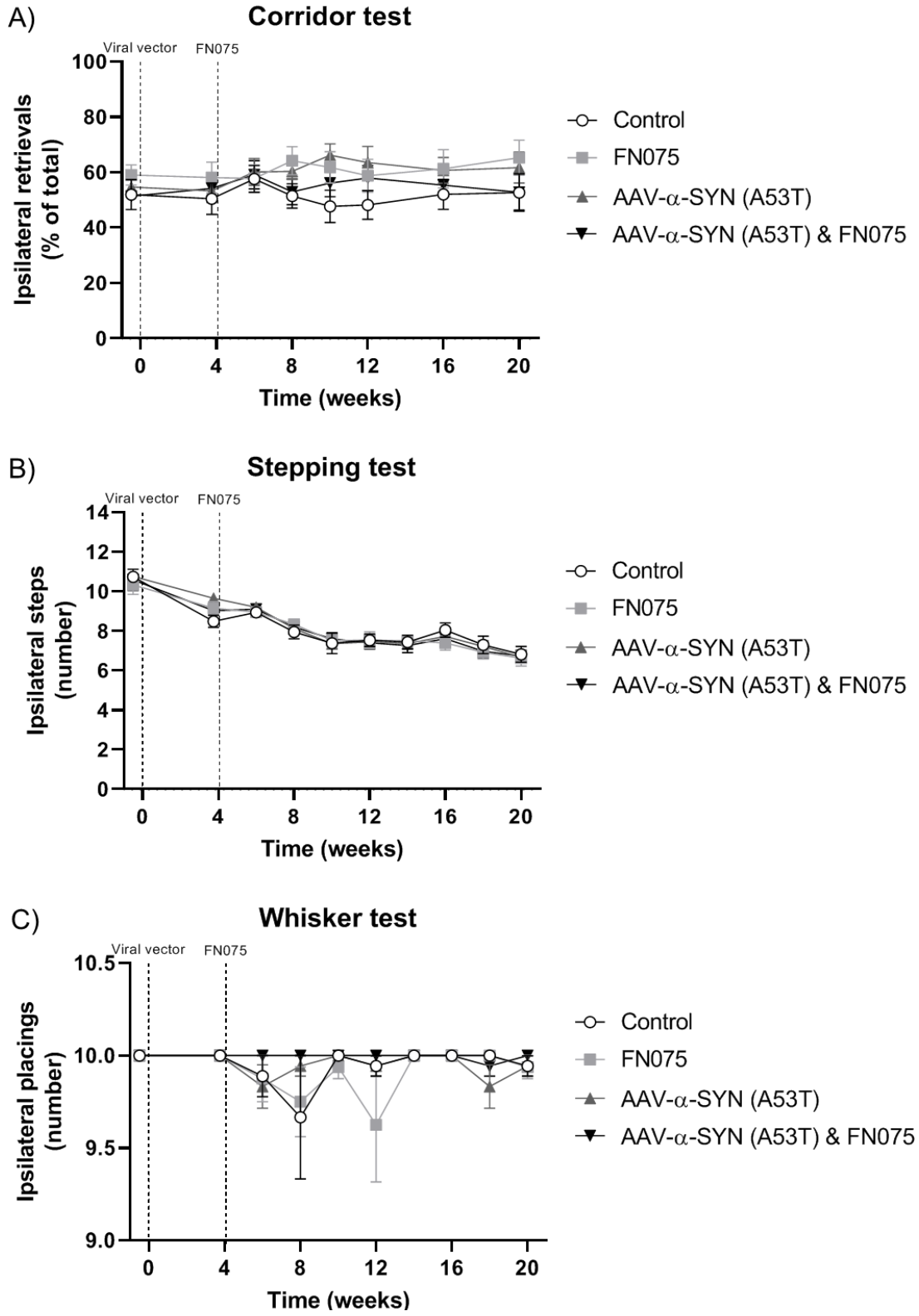

Figure 4.13 Impact of AAV-α-SYN (A53T) alone or in combination with FN075 on ipsilateral motor function. There were no differences in ipsilateral motor function in the Corridor (A), Stepping (B) or Whisker (C) behavioural tests. Data are represented as mean ± SEM with n=8-9 animals per group and were analysed by two-way repeated-measures ANOVA. Dashed lines represent the days of infusion surgeries.

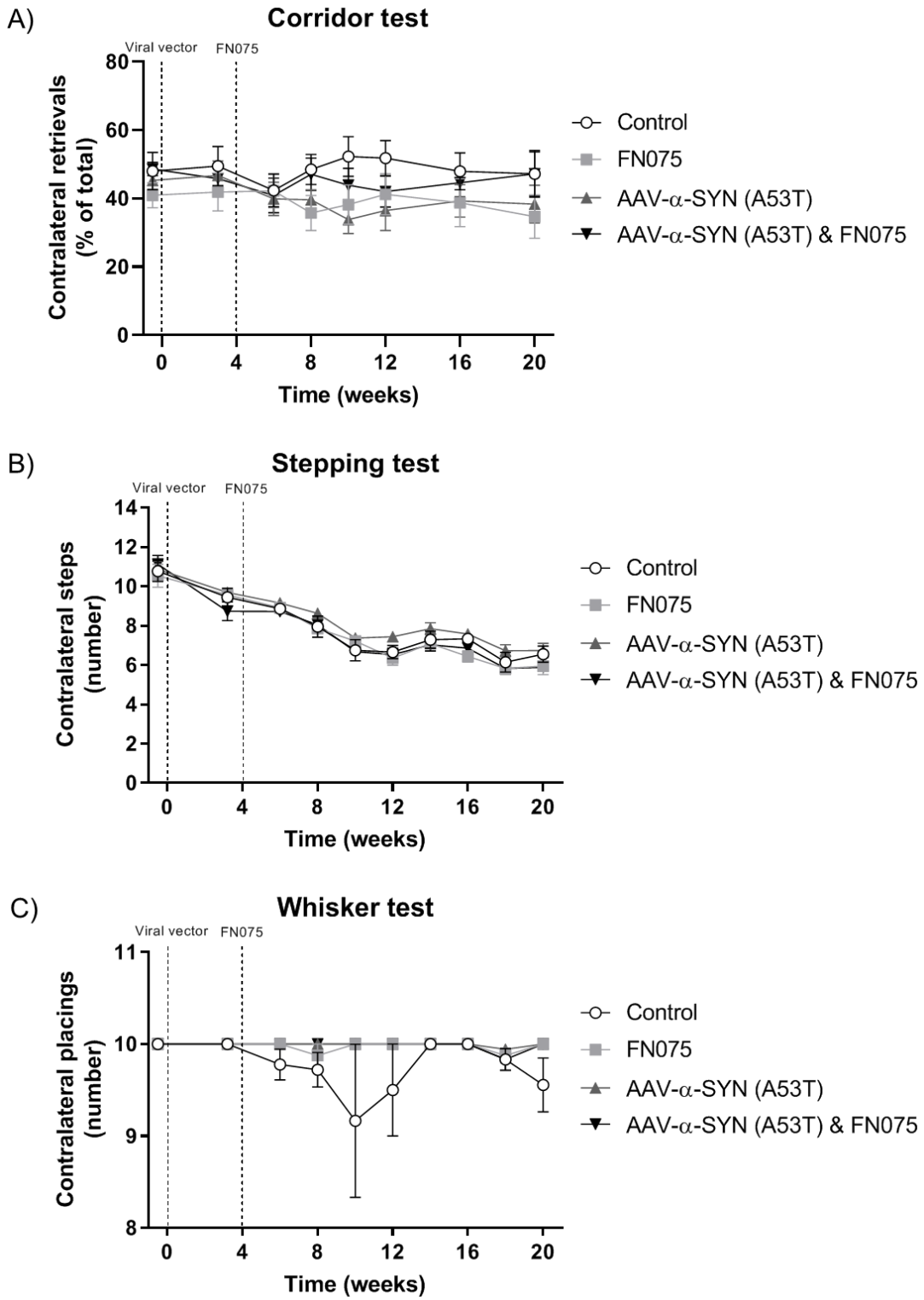

Figure 4.14 Impact of AAV-α-SYN (A53T) alone or in combination with FN075 on contralateral motor function. AAV-α-SYN (A53T), administered alone or with FN075, did not induce contralateral motor dysfunction in the Corridor (A), Stepping (B) or Whisker (C) behavioural tests. Data are represented as mean ± SEM with n=8-9 animals per group and were analysed by two-way repeated-measures ANOVA. Dashed lines represent the days of infusion surgeries.

4.3.2.2 AAV-α-synuclein administration induced significant α-synuclein expression

Once behavioural analyses were complete and the animals were sacrificed, *post mortem* we sought first to examine the expression of α-synuclein in the nigrostriatal pathway in the AAV-α-synuclein-infused animals. Immunohistochemical staining for human α-synuclein confirmed that injection of AAV-α-SYN (A53T) induced widespread human α-synuclein expression in the substantia nigra (Figure 4.15; $F_{(3,31)}$=5.94, P<0.05) and across the midbrain. As expected, α-synuclein expression was only seen in the groups which received the AAV-α-synuclein vector, and not in the groups that received the AAV-GFP vector. The α-synuclein aggregating molecule, FN075, did not affect the density of human α-synuclein staining, either in combination with AAV-α-SYN (A53T) or alone.

In the striatum, AAV-α-SYN (A53T), administered by itself or sequentially with FN075, caused a significant overexpression of human α-synuclein on the ipsilateral side compared to the Control group, but FN075 did not have any exacerbatory effect on expression (Figure 4.16; Group, $F_{(3,31)}$=7.58, P<0.05).

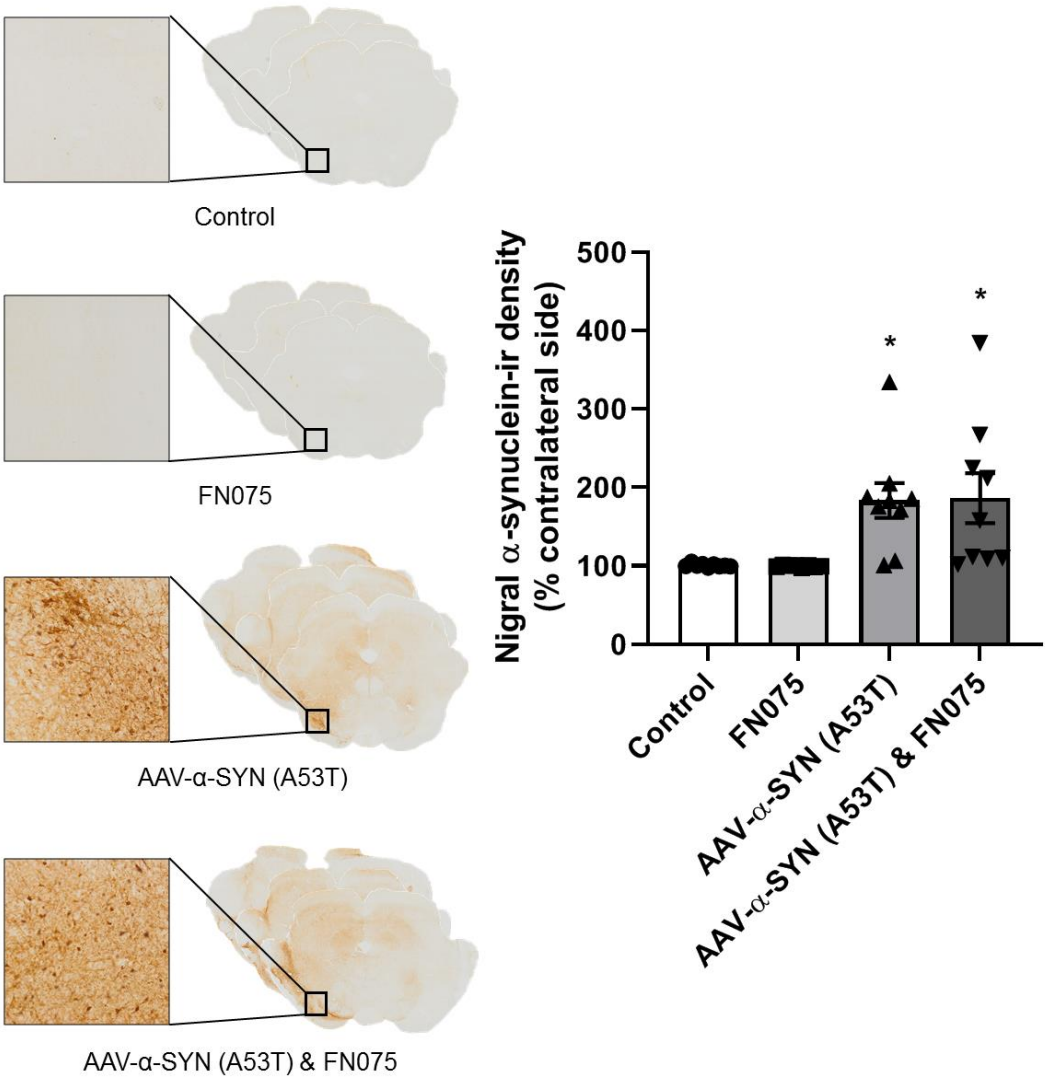

Figure 4.15 Immunohistological assessment of the impact of AAV-α-SYN (A53T) alone or in combination with FN075 on the expression of human α-synuclein in the substantia nigra. AAV-α-SYN (A53T) caused a significant overexpression of human α-synuclein in the substantia nigra, but FN075 had no additive effect. Data are represented as mean ± SEM with n=8-9 animals per group. **P*<0.05 vs. Control by one-way ANOVA with *post hoc* Tukey.

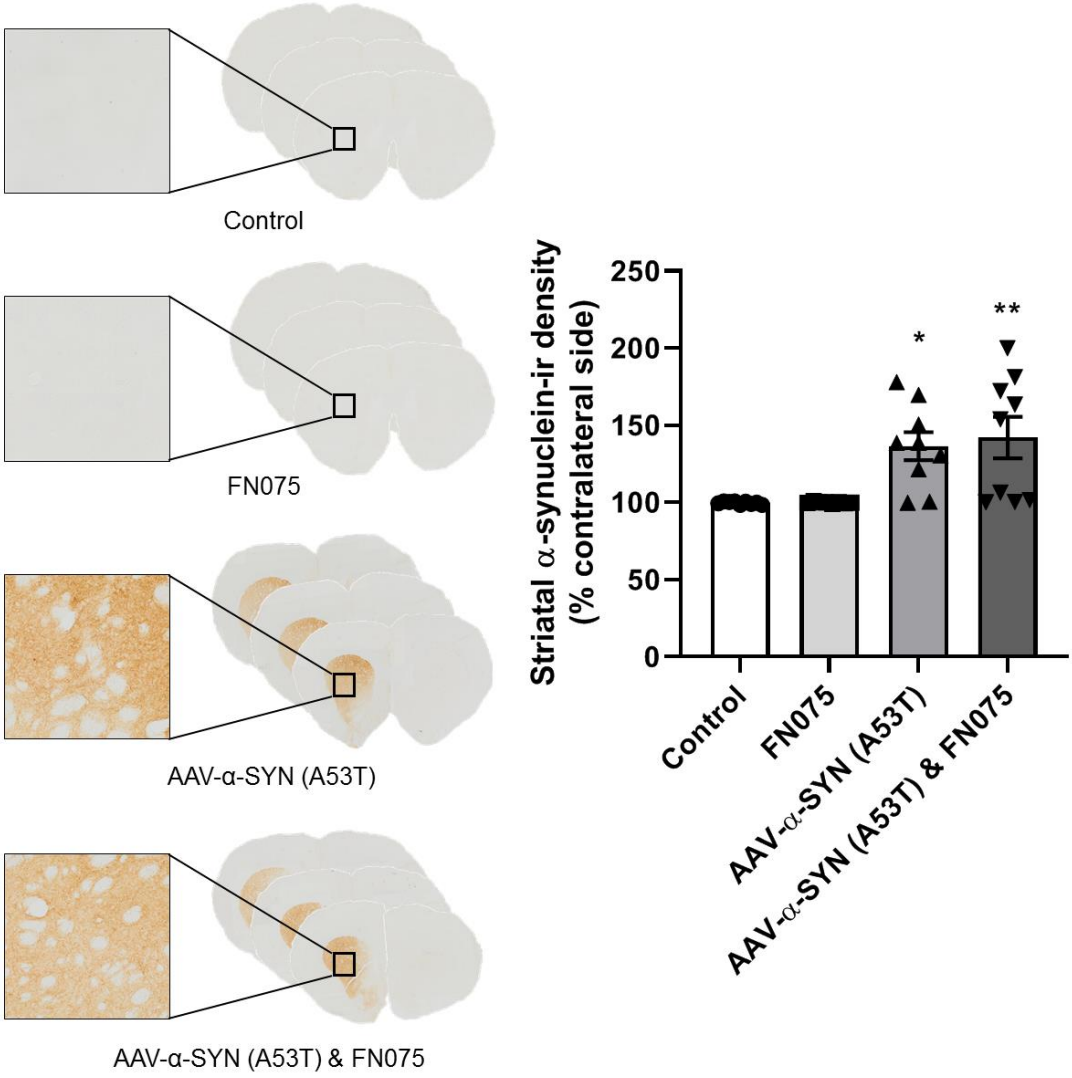

Figure 4.16 Immunohistological assessment of the impact of AAV-α-SYN (A53T) alone or in combination with FN075 on the expression of human α-synuclein in the striatum. AAV-α-SYN (A53T) caused a significant overexpression of human α-synuclein in the striatum, alone or with FN075. Data are represented as mean ± SEM with n=8-9 animals per group. **P*<0.05, ***P*<0.01 vs. Control by one-way ANOVA with *post hoc* Tukey.

4.3.2.3 FN075 significantly increased α-synuclein phosphorylation at serine 129

Following confirmation of human α-synuclein expression in the nigrostriatal pathway, we investigated if FN075 affected the phosphorylation of α-synuclein by utilising an antibody that is specific for α-synuclein that is phosphorylated at the serine 129 residue.

We determined that FN075, in combination with the AAV-α-SYN (A53T) vector, significantly increased the density of pS129-α-synuclein, compared to both the Control group and the AAV-α-SYN (A53T) group (Figure 4.17; Group, $F_{(3,32)}$=4.74, P<0.05). Under high magnification, accumulations of the protein were clearly evident in this combined AAV-α-SYN (A53T) & FN075 group. We carried out a second analysis method by counting the number of these pS129-α-synuclein-positive accumulations on the lesioned side of the brain. We found that FN075 in combination with AAV-α-SYN (A53T) induced a significant increase in the number of accumulations compared to the control group (Figure 4.18; Group, $F_{(3,31)}$=4.85, P<0.05).

Furthermore, we carried out a stain specific for α-synuclein in the filamentous form. Similarly to the previous study, aggregates were visible in some animals under high magnification, suggesting that FN075 in combination with AAV-α-synuclein may induce α-synuclein aggregation on the ipsilesional side (Figure 4.19).

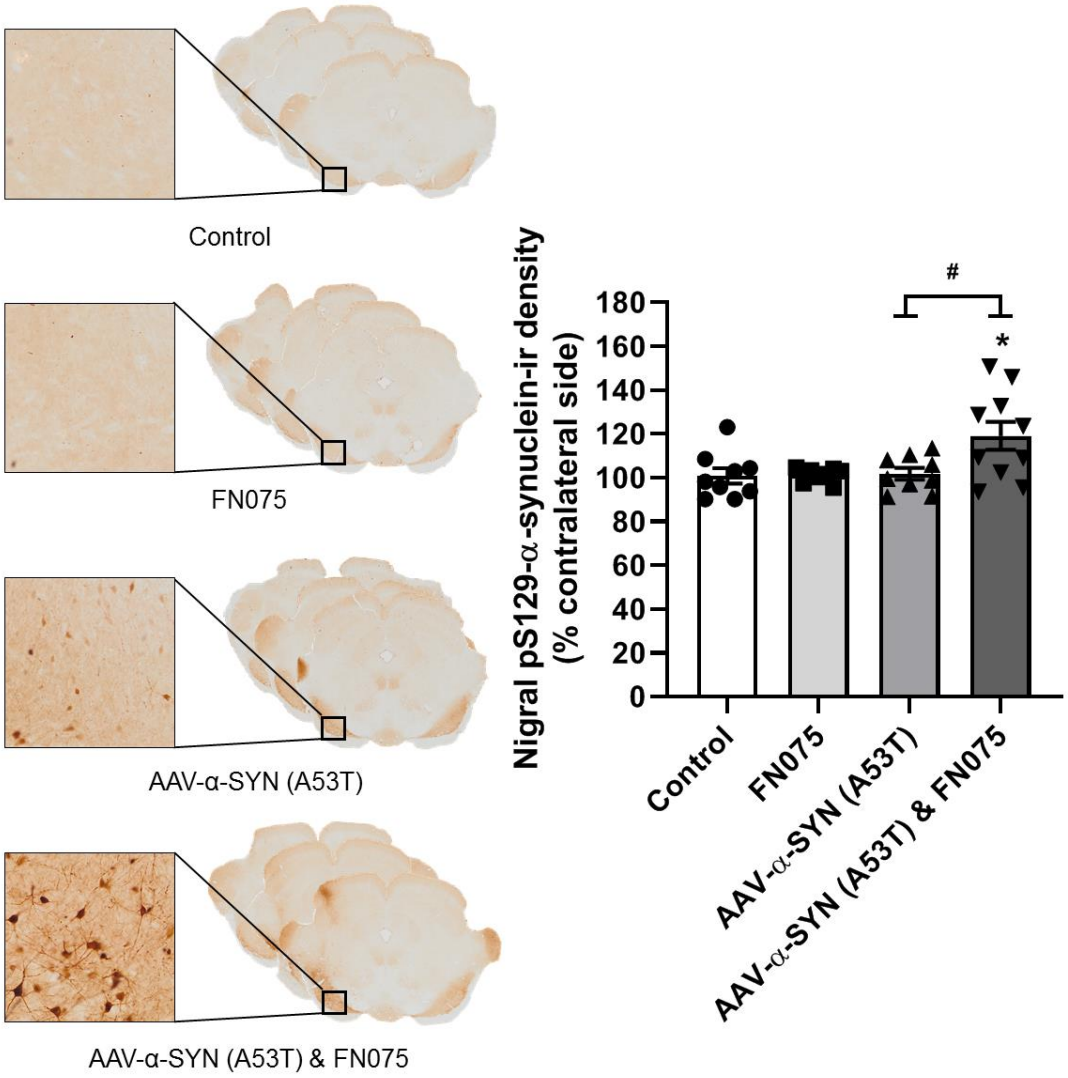

Figure 4.17 Immunohistological assessment of the impact of FN075 on the phosphorylation of α-synuclein at serine 129 in the substantia nigra. FN075 in combination with AAV-α-SYN (A53T) induced a significant increase in the density of phosphorylated α-synuclein in the substantia nigra. Data are represented as mean ± SEM with n=8-10 animals per group. **P*<0.05 vs. Control; $^{\#}P$<0.05 vs. AAV-α-SYN (A53T) by one-way ANOVA with *post hoc* Tukey.

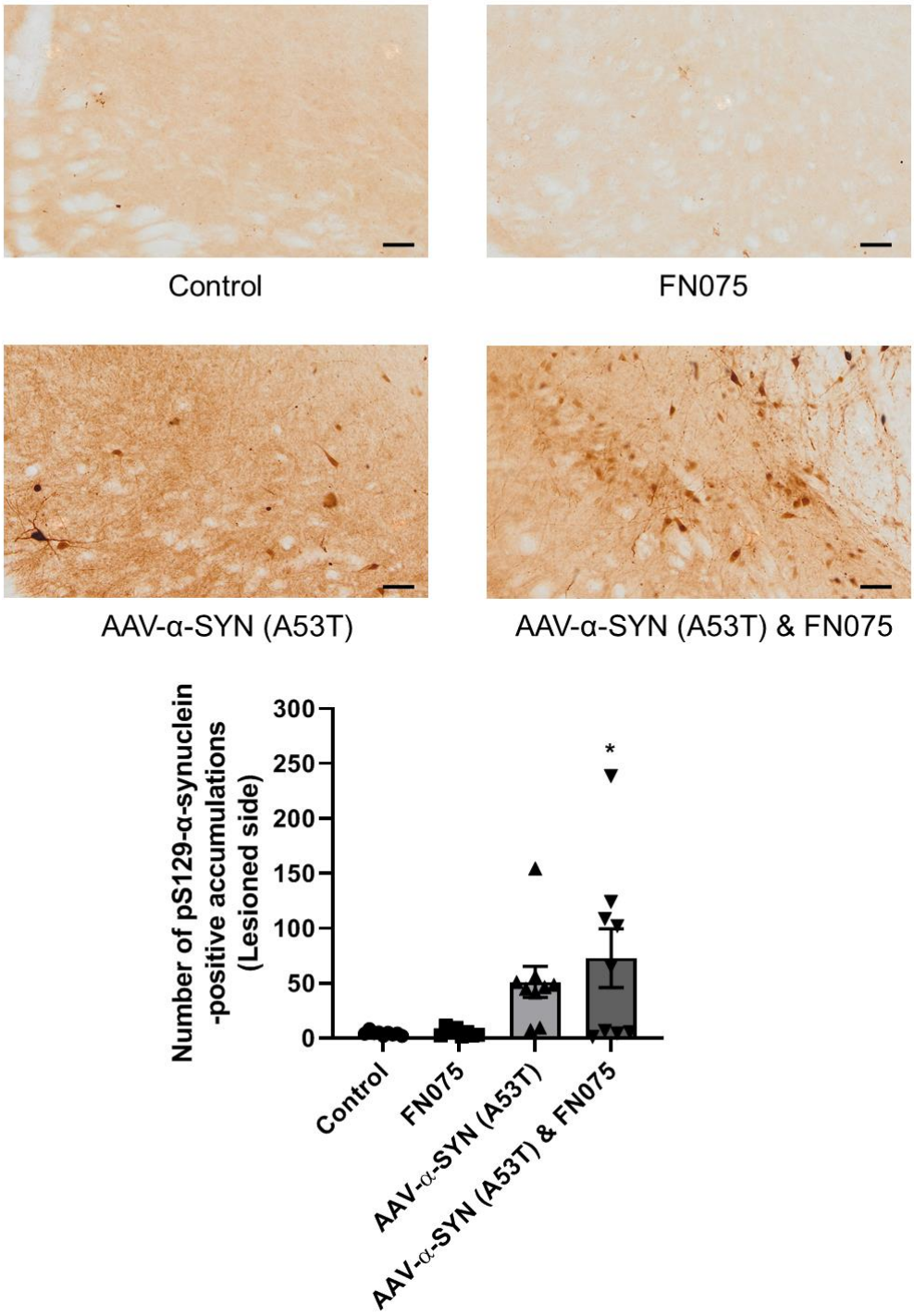

Figure 4.18 Immunohistological assessment of the impact of the small molecule FN075 on the accumulation of phosphorylated α-synuclein in the substantia nigra. FN075 in combination with AAV-α-SYN (A53T) induced a significant increase in the number of pS129-α-synuclein-positive accumulations in the substantia nigra. Scale bars represent 50 µm. Data are represented as mean ± SEM with n=8-10 animals per group. **P*<0.05 vs. Control by one-way ANOVA with *post hoc* Tukey.

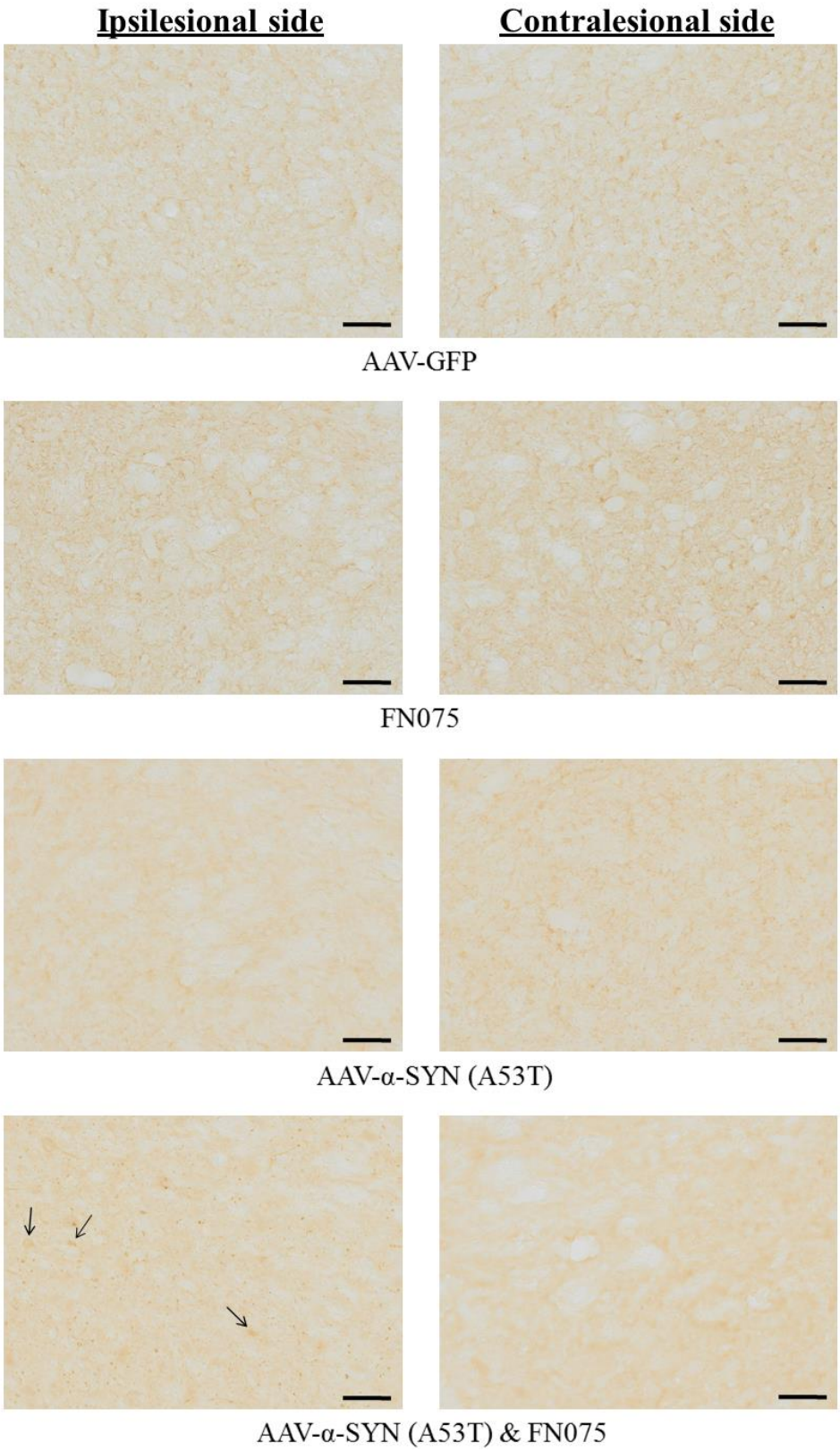

Figure 4.19 Immunohistological visualisation of the impact of the small molecule FN075 on the expression of α-synuclein filaments in the substantia nigra. FN075 in combination with AAV-α-SYN (A53T) induced the aggregation of α-synuclein in the substantia nigra on the ipsilesional side. Scale bars represent 50 µm.

4.3.2.4 AAV-α-synuclein administration did not cause degeneration in the nigrostriatal pathway

Following analyses of α-synuclein expression, we next sought to determine if the overexpression of α-synuclein that we had visualised in the substantia nigra and the striatum was sufficient to induce degeneration of dopaminergic neurons in the nigrostriatal pathway, as the slow progressive death of the neurons in the nigrostriatal pathway is a key pathological characteristic of Parkinson's disease. However, in this study, we did not detect any differences with regard to the number of TH-positive cells in the substantia nigra (Figure 4.20; Group, $F_{(3,31)}$=2.04, P>0.05) and similarly in the striatum, there were no differences observed between groups with regard to the density of staining of the nerve terminals (Figure 4.21; Group, $F_{(3,31)}$=3.26, P>0.05). Further to the striatal analysis, these tyrosine hydroxylase-stained sections were also analysed for the presence of dystrophic neurites. In this study, there were no significant differences between groups regarding the number of dystrophic neurites in the striatum (Figure 4.22; Group, $F_{(3,31)}$=2.69, P>0.05).

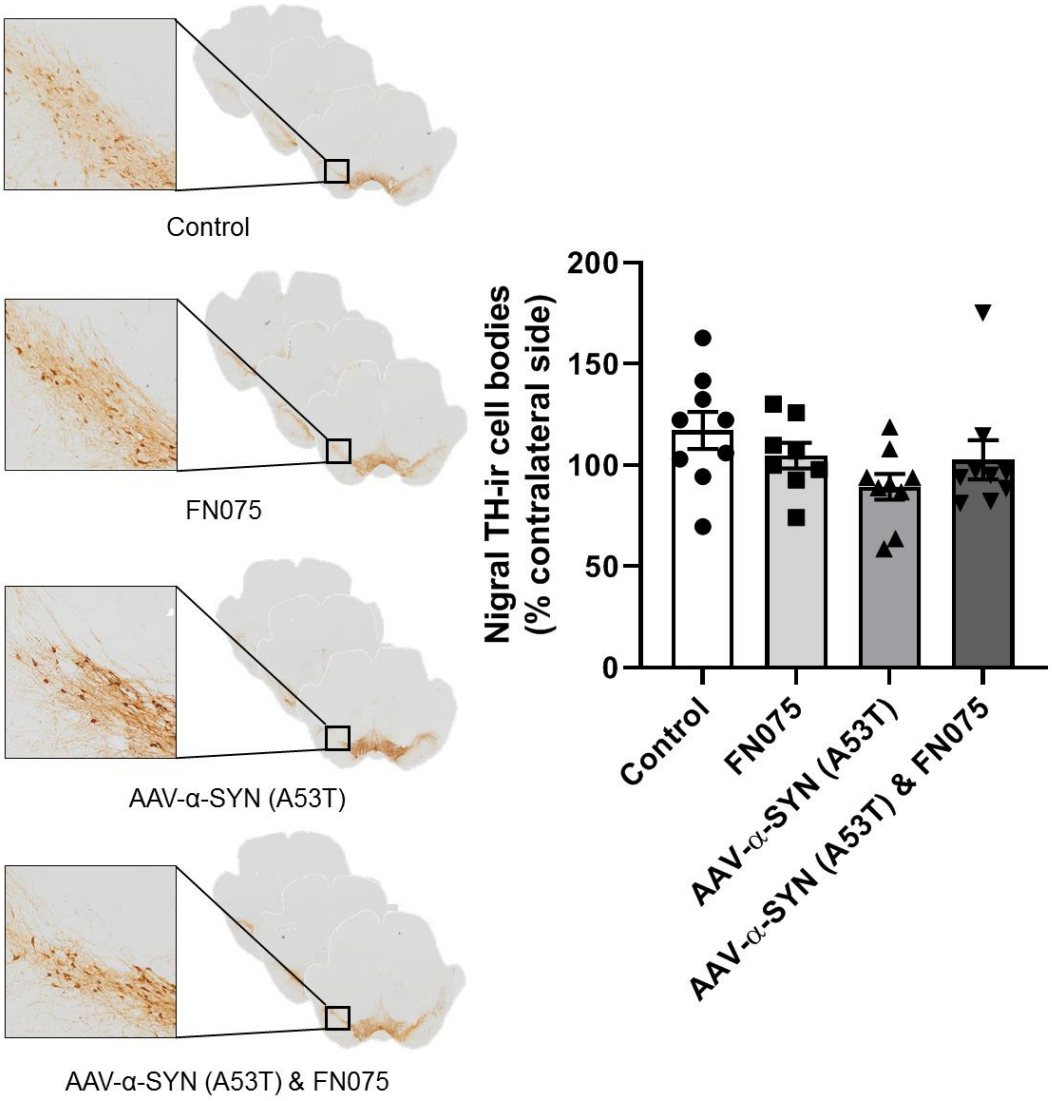

Figure 4.20 Immunohistological assessment of the impact of AAV-α-SYN (A53T) alone or in combination with FN075 on nigral cell counts. AAV-α-SYN (A53T) and/or FN075 did not significantly affect the number of TH-immunoreactive cell bodies in the substantia nigra. Data are represented as mean ± SEM with n=8-9 animals per group and were analysed by one-way ANOVA.

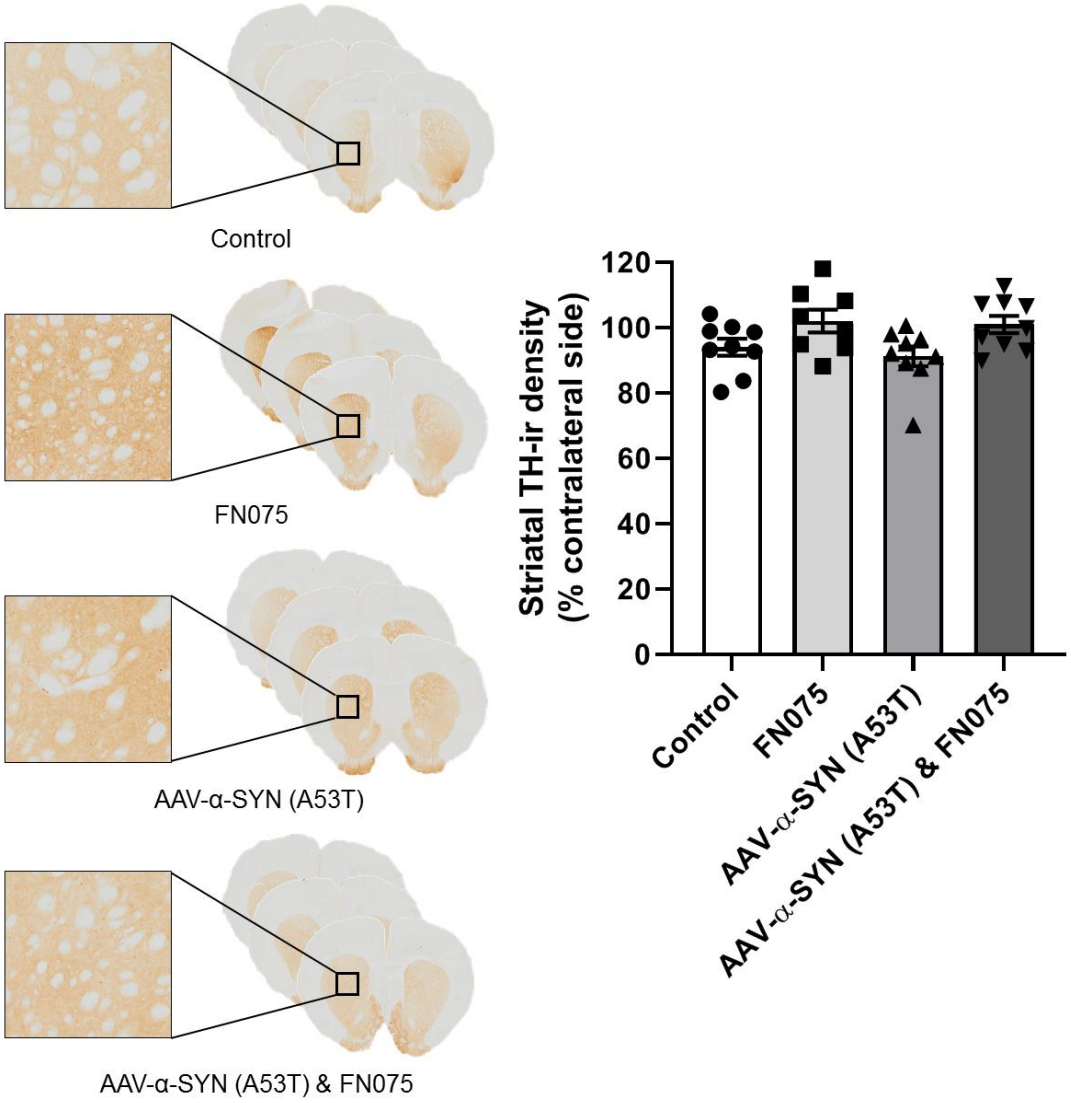

Figure 4.21 Immunohistological assessment of the impact of AAV-α-SYN (A53T) alone or in combination with FN075 on the density of dopaminergic nerve terminals in the striatum. AAV-α-SYN (A53T), alone or with FN075, did not significantly affect the optical density of TH staining in the striatum. Data are represented as mean ± SEM with n=8-9 animals per group and were analysed by one-way ANOVA.

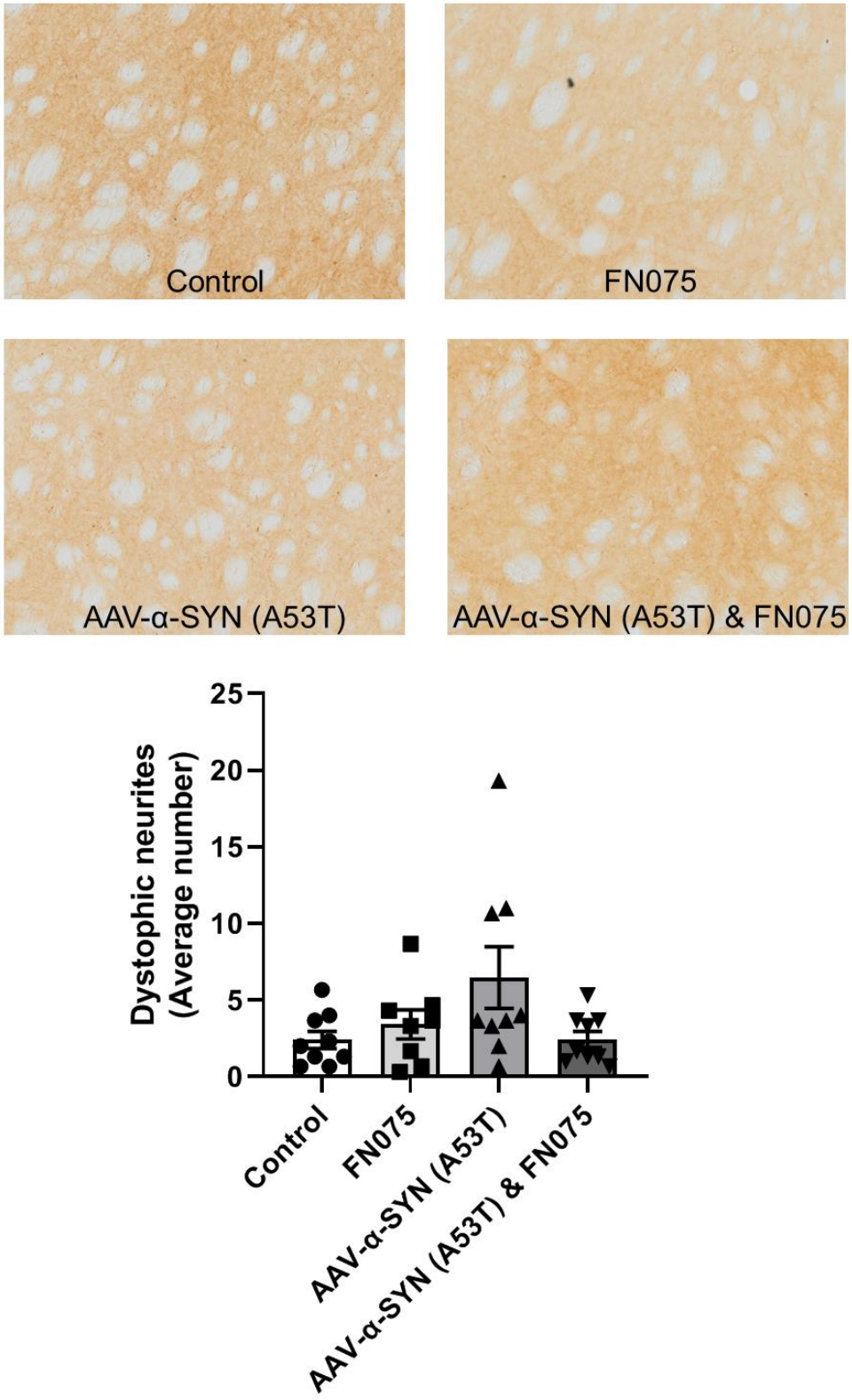

Figure 4.22 Immunohistological assessment of the impact of AAV-α-SYN (A53T) alone or in combination with FN075 on striatal dopaminergic dystrophic neurites. AAV-α-SYN (A53T) and/or FN075 did not significantly affect the number of dystrophic neurites in the striatum. Data are represented as mean ± SEM with n=8-9 animals per group and were analysed by one-way ANOVA.

4.3.2.5 AAV-α-synuclein administration did not affect microgliosis

Although we did not detect any alterations in the integrity of the dopaminergic neurons in the nigrostriatal pathway, we wished to assess if there were any alterations in the microglial response in this pathway by carrying out quantitative OX-42 staining. This stain recognises the CD11b protein that is highly expressed on macrophages and microglia. We did not observe differences in microglial density between groups in either the substantia nigra (Figure 4.23A; Group, $F_{(3,31)}$=0.06, P>0.05) or the striatum (Figure 4.23B; Group, $F_{(3,31)}$=0.61, P>0.05).

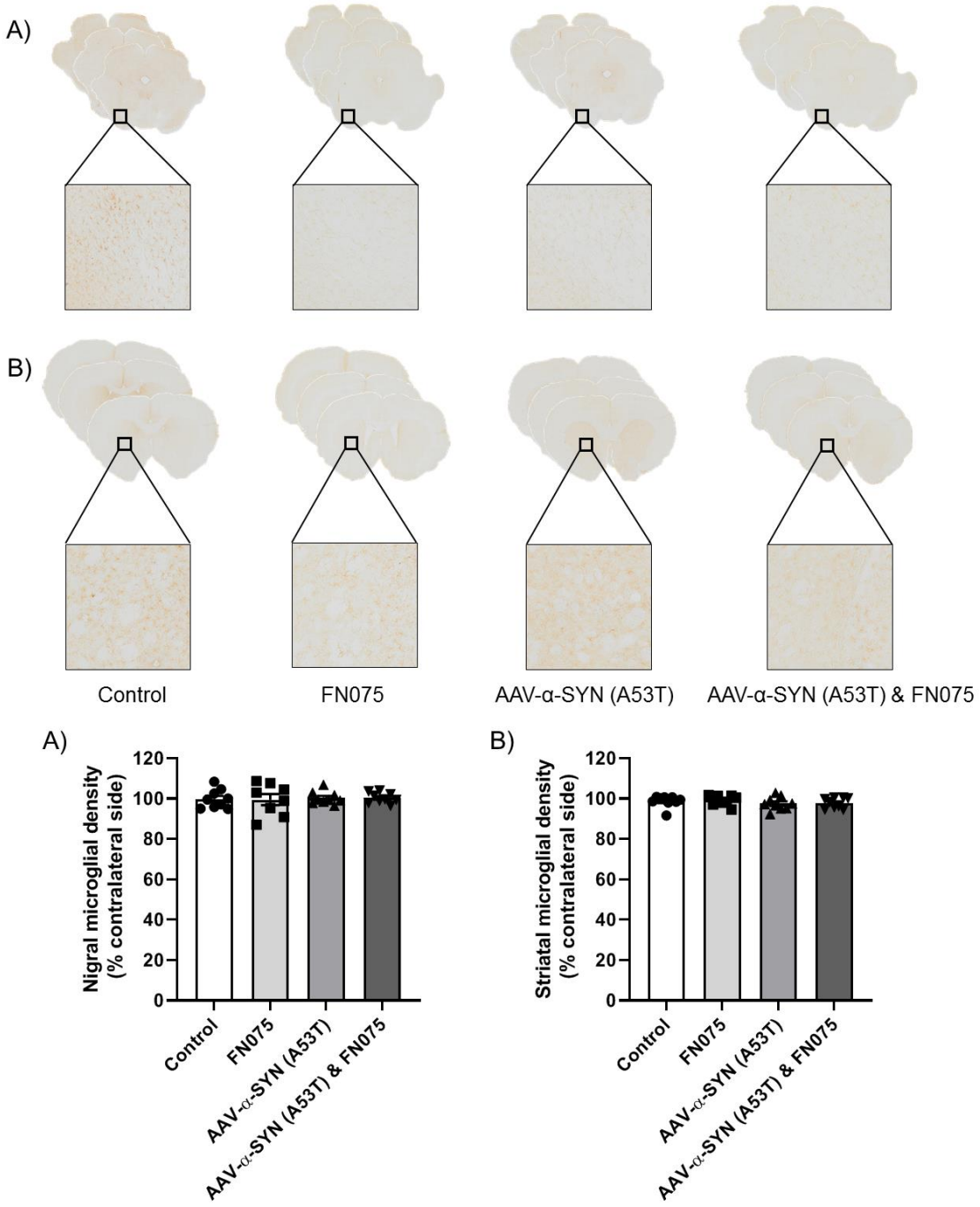

Figure 4.23 Immunohistological assessment of the impact of AAV-α-SYN (A53T) alone or in combination with FN075 on microglial cells. AAV-α-SYN (A53T), with or without FN075, did not affect the optical density of OX-42 staining in the substantia nigra (A) or the striatum (B). Data are represented as mean ± SEM with n=8-9 animals per group and were analysed by one-way ANOVA.

4.3.2.6 AAV-α-synuclein administration did not affect astrocytosis

Further to our analyses of microglial densities, we investigated if either the AAV-A53T-α-synuclein vector or the α-synuclein aggregating molecule FN075 had an effect on the astrocytic response in the nigrostriatal pathway, by carrying out quantitative GFAP staining. This stains for glial fibrillary acidic protein, a filament protein expressed by astrocytes in the CNS. We did not detect any differences between groups with regard to astrocytosis in either the substantia nigra (Figure 4.24A; Group, $F_{(3,31)}$=0.52, P>0.05) or in the striatum (Figure 4.24B; Group, $F_{(3,31)}$=1.82, P>0.05).

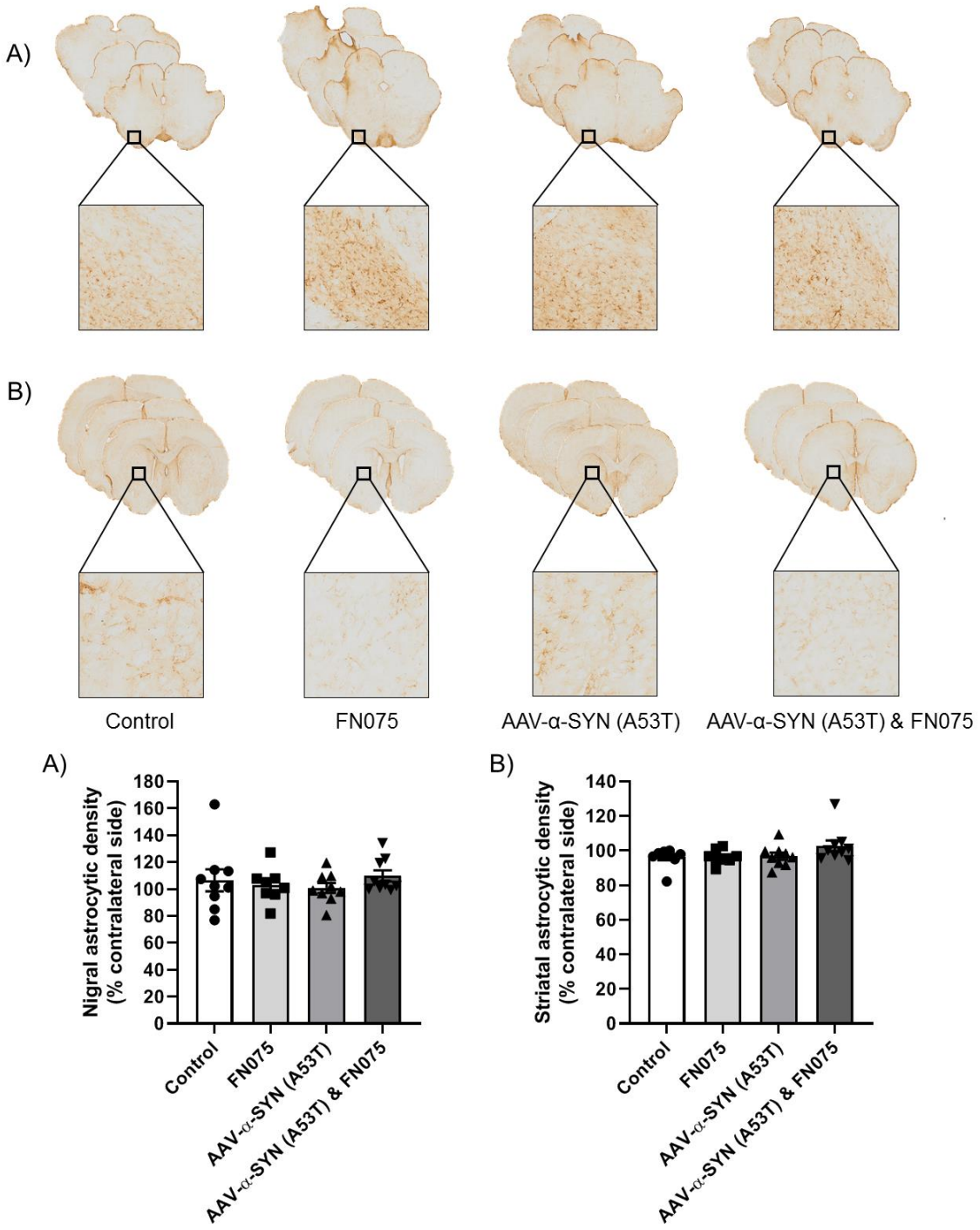

Figure 4.24 Immunohistological assessment of the impact of AAV-α-SYN (A53T) alone or in combination with FN075 on astrocytes. AAV-α-SYN (A53T), administered alone or sequentially with FN075, did not affect the optical density of GFAP staining in the substantia nigra (A) or in the striatum (B). Data are represented as mean ± SEM with n=8-9 animals per group and were analysed by one-way ANOVA.

4.4 DISCUSSION

Most of the currently available animal models of Parkinson's disease involve the administration of a toxin, either systemically or directly into the brain, which causes degeneration of dopaminergic neurons in the nigrostriatal pathway. While these models are undeniably useful in the study of some aspects of the disease, they are not entirely representative with regard to the rapidity at which they cause neuronal death and the mechanism by which this death is caused. The establishment of the AAV-α-synuclein model was a major advancement to the field, as it can induce α-synuclein overexpression and a gradual neuronal loss that is more representative of the changes occurring in the brain in Parkinson's disease patients (Kirik *et al.*, 2002). However, this model is still limited as it takes months to progress, and it is also associated with a very high degree of variability. Therefore, the development of novel animal models of this disease is vital for the future study of the discipline.

Several attempts have been made thus far to improve upon the AAV-α-synuclein Parkinson's disease model. The laboratory of Anders Björklund recently obtained promising results for a potential novel model by a combination of preformed fibrils and AAV-α-synuclein, reproducing Lewy body pathology and progressive dopamine cell loss (Thakur *et al.*, 2017). This synucleinopathy induced a profound inflammatory response, with activation of microglia and lymphocyte infiltration. Furthermore, a study carried out by a previous member of our research group found that the sequential intra-nigral administration of AAV-α-synuclein and the pesticide, rotenone, significantly exacerbated nigrostriatal degeneration and induced a progressive decline in motor function (Mulcahy *et al.*, 2012). Sequential administration of subclinical doses of intra-nigral AAV-α-synuclein and intra-striatal rotenone similarly precipitated motor impairment and degeneration of nigral cell bodies (Naughton *et al.*, 2017). Undoubtedly, there is enormous potential in using a dual approach to modelling Parkinson's disease pathology.

FN075 is a novel small molecule that has been shown to accelerate α-synuclein aggregation, and the fibrils formed by this process are structurally similar to those formed by naturally occurring aggregation (Cegelski *et al.*, 2009; Horvath *et al.*, 2012). Given the aforementioned problems with the AAV-α-synuclein model and the promising results from previous *in vivo* FN075 studies (Chermenina *et al.*, 2015; Olsen *et al.*, 2019), we

decided to attempt to develop a more relevant and more rapidly progressing model of Parkinson's disease by combining AAV-α-synuclein with FN075, the results of which are presented in this chapter. Across two studies, we administered a viral vector that expressed either human wild-type α-synuclein or human α-synuclein with the A53T mutation, and then four weeks later administered the peptidomimetic small molecule FN075. We assessed lateralised motor performance throughout the studies and assessed α-synuclein expression, dopaminergic degeneration and neuroinflammation *post mortem*. In both studies, we observed that the α-synuclein viral vector induced a significant overexpression of human α-synuclein in the nigrostriatal pathway. Furthermore, in both studies, FN075 in combination with the α-synuclein viral vectors caused a significant increase in phosphorylation of α-synuclein, as measured by both optical density and by counts of pS129-α-synuclein-positive accumulations. However, this α-synuclein overexpression and pathology was not sufficient to induce nigrostriatal degeneration, which explains the lack of noticeable impairments in the lateralised motor tests.

As previously stated, the degeneration of the dopaminergic neurons in the nigrostriatal pathway is the main pathological characteristic of the Parkinsonian brain, and the impairments in motor function that are the primary clinical manifestations in Parkinson's disease patients are a consequence of the loss of these neurons. Therefore, when discussing an animal model of the disease, the loss of these neurons is the key feature that needs to be replicated in order to be considered a valid model. In the first study, we used a vector that expressed the wild-type form of α-synuclein but when we did not observe dopaminergic degeneration using this vector, we carried out the second study, in which the AAV vector expressed the A53T mutated form of human α-synuclein. This mutation is associated with an autosomal dominant familial form of Parkinson's disease (Polymeropoulos *et al.*, 1997), and has been seen to accelerate fibril formation compared to the wild-type form (Conway *et al.*, 1998). Furthermore, in a comparison *in vivo* rat study, an A53T AAV vector induced more motor impairment and increased dopaminergic cell loss compared to a wild-type vector (Lu *et al.*, 2015). However, as with our wild-type study, although administration of the A53T α-synuclein vector resulted in widespread α-synuclein expression, it did not induce dopaminergic degeneration. These results emphasise the variability that is associated with this model. There is a vast number of factors that differ between the viral vectors used by distinct research groups, including

the serotype, the promotor, and the titres, which all influence the ability of the vector to induce specific nigrostriatal degeneration. A viral vector that has been shown to be consistently capable of inducing neuronal death would be required in order to fully ascertain the viability of a combined model with FN075, but this inconsistency between viral vectors serves to highlight the faults in the AAV-α-synuclein model and the requirement for a novel improved Parkinson's disease model.

As expected, we observed that the α-synuclein aggregating molecule FN075 did not alter the total amount of α-synuclein, but we observed that it did affect the pathology of the α-synuclein that was being expressed by the viral vector. In the two studies presented in this chapter, FN075 in combination with the AAV-α-synuclein vectors significantly increased phosphorylated α-synuclein compared to the control, as measured by both the optical density analyses and the pS129-α-synuclein accumulation counts. The phosphorylation of α-synuclein is a characteristic trait of Lewy bodies, with 90% of α-synuclein deposited in Lewy bodies extensively phosphorylated at serine 129. In comparison, only 4% of α-synuclein in healthy brains is phosphorylated at this residue (Fujiwara *et al.*, 2002; Anderson *et al.*, 2006). Therefore, it is promising that our novel combined model is consistent with regard to this pathophysiological feature.

Neuroinflammation is a key feature of Parkinson's disease and has been proposed to contribute to the disease in a self-sustaining cyclical manner: the death of neurons causes the activation of neuroinflammatory cells which induces further neuronal death (Frank-Cannon *et al.*, 2009; Tansey and Goldberg, 2010). Therefore in the absence of neuronal death, a lack of a neuroinflammatory response would not be unexpected. Furthermore, relative to other neurotoxic models, the neuroinflammatory response to AAV-driven α-synuclein expression is relatively mild, with less overt microgliosis evident (Sanchez-Guajardo *et al.*, 2010). Indeed, microglia have been shown to acquire distinct activation profiles depending on whether α-synuclein pathology induced cell death (Sanchez-Guajardo *et al.*, 2010). Given these findings, and the absence of significant neuronal death in both studies presented here, it was not surprising that our quantitative immunohistological analyses for microglia and astrocytes did not reveal a conspicuous neuroinflammatory response.

In summary, it is not possible to adjudicate regarding the feasibility of a combined AAV-α-synuclein and FN075 model from the results presented in this chapter, as in order to draw any conclusions about this novel combined model an AAV-α-synuclein vector that independently induces significant dopaminergic degeneration is required. With such a vector, any additive or exacerbatory effects by subsequent exposure to FN075 can be properly elucidated. However, the presence of the large intracellular accumulations of phosphorylated α-synuclein which were formed by the combination of AAV-α-synuclein and FN075 is a promising result, and this potential model decidedly warrants further investigation. However, this model needs to be developed further (with regard to the specific vector used and its titre, the dose of FN075 administered, and the timings between the two lesion surgeries) before it can be utilised beneficially for studies of the endocannabinoid system. Therefore, for the subsequent chapters in which we investigate the potential of targeting the CB_2 receptor for disease modulation, we utilised models that have been substantiated previously.

Chapter 5: Pharmacological targeting of the CB_2 receptor in a viral priming Parkinson's disease model

5.1 INTRODUCTION

The most widely accepted view of the aetiology of Parkinson's disease is that it is caused by interactions between environmental exposures and a person's genetics (Gao and Hong, 2011). An association between viral infection and Parkinson's disease was first proposed over a century ago, when an epidemic of post-encephalitic Parkinson's cases followed the Spanish influenza outbreak of 1918-1920 (Ravenholt and Foege, 1982; Hoffman and Vilensky, 2017). Henceforth, numerous other viral infections have been linked to an increased incidence of the disease, including herpes simplex virus (HSV), influenza virus A, and mumps (Marttila *et al.*, 1977; Marttila and Rinne, 1978; Harris *et al.*, 2012; Vlajinac *et al.*, 2013; Olsen *et al.*, 2018). A recent meta-analysis found that overall, infectious disease (both bacterial and viral), increased the risk of Parkinson's disease by 20% (Meng *et al.*, 2019). We are presently still amidst the COVID-19 viral global pandemic, but already there have been several case reports of COVID-19 induced Parkinsonism (Cohen *et al.*, 2020; Faber *et al.*, 2020; Méndez-Guerrero *et al.*, 2020). Researchers have suggested that a Parkinson's epidemic may be the 'third wave' of the COVID-19 crisis (Beauchamp *et al.*, 2020). Therefore, it is all the more critical now that we understand the link between viral infections and the development of Parkinson's disease, and that we determine if an intervening treatment could halt the onset of such Parkinsonian cases.

There is evidence to suggest that viral infections 'prime' microglia, the innate immune cells of the brain, making them more responsive to subsequent pathological challenges (Deleidi *et al.*, 2010; Bobyn *et al.*, 2012; Olsen *et al.*, 2019). Thus, a viral infection can drive a chronic inflammatory response in the brain and initiate the pathological self-sustaining cycle of neuroinflammation and neurodegeneration that is well documented in Parkinson's disease (Frank-Cannon *et al.*, 2009; Tansey and Goldberg, 2010). Suppositionally, such an inflammatory response and hence the onset of Parkinson's disease could be halted with an anti-neuroinflammatory therapy regimen.

The cannabinoid CB_2 receptor has emerged as a potential target for anti-inflammatory therapy in Parkinson's disease in recent years due to several converging lines of evidence. A pronounced upregulation of CB_2 receptors on microglia has been identified in patients in the substantia nigra (Gómez-Gálvez *et al.*, 2016). A similar increase in expression has also been observed in other neurodegenerative diseases, such as Alzheimer's disease and Huntington's disease (Benito *et al.*, 2003; Palazuelos *et al.*, 2009). This has been corroborated by animal studies, as a pronounced increase in CB_2 receptor expression has been noted in both neurotoxic and inflammatory Parkinson's disease models, with a more marked increase in inflammatory models, highlighting the potential of targeting this receptor for anti-inflammatory benefits (Concannon *et al.*, 2015b; Concannon *et al.*, 2016). Numerous cannabinoids have been tested *in vivo*, and have been shown to have profound immunomodulatory and anti-inflammatory effects (García-Arencibia *et al.*, 2007; Price *et al.*, 2009; García *et al.*, 2011; Javed *et al.*, 2016). However, to date, no research has been undertaken to assess the effectiveness of CB_2 receptor activation on curtailing viral priming and thus potentially averting the development of a Parkinson's disease pathology following infection. A former member of our group recently published a paper detailing a novel animal Parkinson's disease model combining viral priming with α-synuclein aggregation (Olsen *et al.*, 2019). They found that in rats, sequential exposure to the viral mimetic poly I:C and the α-synuclein aggregating molecule FN075 induced a substantial increase in nigral microgliosis and astrocytosis and subsequently a significant degeneration of dopaminergic nigrostriatal neurons. Therefore, we decided that this innovative model was optimal to determine if a cannabinoid intervention could diminish the neuroinflammatory response induced by viral priming and thus curtail the subsequent degeneration of the dopamine neurons.

There are many CB_2 agonists currently available that have exhibited anti-inflammatory activity. These include both synthetic and natural compounds that illustrate varying degrees of selectivity for the CB_2 receptor over the CB_1 receptor. One such compound is the dietary cannabinoid, β-caryophyllene (BCP). It is present in the essential oils of many edible plants including cloves, oregano, cinnamon and black pepper. This molecule selectively binds to the CB_2 receptor (K_i = 155 ± 4 nM) and has been shown to exhibit anti-inflammatory activity (Gertsch *et al.*, 2008). It has previously been used in murine models of Parkinson's disease and provided neuroprotective effects in both an MPTP

mouse model (Viveros-Paredes *et al.*, 2017) and a rotenone rat model (Ojha *et al.*, 2016). In addition to having effects at the CB_2 receptor, BCP has also been shown to have activity at the peroxisome proliferator-activated receptor-γ (PPARγ). The beneficial anti-inflammatory effects observed in a small number of studies, including in a colitis model and an Alzheimer's disease model, were attributed to its activity at both the CB_2 receptor and the PPARγ receptor (Bento *et al.*, 2011; Cheng *et al.*, 2014; Youssef *et al.*, 2019).

Taking all the above evidence into consideration, we hypothesised that chronic administration of a CB_2 agonist could ameliorate the viral priming effect, prevent the microglial hyper-responsiveness to subsequent Parkinsonian challenges and thus inhibit the development of Parkinson's disease. Therefore, the aim of this chapter specifically was to determine if administration of the dietary cannabinoid BCP could reduce neuroinflammatory cell activation and provide neuroprotection in poly I:C and FN075 infused rats.

5.2 METHODS AND MATERIALS

All methods are described in detail in Chapter 2.

BCP was sourced from Tokyo Chemical Industries U.K. Ltd (C0796).

5.2.1 EXPERIMENTAL DESIGN

The work presented in this chapter used an animal model of Parkinson's disease to assess the potential of targeting the CB_2 receptor to impede the viral priming effect on microglia, to reduce the hyper-responsiveness of microglia to subsequent insults and to prevent the development of Parkinson's disease. To investigate this, 32 male rats (Charles River, U.K.) received a unilateral intra-nigral lesion (at the stereotaxic coordinates: AP -5.3, ML +2.0, DV -7.2) of the viral mimetic poly I:C (30 μg in 4 μl 0.9% w/v saline) or its vehicle (saline). Two weeks later, the animals all received an infusion of FN075 (1.926 μg in 4 μl DMSO/imidazole/PBS) at the same site. During the intermediate time between the surgeries, the rats received daily injections intraperitoneally (i.p.) of BCP or its vehicle (ethanol: cremophor: saline at a ratio of 1:1:18). This produced four experimental groups, as described in the table below (Table 5.1). The intake of food and water, as well as the weights of the animals, were monitored throughout the time the rats received the drug intervention.

Group	1st surgery	Drug treatment	2nd surgery	n
Control	Saline	Vehicle	FN075	8
FN075 & BCP	Saline	BCP	FN075	8
Poly I:C & FN075	Poly I:C	Vehicle	FN075	8
Poly I:C & FN075 & BCP	Poly I:C	BCP	FN075	8

Table 5.1 Groups used in neuroprotection study. Animals received an intra-nigral injection of poly I:C (30 μg) or vehicle, before being injected with BCP or vehicle intraperitoneally once daily for 14 days. At the end of the cannabinoid treatment period, the rats received an intra-nigral injection of FN075 (1.926 μg).

The original plan for this study involved administering 30 mg/kg of BCP (dose chosen based on previously published *in vivo* studies using this drug (Abbas *et al.*, 2013; Ojha *et al.*, 2016; Viveros-Paredes *et al.*, 2017)) for the two-week duration of dosing and sacrificing the animals six weeks following the FN075 surgeries (Figure 5.1), mirroring the time-course employed in the viral priming model used previously (Olsen *et al.*, 2019). However, seven days into dosing, the rats in the drug treatment groups were not gaining weight at the same rate as the animals that were receiving the vehicle and so the decision was made to reduce the dose to 20 mg/kg for the remainder of the dosing period, and subsequently, the rats began to gain weight normally. However, eight days following the end of dosing, a rat that had received BCP had to be sacrificed due to suspected liver failure, and the next day another animal had to be culled for the same reason. Several other rats that had received the drug presented pale and piloerect, and therefore the decision was made to sacrifice all the animals at this point (10 days following final drug injection) for the sake of animal welfare (Figure 5.2). We proceeded with *post mortem* immunohistological analyses as planned to determine if there were alterations in neuroinflammation or neurodegeneration at this earlier time-point.

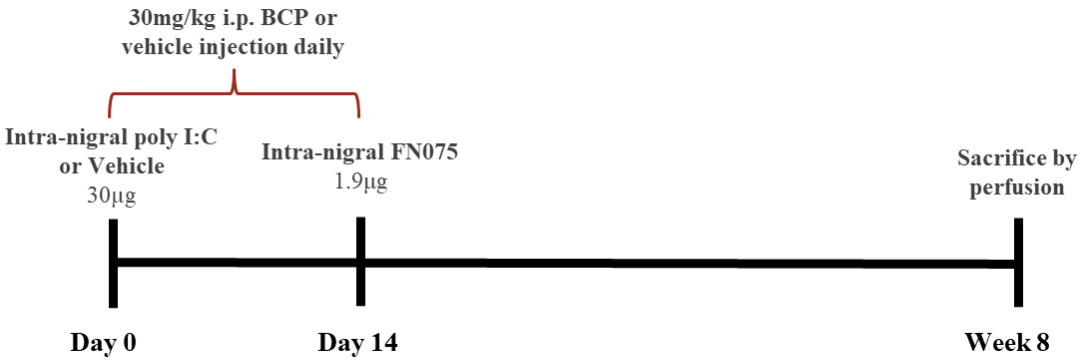

Figure 5.1 Planned timeline of the study. The original plan for the experimental design of this study based on Olsen *et al.* (2019).

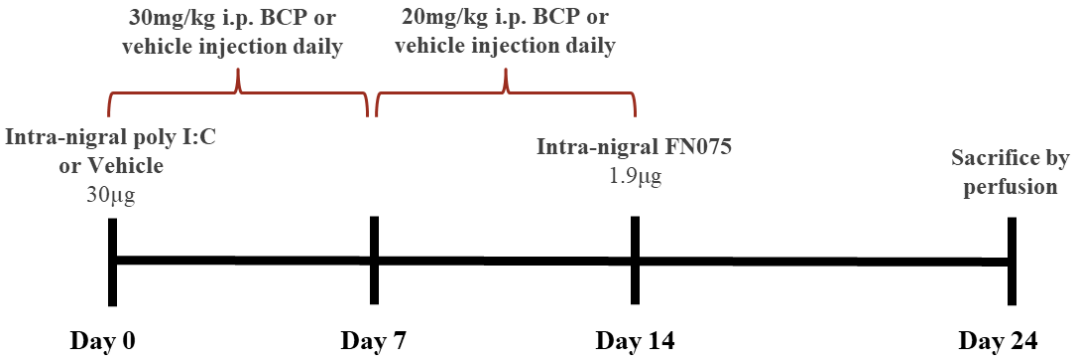

Figure 5.2 Actual timeline of the study executed.

5.3 RESULTS

5.3.1 THE CB_2 AGONIST BCP DID NOT ALTER MICROGLIOSIS

Post mortem, we first sought to investigate if the molecules poly I:C and FN075 induced any changes in microglial cell numbers in the substantia nigra and the striatum, and if BCP had any effect on the neuroinflammatory response. However, although overt microgliosis was visible in some animals in the region of the toxin needle tract in the substantia nigra, we did not observe any significant differences between groups with regard to microgliosis in either the substantia nigra (Figure 5.3; Group, $F_{(3,25)}$=0.71, P>0.05) or the striatum (Figure 5.4; Group, $F_{(3,25)}$=1.38, P>0.05.

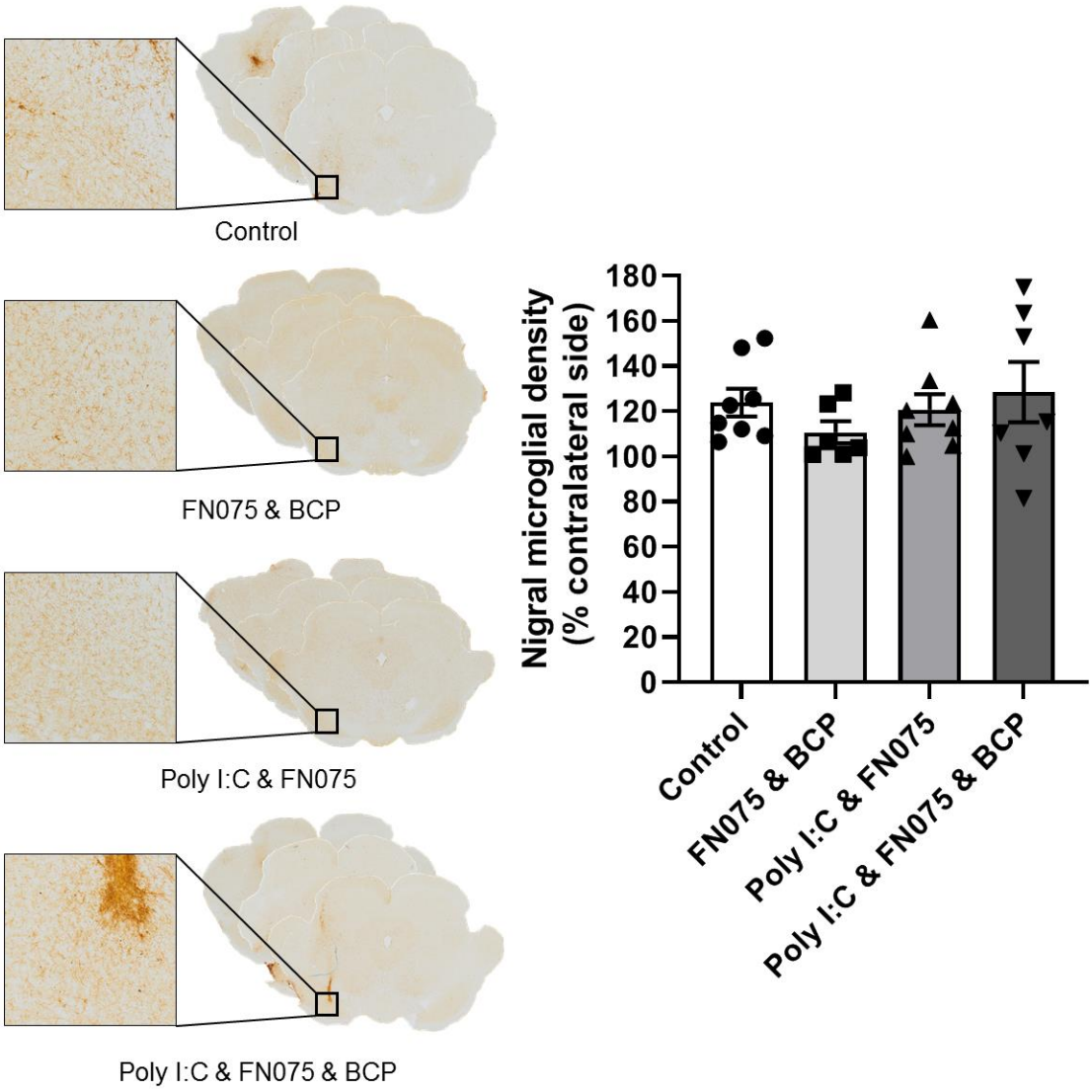

Figure 5.3 Immunohistological assessment of BCP-induced effects on nigral microgliosis. The combination of poly I:C and FN075 did not significantly affect the density of microglia in the substantia nigra and administration of BCP did not alter this. Data are represented as mean ± SEM with n=6-8 animals per group and were analysed by one-way ANOVA.

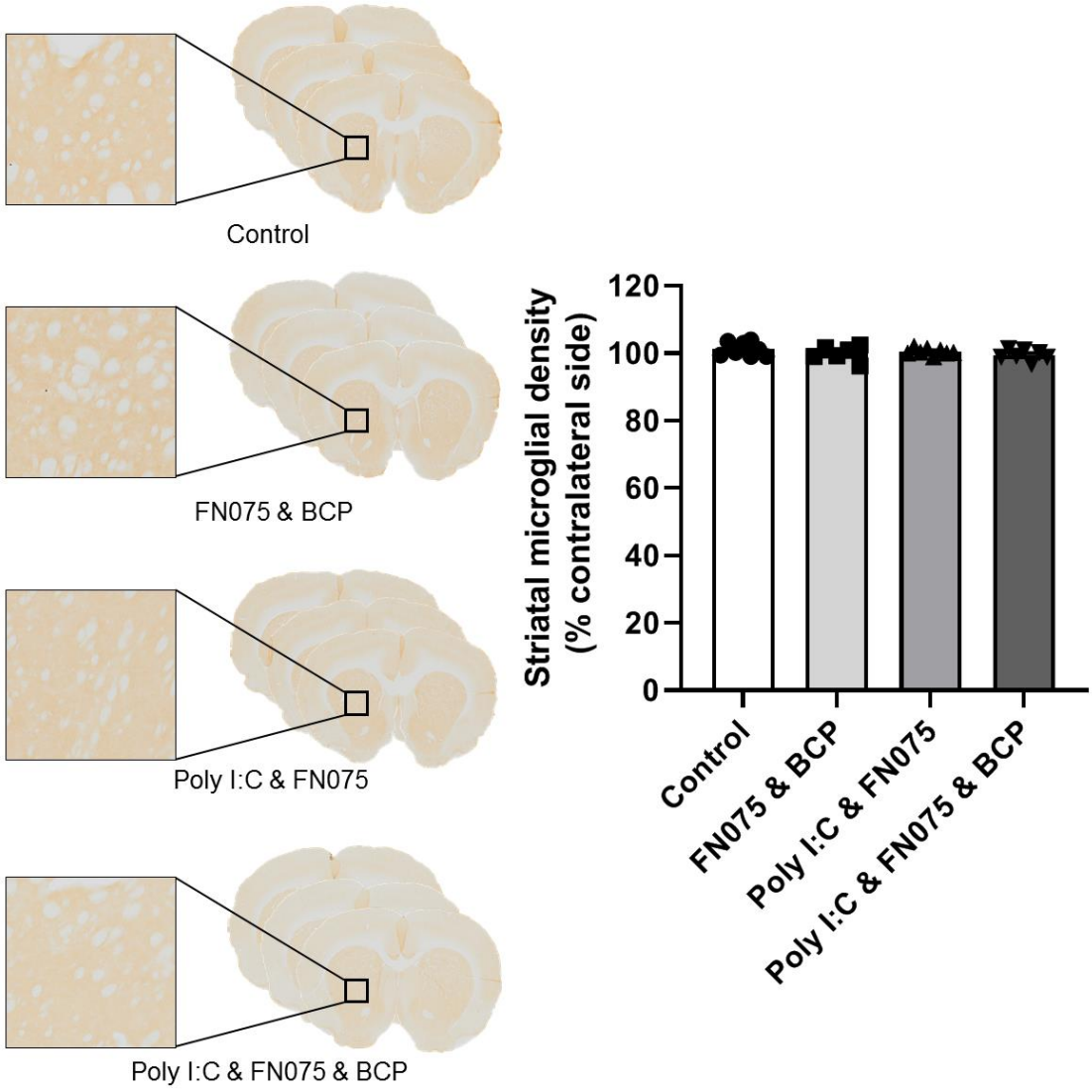

Figure 5.4 Immunohistological assessment of BCP-induced effects on striatal microgliosis. The combination of poly I:C and FN075 did not significantly affect the density of microglia in the striatum and BCP did not alter this. Data are represented as mean ± SEM with n=6-8 animals per group and were analysed by one-way ANOVA.

5.3.2 THE CB_2 AGONIST BCP DID NOT ALTER ASTROCYTOSIS

Following our analyses of microgliosis, we sought to determine if there was an astrocytic response along the nigrostriatal pathway to the viral-like inflammagen poly I:C and the α-synuclein aggregating molecule FN075, and if BCP altered this response. In the substantia nigra, no differences in astrocytosis were seen between groups (Figure 5.5; Group, $F_{(3,25)}$=0.53, P>0.05). In the striatum, while there was a trend for increased astrocytosis in the combined poly I:C and FN075 group compared to the Control group,

this did not reach significance, and no differences were detected between other groups (Figure 5.6; Group, $F_{(3,25)}$=2.19, P>0.05).

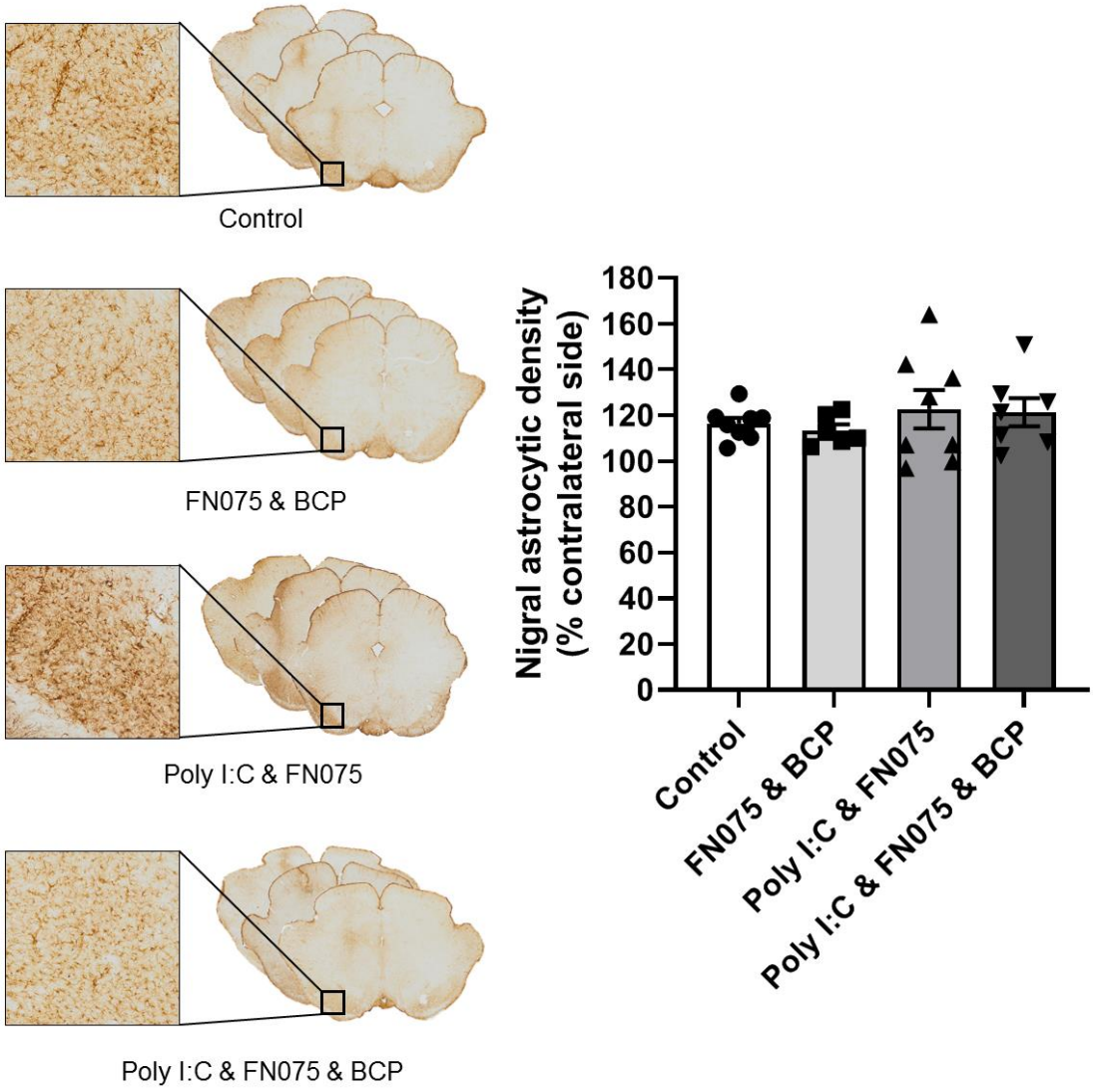

Figure 5.5 Immunohistological assessment of BCP-induced effects on nigral astrocytosis. The combination of poly I:C and FN075 did not significantly affect the density of astrocytic cells in the substantia nigra and BCP had no effect. Data are represented as mean ± SEM with n=6-8 animals per group and were analysed by one-way ANOVA.

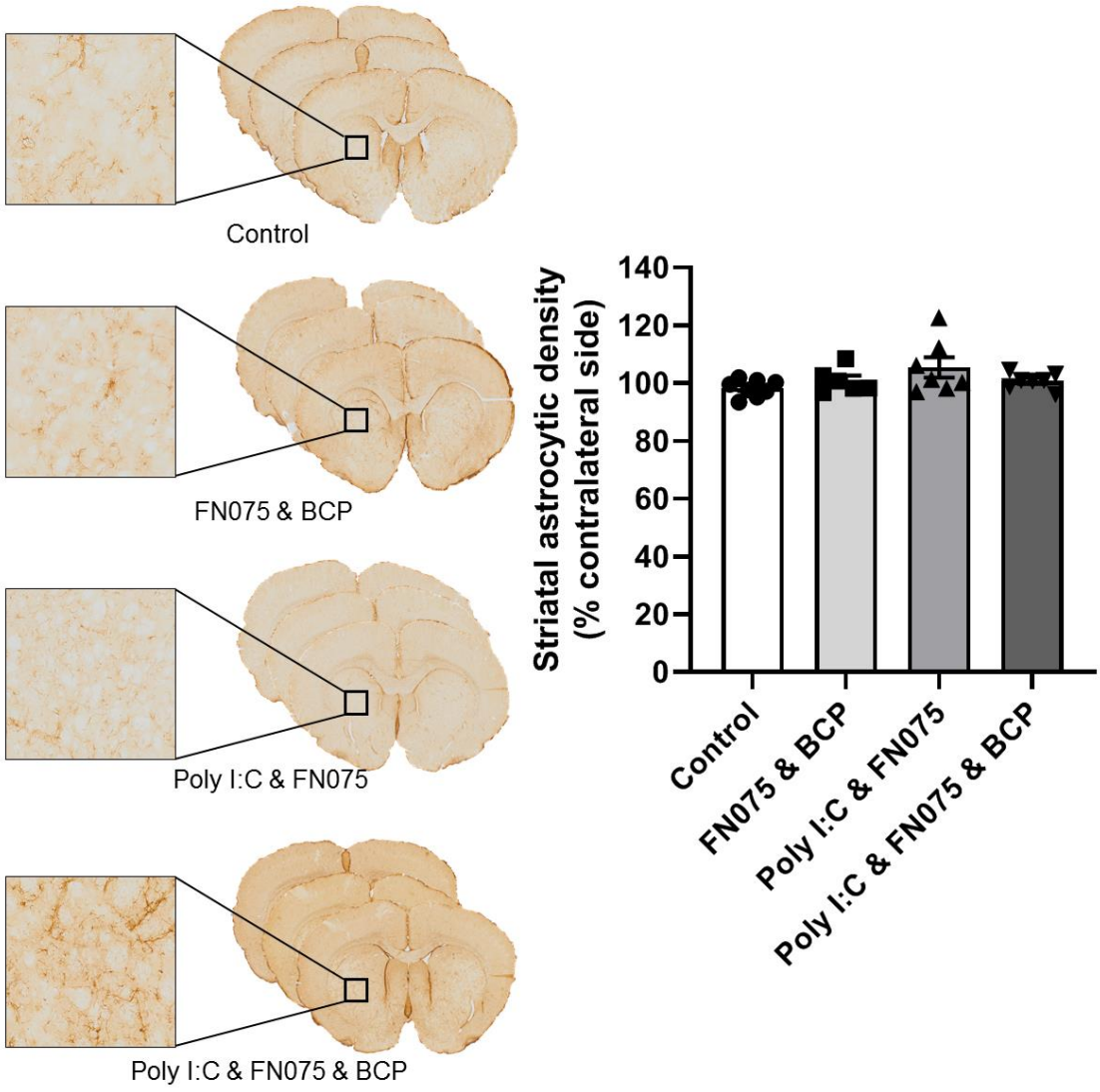

Figure 5.6 Immunohistological assessment of BCP-induced effects on striatal astrocytosis. The combination of poly I:C and FN075 did not significantly affect the density of astrocytes in the striatum, and BCP did not alter this. Data are represented as mean ± SEM with n=6-8 animals per group and were analysed by one-way ANOVA.

5.3.3 POLY I:C AND FN075 DID NOT CAUSE DEGENERATION IN THE NIGROSTRIATAL PATHWAY

Following our analyses of the neuroinflammatory response in the nigrostriatal pathway, we next sought to investigate if the combination of the viral mimetic poly I:C and the α-synuclein aggregating molecule FN075 induced degeneration of dopaminergic neurons in the nigrostriatal pathway and if the administration of the CB_2 receptor agonist BCP conferred neuroprotection to these neurons. The loss of these neurons is a key

pathological feature of Parkinson's disease and is necessary to test the neuroprotective effect of a drug intervention. However, in this study, the viral mimetic poly I:C and the α-synuclein aggregating molecule FN075 did not significantly alter the number of dopamine neuronal cell bodies in the substantia nigra (Figure 5.7; Group, $F_{(3,25)}$=0.64, P>0.05), and treatment with BCP did not affect these dopaminergic cell numbers. In the striatum, there were no differences observed between any groups with regard to the density of staining of the dopaminergic nerve terminals (Figure 5.8; Group, $F_{(3,25)}$=0.17, P>0.05).

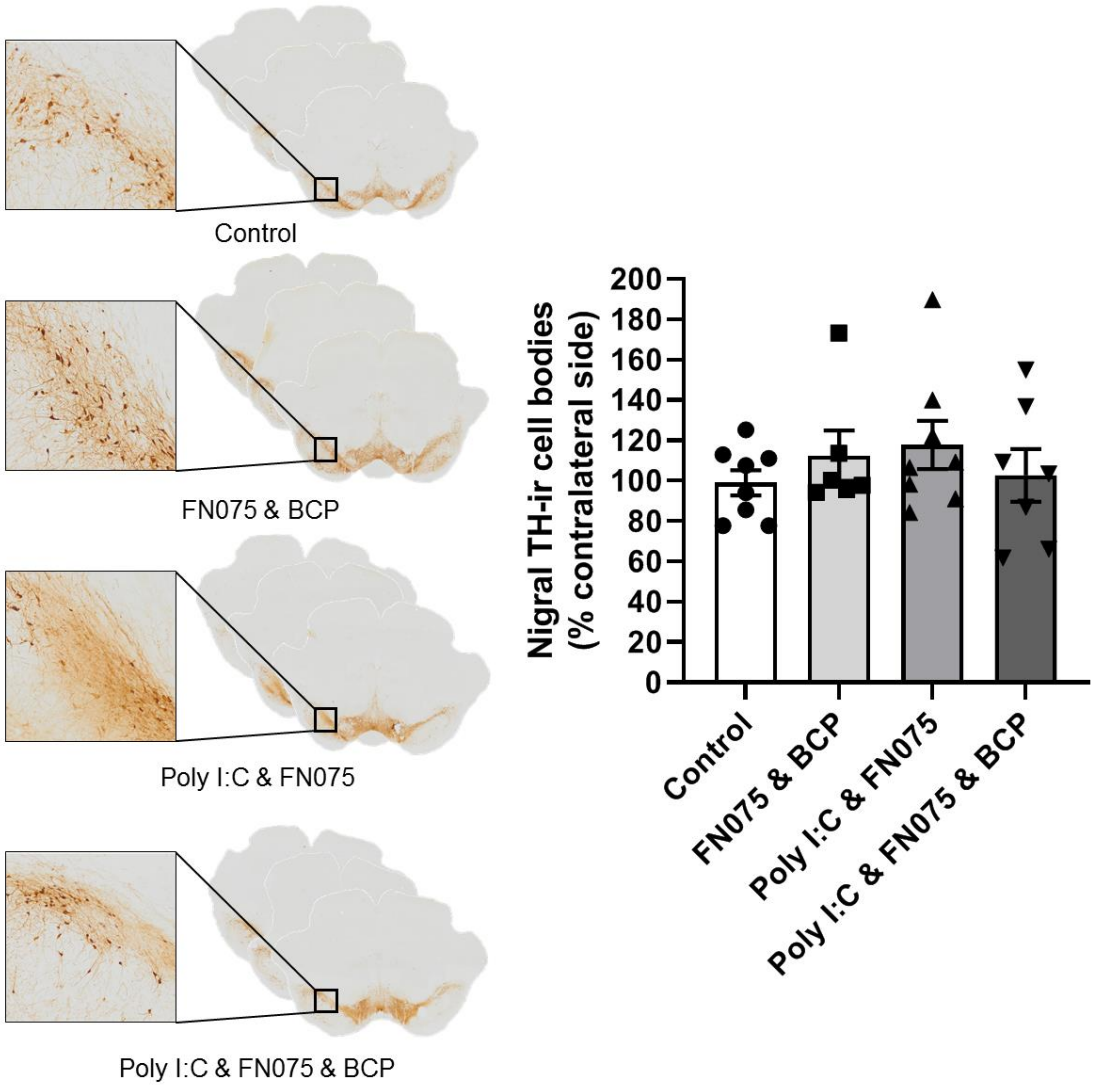

Figure 5.7 Immunohistological assessment of BCP-induced effects on nigral cell counts. The combination of poly I:C and FN075 did not significantly affect the number of cells in the substantia nigra, and BCP did not alter this. Data are represented as mean ± SEM with n=6-8 animals per group and were analysed by one-way ANOVA.

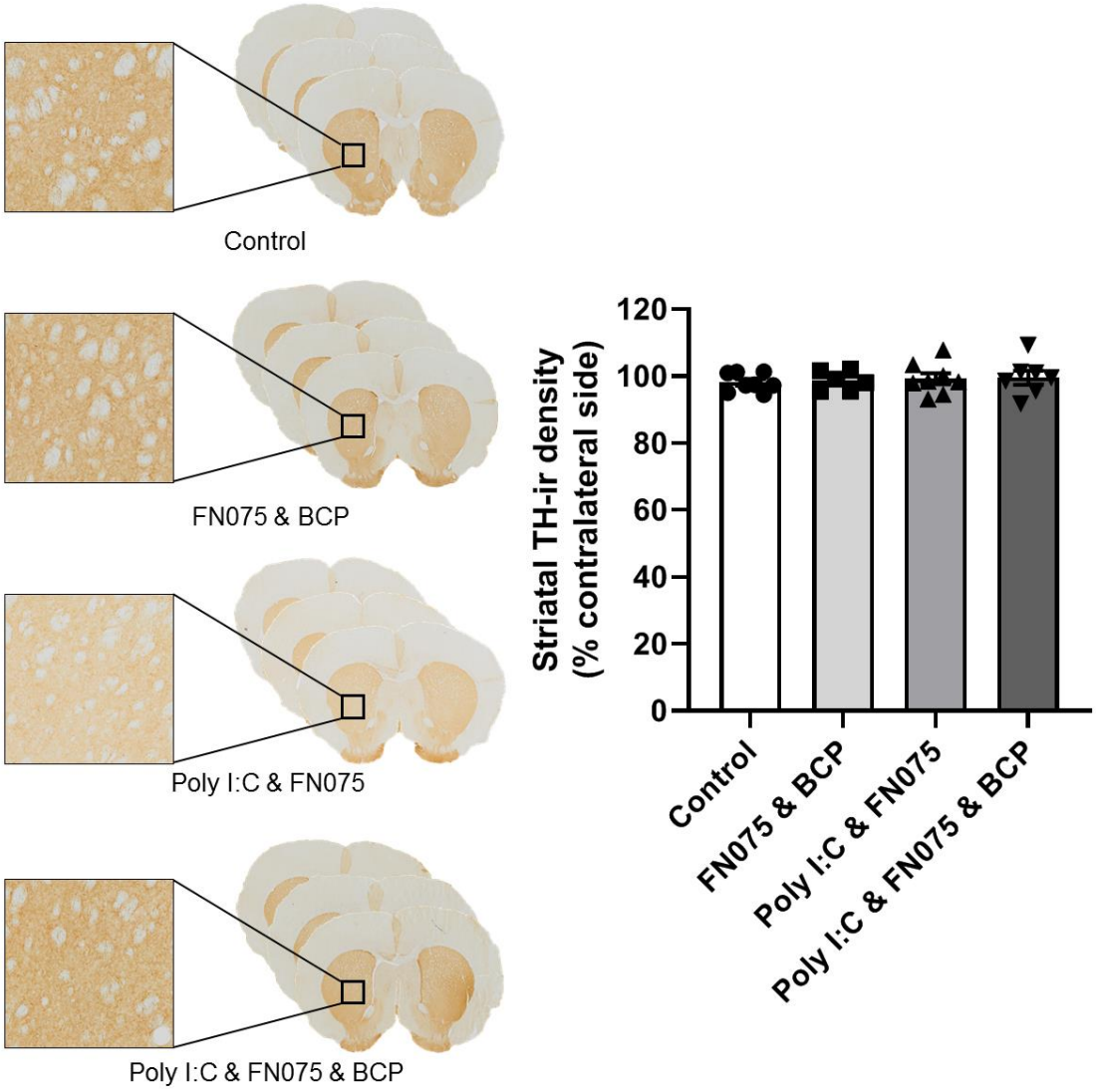

Figure 5.8 Immunohistological assessment of BCP-induced effects on striatal integrity. Poly I:C and FN075 did not alter striatal TH density, and BCP did not affect this. Data are represented as mean ± SEM with n=6-8 animals per group and were analysed by one-way ANOVA.

5.4 DISCUSSION

Viral infection has been demonstrated to be linked to an increased risk of developing Parkinson's disease (reviewed in Olsen *et al.*, 2018). Given the scale of the current global viral pandemic, a Parkinson's epidemic may feasibly ensue in the coming decades. Viral infections have been suggested to 'prime' the microglial cells in the brain, making them more responsive to subsequent Parkinsonian challenges (Deleidi *et al.*, 2010; Bobyn *et al.*, 2012; Olsen *et al.*, 2019). This hyper-responsiveness can thus result in the initiation of a detrimental self-sustaining cycle of neuroinflammation and neurodegeneration (Frank-Cannon *et al.*, 2009; Tansey and Goldberg, 2010).

The activation of the cannabinoid CB_2 receptor has been shown to result in a less active microglial phenotype (reviewed in Mecha *et al.*, 2016) and in recent years, many studies have investigated the potential of targeting the CB_2 receptor in animal models of neurodegeneration, with agonism of the receptor demonstrating immunosuppressive and anti-inflammatory effects (García-Arencibia *et al.*, 2007; Price *et al.*, 2009; García *et al.*, 2011; Javed *et al.*, 2016). However, no research (that we know of) has been carried out to test the effectiveness of CB_2 receptor activation on curtailing viral priming and thus conceivably preventing the development of Parkinson's disease pathology in an animal model. Therefore, we decided to investigate the potential of pharmacological targeting of the CB_2 receptor to reduce viral priming and hinder the onset of Parkinson's disease, using an innovative new model designed by a former member of our research group that combines priming using a viral mimetic, poly I:C, with α-synuclein aggregation by the small molecule FN075 (Olsen *et al.*, 2019).

The original experimental plan involved following the timeline used by Olsen *et al.*, and sacrificing the animals for *post mortem* analyses six weeks following the FN075 surgeries, a.k.a. eight weeks after the poly I:C surgeries (Olsen *et al.*, 2019). However, unexpected adverse effects associated with the group of animals who received the BCP drug treatment involved not only an adjustment of the dose but also an abrupt cessation of the study. This side effect was completely unforeseen, as this drug has been used in previous studies in rodents for longer periods of time and at higher doses without any reports of adverse effects (Basha and Sankaranarayanan, 2016; Ojha *et al.*, 2016; Ames-Sibin *et al.*, 2018). A toxicity study in female *Swiss* mice carried out by Oliveira *et al.*

(2018) reported an absence of any adverse effects, including any hepatic effects, with chronic dosing on a regimen of 2000 mg/kg orally for 28 days. We sourced our BCP from TCI Chemicals Industry, which was used previously in our group with no ill effects, albeit at a lower dose of 10 mg/kg (Concannon, 2016). However, for this study we purchased a new vial and given the evidence from the aforementioned studies utilising BCP, we suspect that this vial may have been contaminated at some stage of the production process and it was this contaminant rather than BCP which was the reason for the adverse effects. The company assures the purity to be >90%, and the purity of the particular vial we used was significantly higher, at 95.4%. However, we do not know the purity of the vial that was used *in vivo* by our group previously as it has since been discarded. The toxicological information available from the company safety data sheet is extremely limited, and contains no information regarding repeated exposure. We intend to contact TCI Chemicals Industry to inform them of this adverse event to prevent it from occurring in the future and to perhaps resolve the issue.

Despite the alterations in the experimental design of this study, we carried out the *post mortem* immunohistochemical analyses as planned to determine if BCP induced any anti-inflammatory effects or neuroprotective effects at this earlier time-point. Firstly, we investigated whether there were any alterations in the neuroinflammatory response in the substantia nigra or the striatum by carrying out OX-42 and GFAP staining for microglia and astrocytes. Poly I:C is a viral mimetic and a TLR3 agonist and has been demonstrated to produce a sustained inflammatory response after injection into the substantia nigra (Deleidi *et al.*, 2010). Furthermore, this model was previously shown to result in an exacerbation of neuroinflammation from sequential poly I:C and FN075 administration (Olsen *et al.*, 2019). In our study, poly I:C in combination with FN075 did not induce differences with regard to either microglial density or astrocytic density in the substantia nigra. A slight trend was seen for increased astrocytosis in the striatum in the combined poly I:C and FN075 group, but this did not reach significance. Moreover, the CB_2 agonist BCP did not alter the levels of microgliosis or astrocytosis in either region.

Following our analyses of neuroinflammation, we next sought to investigate if there were any differences induced in the integrity of the dopaminergic neurons of the nigrostriatal pathway. We found that the sequential administration of the viral-like inflammagen poly I:C with the α-synuclein aggregating molecule FN075 did not significantly alter the

number of tyrosine hydroxylase-positive cell bodies in the substantia nigra or the density of the terminals of these neurons in the striatum. This is contrary to the results from the modelling paper published by Olsen *et al.*, who reported an enhanced neuroinflammatory response and increased degeneration of nigrostriatal dopamine neurons by the combination of the two toxins (Olsen *et al.*, 2019). This is likely due to the necessary curtailment of the study duration but means that we were unable to determine if BCP had an anti-inflammatory or anti-priming microglia effect in this study.

In summary, it is not possible to draw conclusions about the potential of the cannabinoid agonist BCP to ameliorate the viral priming effect and prevent the development of Parkinson's disease from the results in this study, as due to the abridged study duration the model did not induce a microglial priming effect as expected. However, the potential of CB_2 receptor activation to prevent the development of Parkinson's disease post-viral infection remains an important line of inquiry and should be investigated further, as it is particularly relevant given the ongoing global viral pandemic.

Chapter 6: Pharmacological targeting of the CB_2 receptor in an inflammatory Parkinson's disease model

6.1 INTRODUCTION

In recent decades, it has become increasingly apparent that chronic neuroinflammation plays a critical part in the progression of Parkinson's disease pathology. Neurotoxicity and neuroinflammation drive each other in a pernicious self-sustaining cycle that is detrimental to the survival of the dopaminergic neurons (Frank-Cannon *et al.*, 2009; Tansey and Goldberg, 2010). Therefore, the concept of anti-inflammatory therapy for Parkinson's disease emerged with the aim of halting this cycle and perhaps hinder the progression of the disease. Increasing evidence shows that the cannabinoid system has a major role in the modulation of inflammation, and thus has the potential to be of therapeutic benefit in diseases with an inflammatory component.

The proposal of targeting the cannabinoid CB_2 receptor in Parkinson's disease is based on several converging lines of evidence. An upregulation of the expression of the receptor on microglia has been noted in patients of numerous neurodegenerative diseases, including Parkinson's disease (Gómez-Gálvez *et al.*, 2016), as well as Alzheimer's disease and Huntington's disease (Benito *et al.*, 2003; Palazuelos *et al.*, 2009; Solas *et al.*, 2013). Similarly, an increase in CB_2 receptor expression has been seen in animal models of Parkinson's disease induced by a wide variety of stimuli (García *et al.*, 2011; Concannon *et al.*, 2015b; Concannon *et al.*, 2016). Interestingly, a more pronounced upregulation of the receptor was observed in response to the bacterial and viral inflammagens, LPS and poly I:C respectively, compared to the direct neurotoxins 6-OHDA and rotenone (Concannon *et al.*, 2015b; Concannon *et al.*, 2016). Moreover, an aggravation of inflammation and neuronal death due to genetic inactivation of the CB_2 receptors has been reported in the inflammatory LPS model, but not in the 6-OHDA neurotoxin model (García *et al.*, 2011; Gómez-Gálvez *et al.*, 2016). These studies highlight the connection between the CB_2 receptor and the inflammatory pathology of neurodegenerative disease, hence the rationale behind why we decided to use an inflammatory model of Parkinson's disease in this study.

Pharmacological targeting of the cannabinoid system in animal models of neurodegenerative disease provides further corroboration that the modulation of this system has major therapeutic potential. In a rat model of Alzheimer's disease, treatment with the synthetic cannabinoid WIN55,212-2 improved memory function and caused reductions in the elevation of pro-inflammatory markers (Fakhfouri *et al.*, 2012). In the Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) model of MS, treatment with WIN55,212-2 or the CB_2 agonist JWH015 caused a pronounced reduction in microglial activation and induced long-term improvement in motor function (Arevalo-Martin *et al.*, 2003). In Parkinson's disease models, several studies have reported an anti-inflammatory effect upon CB_2 receptor activation (Price *et al.*, 2009; Chung *et al.*, 2016; Javed *et al.*, 2016; Ojha *et al.*, 2016). García and colleagues found that chronic administration of Δ9-THCV attenuated the loss of dopaminergic neurons in both the 6-OHDA and LPS model. This effect was also elicited in the LPS model by the CB_2 selective agonist HU-308, suggesting that it was CB_2 receptor-mediated (García *et al.*, 2011). However, HU-308 did not induce a significant neuroprotective effect in 6-OHDA lesioned rats (García-Arencibia *et al.*, 2007), affirming that much remains to be elucidated regarding the potential of the CB_2 receptor for anti-inflammatory disease modulation. We thus decided to assess if activation of the CB_2 receptor in an inflammatory model of Parkinson's disease could curb the neuroinflammatory response and hence afford neuroprotection to the nigrostriatal dopamine neurons.

There are many CB_2 agonists currently available, both natural and synthetic, that have exhibited anti-inflammatory activity. These agonists have varying degrees of selectivity for the CB_2 receptor over the CB_1 receptor. JWH133 is a potent CB_2 selective agonist (K_i = 3.4 nM) and is approximately 200-fold selective for the CB_2 receptor over the CB_1 receptor (Huffman *et al.*, 1999). *In vitro*, JWH133 was demonstrated to block Aβ peptide-induced activation of microglia, decreasing the release of the pro-inflammatory cytokine TNF-α and reducing mitochondrial activity (Ramirez *et al.*, 2005). JWH133 also inhibited the ATP-induced increase in intracellular calcium in primary microglia cells, as well as decreasing LPS-induced nitrite generation (Martin-Moreno *et al.*, 2011). *In vivo*, JWH133 was observed to reduce neuroinflammation and neuronal death following a cerebral injury, such as a stroke or haemorrhage (Zarruk *et al.*, 2012; Tao *et al.*, 2015; Lin *et al.*, 2017). With regard to neurodegenerative disease, JWH133 lessened the increase in the

levels of the pro-inflammatory cytokine TNF-α in a transgenic mouse model of Alzheimer's disease that overexpressed a mutant form of the amyloid precursor protein. It also lowered microglial cell density and abated cognitive deficits in the mice, as measured by the novel object recognition test (Martín-Moreno *et al.*, 2012). In the okadaic acid (OKA) Alzheimer's disease model, administration of JWH133 attenuated the OKA-induced rise in IL-1β levels, and also prevented the spatial memory impairment as measured by the Morris water maze test (Çakır *et al.*, 2019). In an MPTP mouse model of Parkinson's disease, agonism with JWH133 protected the dopaminergic striatal fibres from degeneration, an effect that was associated with a reduction in microglial activation and an inhibition of inflammatory cytokine release. These effects were reversed upon treatment with the selective CB_2 receptor antagonist AM630, confirming the involvement of the CB_2 receptor (Chung *et al.*, 2016).

Taking the aforementioned evidence into consideration, we hypothesised that activation of the CB_2 cannabinoid receptor with the potent CB_2 agonist JWH133 could modulate the neuroinflammatory pathology of Parkinson's disease and thus potentially reduce the extent of neurodegeneration. Therefore, the aim of this chapter was to determine if targeting the CB_2 receptor using the selective CB_2 agonist JWH133 could reduce neuroinflammation and provide functional neuroprotection in the inflammatory LPS model of Parkinson's disease.

6.2 METHODS AND MATERIALS

All methods are described in detail in Chapter 2.

JWH133 was sourced from Tocris, U.K. (1343).

6.2.1 EXPERIMENTAL DESIGN

The work presented in this chapter assessed the potential of targeting the CB_2 receptor to reduce neuroinflammation and confer neuroprotection in the LPS model of Parkinson's disease. Due to the issues we experienced with BCP as outlined previously, we used a different CB_2 agonist in this study, JWH133. To investigate this, 32 female rats (Charles River, U.K.) received a unilateral intra-nigral lesion (at the stereotaxic coordinates: AP -5.3, ML +2.0, DV -7.2) of the bacterial endotoxin, LPS (10 μg in 2 μl 0.9% w/v sterile saline) or its vehicle (saline). Beginning on the day of surgery, the rats received twice-daily injections subcutaneously of JWH133 (1 mg/kg/dose) (dose chosen based on previous *in vivo* studies using this drug (Muñoz-Luque *et al.*, 2008; Murikinati *et al.*, 2010; Concannon, 2016; Çakır *et al.*, 2019)) or its vehicle (ethanol: cremophor: saline at a ratio of 1:1:18), continuing for 14 days. Thus, there were four groups: Control, JWH133, LPS, and LPS & JWH133 (Table 6.1). The weights of the animals were monitored throughout the study. Animals also underwent Corridor, Stepping and Whisker behavioural tests throughout the study to assess the impact of LPS and the drug treatment on motor function. At the end of the drug intervention, the rats were sacrificed (Day 14 post-surgery) via transcardial perfusion for *post mortem* immunohistological analyses.

Group	Surgery	Drug treatment	n
Control	Saline	Vehicle	6
JWH133	Saline	JWH133	6
LPS	LPS	Vehicle	10
LPS & JWH133	LPS	JWH133	10

Table 6.1 Groups used in neuroprotection study. Animals received an intra-nigral lesion of LPS (10 μg) or its vehicle by stereotaxic surgery. JWH133 (1 mg/kg/dose) or its vehicle was injected subcutaneously twice-daily for 14 days beginning on the morning of LPS lesion surgery.

6.3 RESULTS

6.3.1 UNILATERAL, INTRA-NIGRAL LPS ADMINISTRATION DID NOT INDUCE CONTRALATERAL MOTOR IMPAIRMENT

Before embarking on *post mortem* immunohistological analyses, we first sought to determine if a deterioration in motor function was induced by the bacterial inflammagen LPS, and if the administration of the CB_2 selective agonist JWH133 had an effect on motor capacity. The lesion surgeries were performed unilaterally so any impairment would be restricted to one side of the body (contralateral to the site of injection), thus allowing the ipsilateral side to serve as an internal control.

As expected, there were no differences in ipsilateral motor function between groups in the Corridor test of sensorimotor neglect (Figure 6.1A; Group x Time, $F_{(6,56)}$=0.41, P>0.05), the Stepping test of forelimb akinesia (Figure 6.1B; Group x Time, $F_{(21,196)}$=1.27, P>0.05), or the Whisker test of sensorimotor integration (Figure 6.1C; Group x Time, $F_{(21,196)}$=0.00, P>0.05). In the Stepping test, there was a significant effect of time, suggesting a habituation effect as the animals became more accustomed to the testing (Figure 6.1B; Time, $F_{(4.81,134.7)}$=34.58, P<0.05)

With regard to contralateral motor function, no significant differences were detected between groups at any time-point in the Corridor test (Figure 6.2A; Group x Time, $F_{(6,56)}$=0.41, P>0.05). In the Stepping test (Figure 6.2B; Group x Time, $F_{(21,196)}$=1.176, P>0.05), there was a trend for some groups to make fewer steps but this did not reach significance. There was also a significant time effect in this test (Figure 6.2B; Time, $F_{(3.94,110.4)}$=14.32, P<0.05), indicating a training effect similar to the ipsilateral side. In the Whisker test (Figure 6.2C; Group x Time, $F_{(21,196)}$=2.69, P>0.05) there appeared to be a slight impairment in the groups which received LPS compared to the Control and JWH133 groups. However, this did not reach significance and JWH133 had no effect.

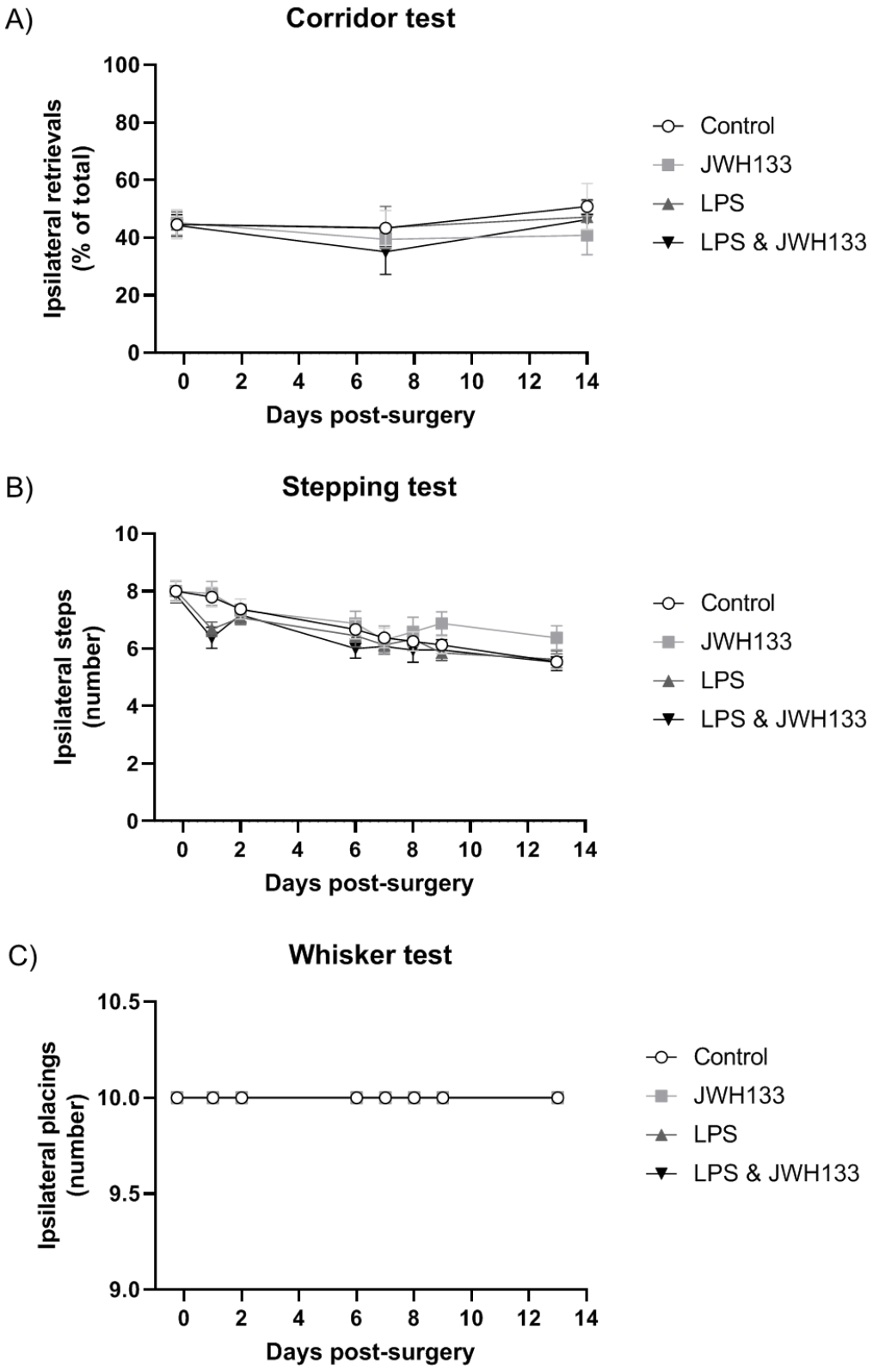

Figure 6.1 Impact of LPS administration and JWH133 treatment on ipsilateral motor function. There were no differences in ipsilateral motor function in the Corridor (A), Stepping (B) or Whisker (C) behavioural tests. Data are represented as mean ± SEM with n=6-10 animals per group and were analysed by two-way repeated-measures ANOVA.

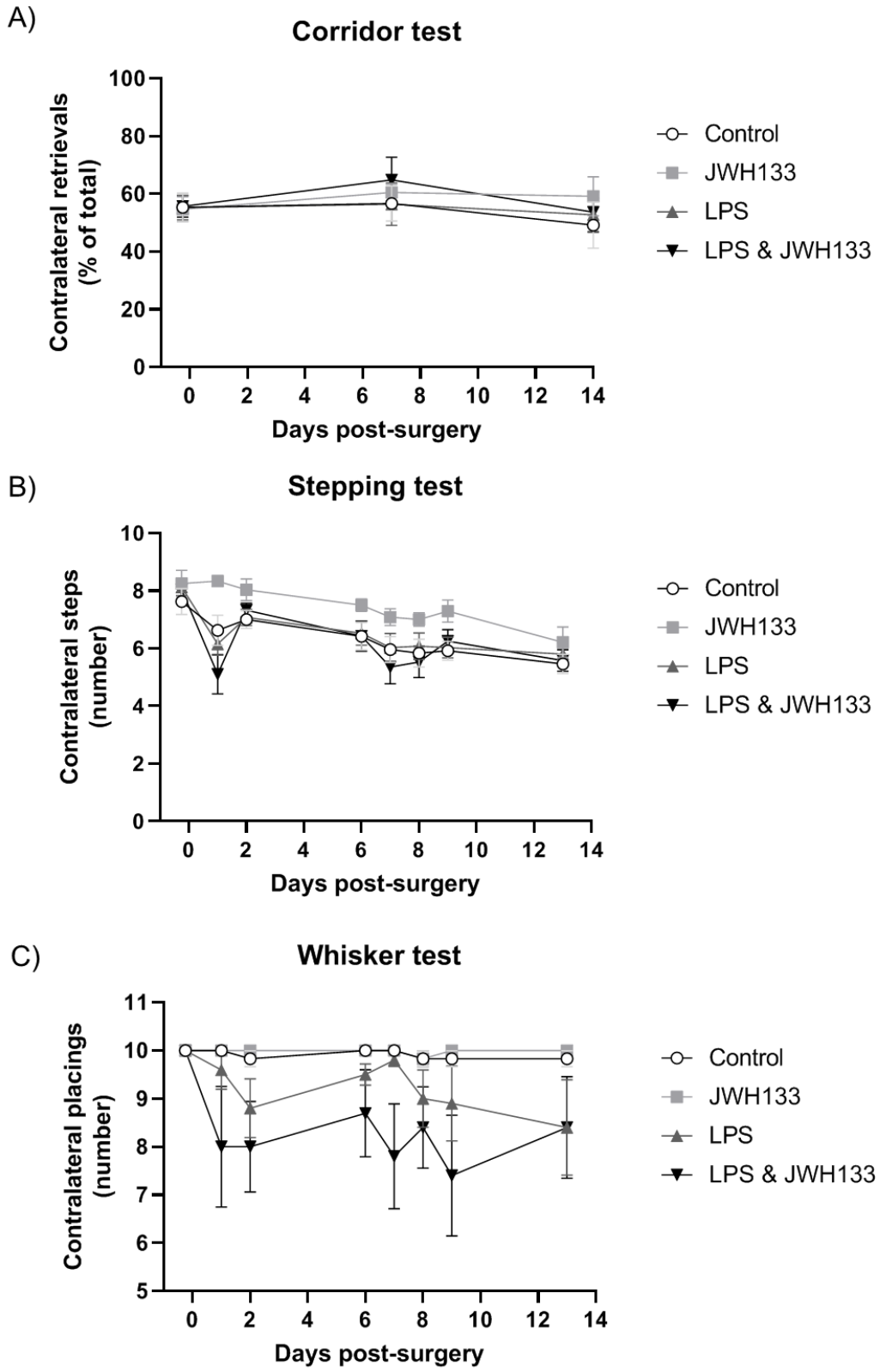

Figure 6.2 Impact of LPS administration and JWH133 treatment on contralateral motor function. LPS did not induce significant contralateral motor impairments compared to Control in the Corridor (A), Stepping (B) or Whisker (C) behavioural tests. Data are represented as mean ± SEM with n=6-10 animals per group and were analysed by two-way repeated-measures ANOVA.

6.3.2 THE CB_2 AGONIST JWH133 DID NOT ALTER LPS-INDUCED MICROGLIOSIS IN THE NIGROSTRIATAL PATHWAY

Post mortem, we first sought to investigate if the bacterial inflammagen LPS induced a neuroinflammatory response in the nigrostriatal pathway and if JWH133 had an anti-inflammatory effect, which we achieved by analysing the density of microglia in the substantia nigra and the striatum. In the substantia nigra, there was a significant increase in microgliosis seen in the LPS group compared to the Control group (Figure 6.3; Group, $F_{(3,28)}$=3.70, P<0.05), an effect which was not seen in the LPS & JWH133 group. In the striatum, there were no differences in microglial density between groups (Figure 6.4; Group, $F_{(3,27)}$=2.62, P>0.05).

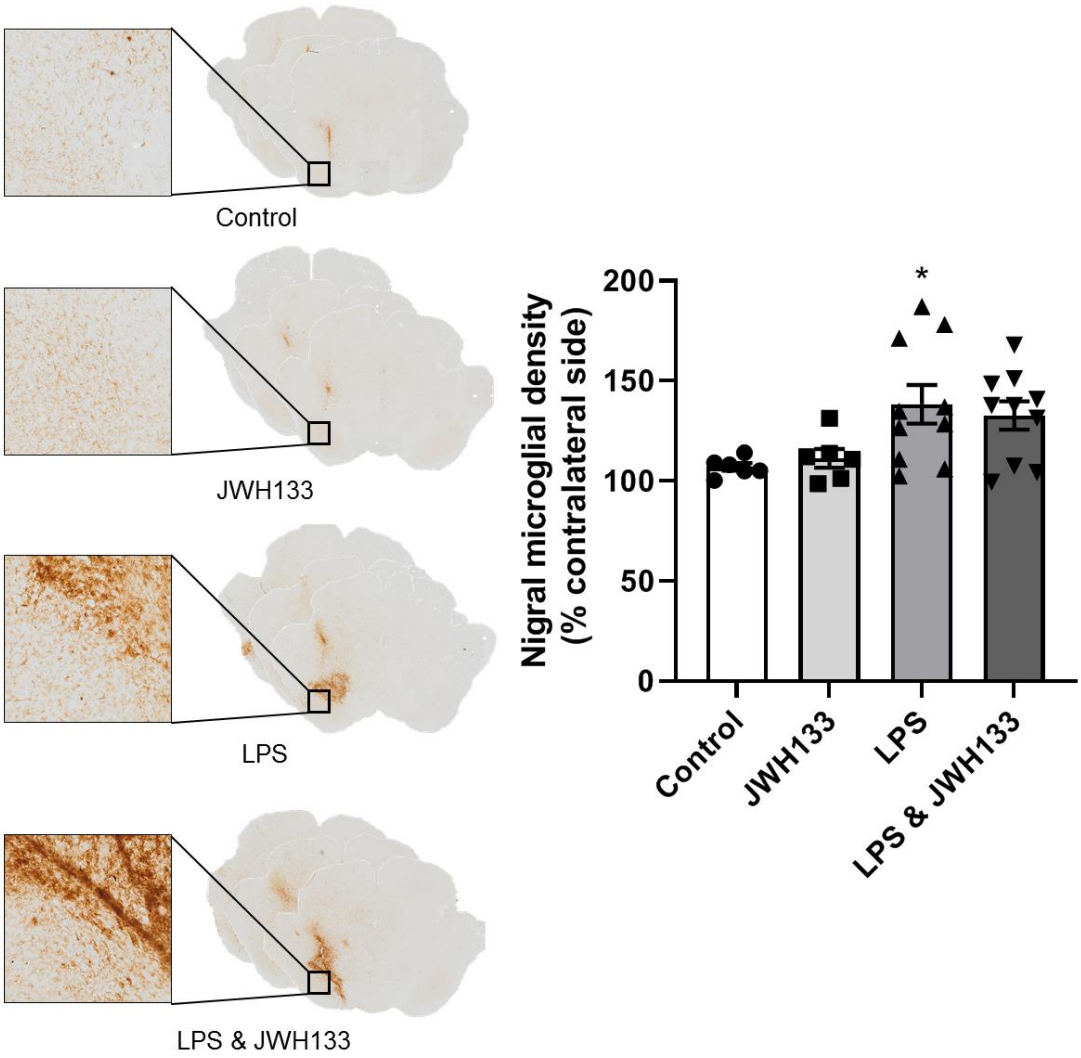

Figure 6.3 Immunohistological assessment of JWH133-induced effects on nigral microgliosis. LPS significantly increased the density of microglia in the substantia nigra, but JWH133 treatment did not alter LPS-induced microgliosis. Data are represented as mean ± SEM with n=6-10 animals per group. *P<0.05 vs. Control by one-way ANOVA with *post hoc* Tukey.

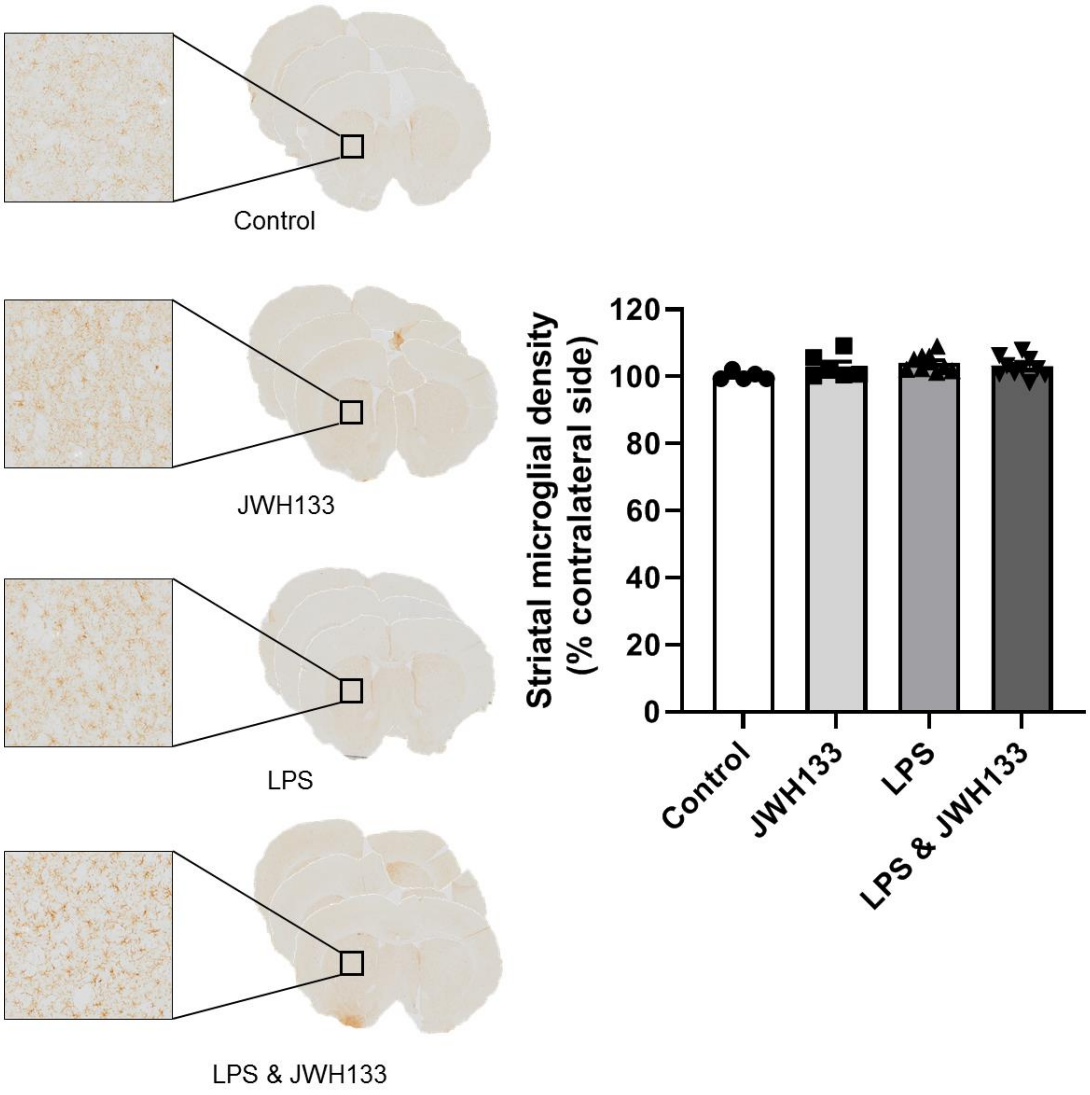

Figure 6.4 Immunohistological assessment of JWH133-induced effects on striatal microgliosis. LPS did not significantly affect the density of microglia in the striatum, and JWH133 did not alter this. Data are represented as mean ± SEM with n=6-10 animals per group and were analysed by one-way ANOVA.

6.3.3 THE CB_2 AGONIST JWH133 DID NOT ALTER LPS-INDUCED ASTROCYTOSIS IN THE NIGROSTRIATAL PATHWAY

Further to our investigation of the microglial response to LPS and JWH133 in the nigrostriatal pathway, we sought to investigate if the toxin or the CB_2 agonism treatment had an effect on the astrocytic response in the substantia nigra or the striatum. In the substantia nigra (Figure 6.5; Group, $F_{(3,28)}$=1.04, P>0.05), there were no differences between groups with regard to astrocytic density. However, in the striatum, LPS induced

a significant increase in astrocytosis compared to controls (Figure 6.6; Group, $F_{(3,28)}$=5.51, P<0.05), but administration of the CB_2 agonist JWH133 did not affect this.

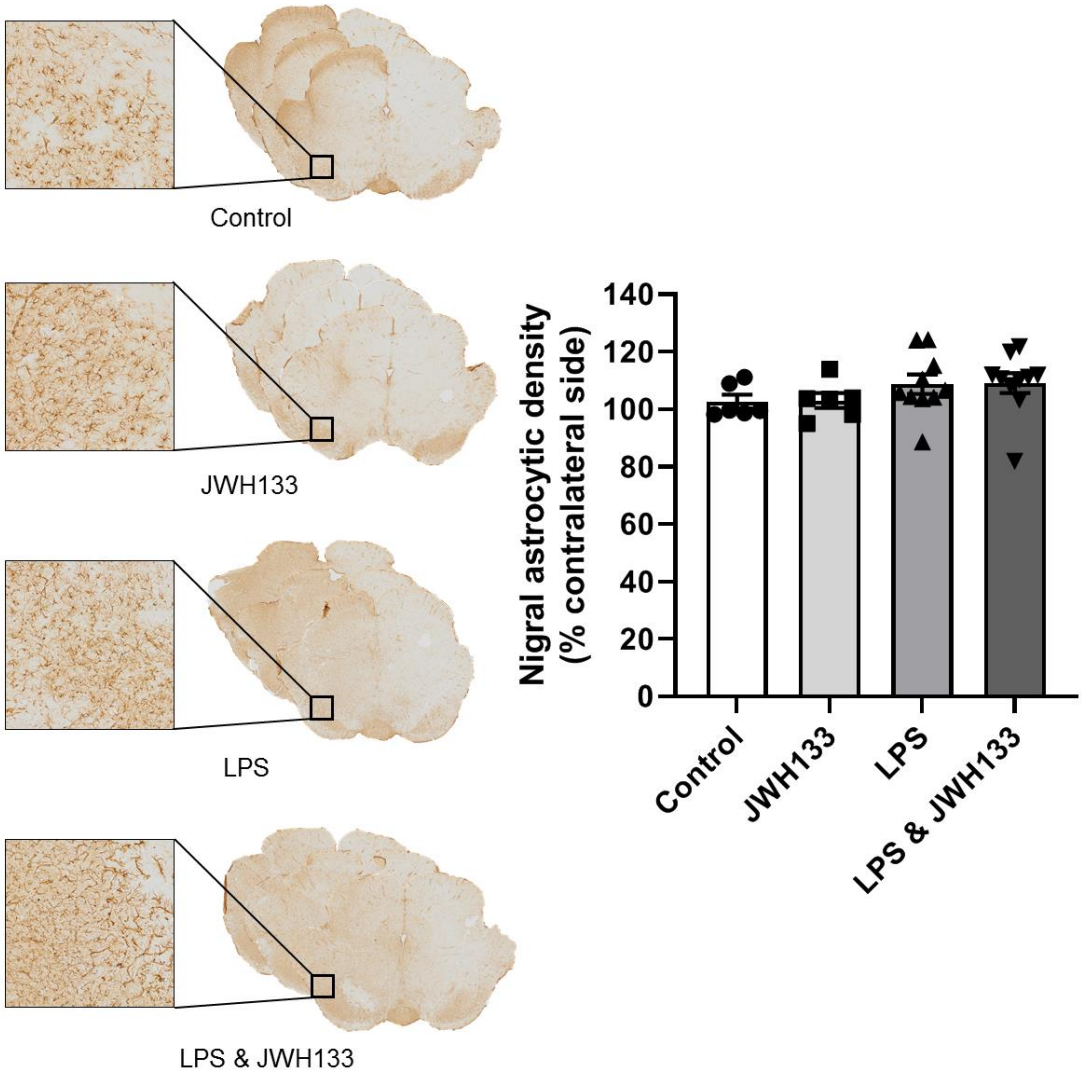

Figure 6.5 Immunohistological assessment of JWH133-induced effects on nigral astrocytosis. LPS did not significantly affect the density of astrocytes in the substantia nigra, and JWH133 did not alter this. Data are represented as mean ± SEM with n=6-10 animals per group and were analysed by one-way ANOVA.

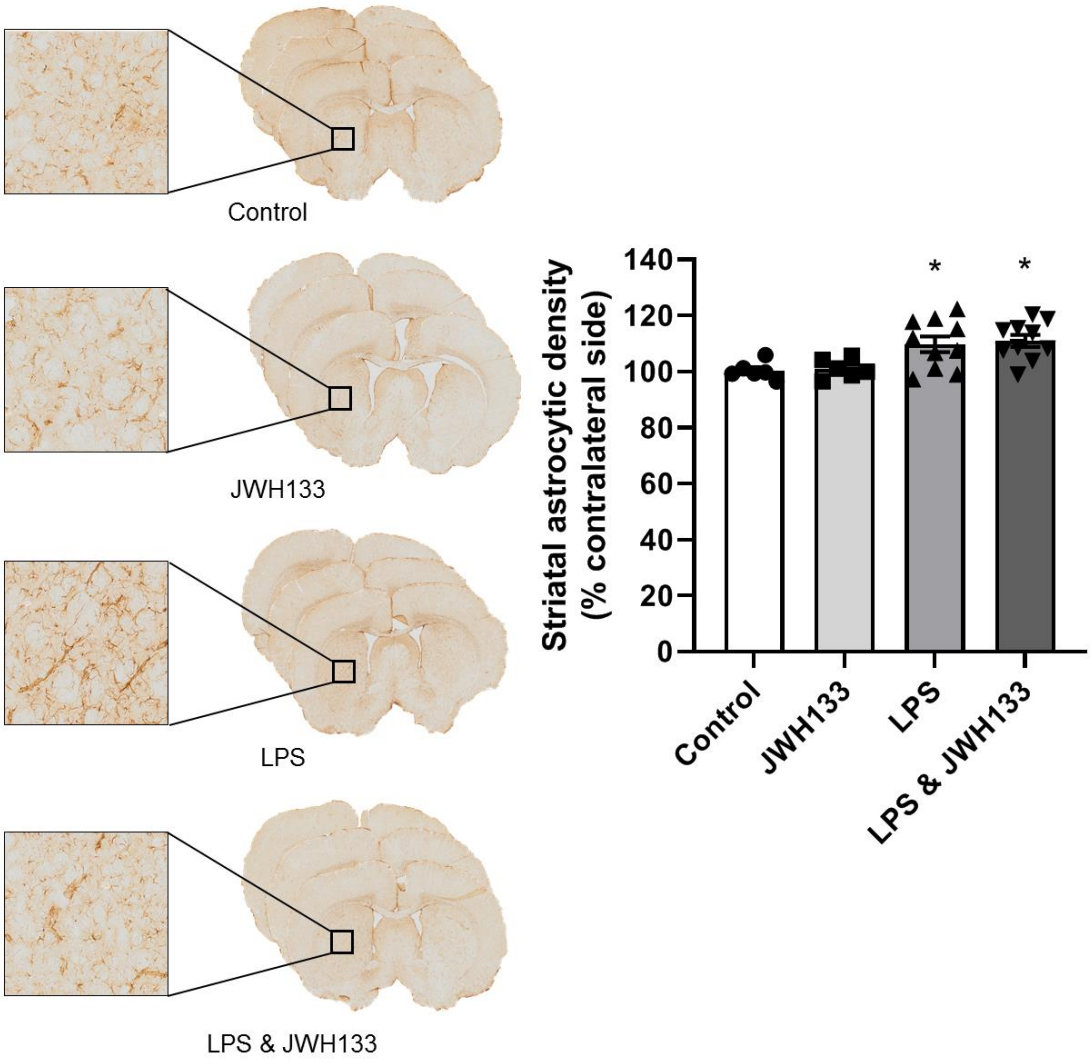

Figure 6.6 Immunohistological assessment of JWH133-induced effects on striatal astrocytosis. LPS significantly increased the density of astrocytes in the striatum, but JWH133 did not alter LPS-induced astrocytosis. Data are represented as mean ± SEM with n=6-10 animals per group. **P*<0.05 vs. Control by one-way ANOVA with *post hoc* Tukey.

6.3.4 THE CB_2 AGONIST JWH133 DID NOT AFFECT LPS-INDUCED DEGENERATION OF NIGRAL CELL BODIES

Following our analyses of neuroinflammatory cell density, we sought to investigate if the LPS-induced inflammatory response resulted in degeneration of the dopaminergic neurons in the nigrostriatal pathway and if the administration of the selective CB_2 receptor agonist JWH133 conferred protection to these neurons. Animals that received an intra-nigral infusion of LPS had a significant reduction in TH-positive cell bodies in the substantia nigra (Figure 6.7; Group, $F_{(3,27)}$=4.84, P<0.05). However, this LPS-induced nigral cell death was not prevented by chronic treatment with JWH133. In the striatum (Figure 6.8; Group, $F_{(3,28)}$=1.39, P>0.05), there were no differences observed between groups with regard to the density of staining of the nerve terminals.

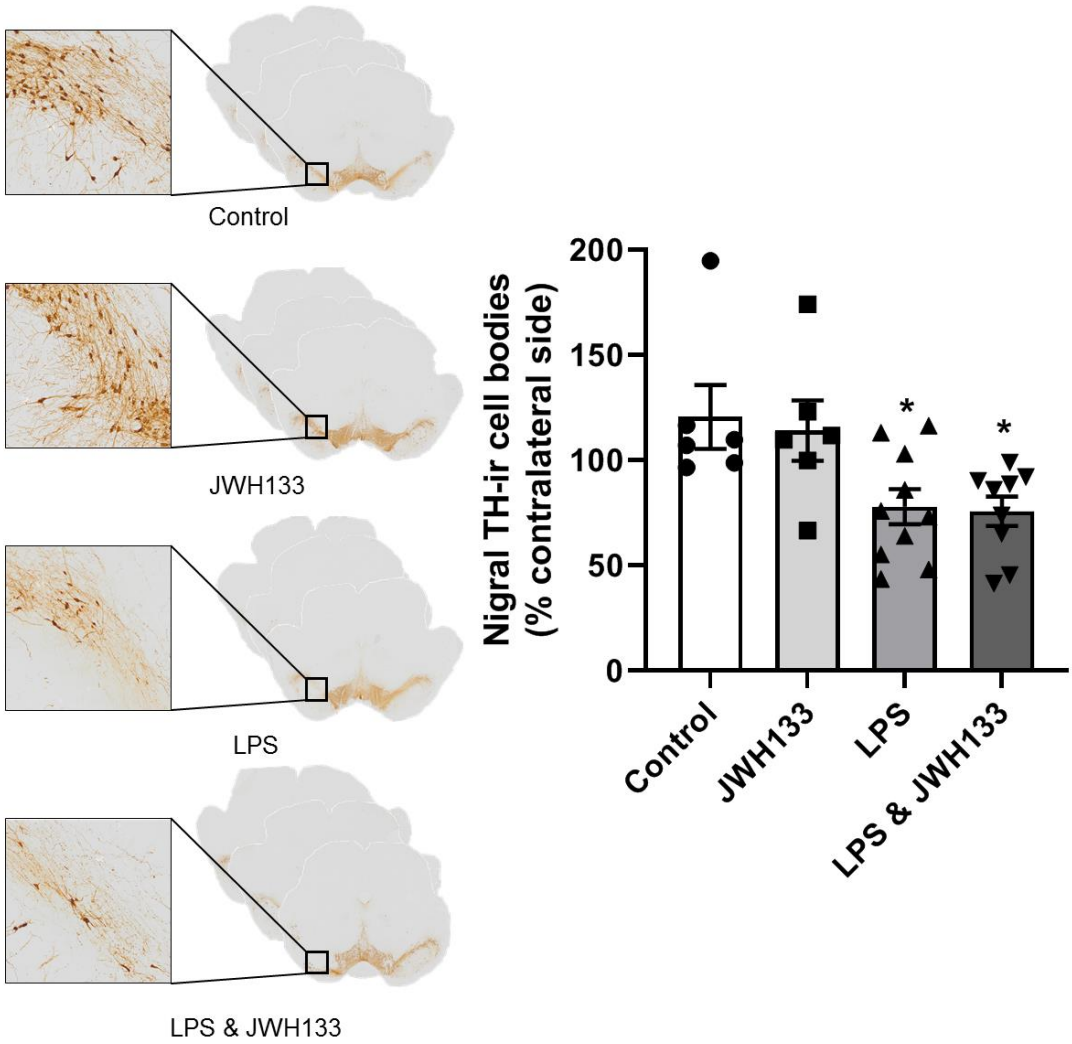

Figure 6.7 Immunohistological assessment of JWH133-induced effects on nigral cell counts. LPS induced a reduction in dopaminergic cell bodies in the substantia nigra. Chronic treatment with JWH133 did not protect dopaminergic cell bodies from LPS-induced degeneration. Data are represented as mean ± SEM with n=6-10 animals per group. **P*<0.05 vs. Control by one-way ANOVA with *post hoc* Tukey.

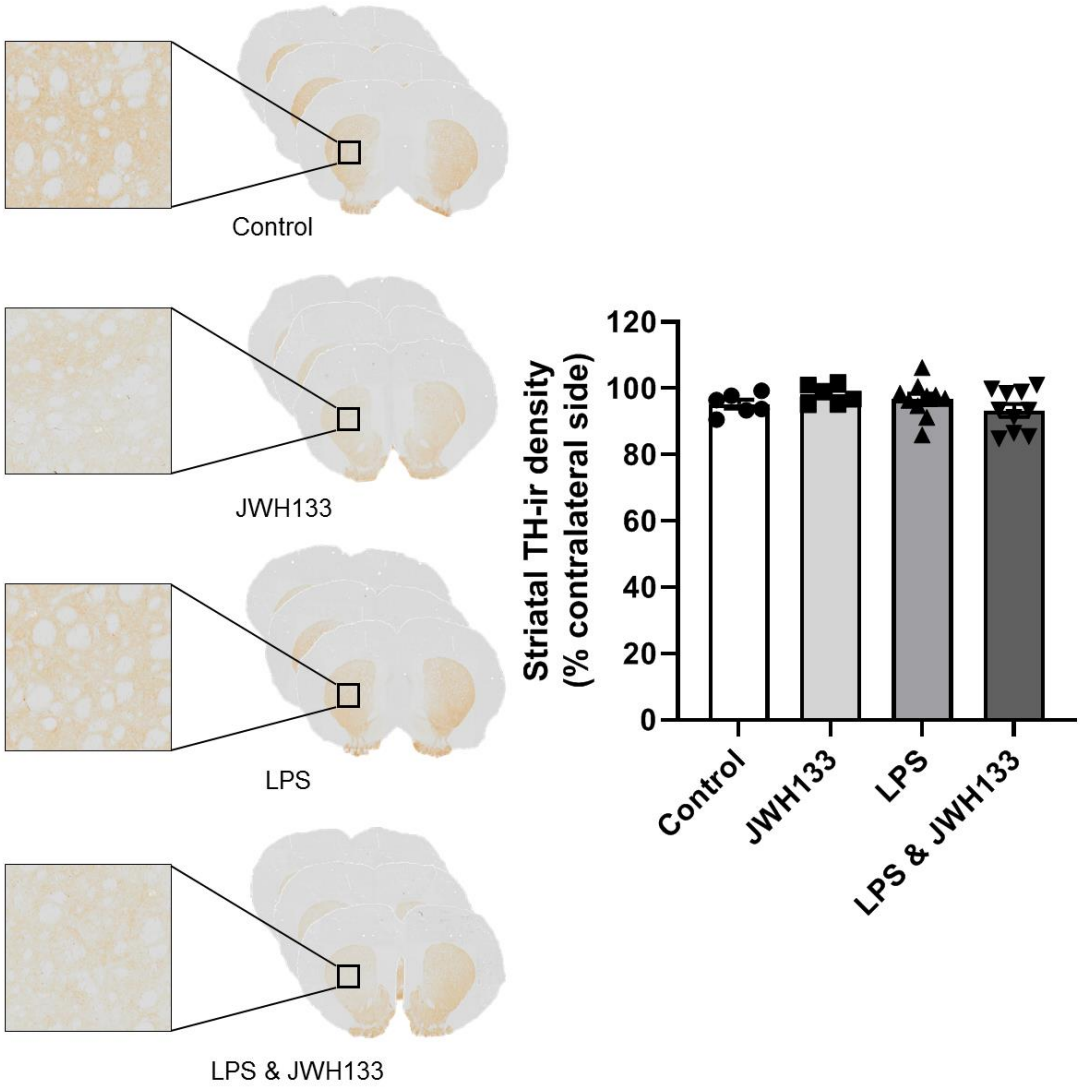

Figure 6.8 Immunohistological assessment of JWH133-induced effects on striatal integrity. LPS did not affect striatal TH density, and JWH133 did not alter this. Data are represented as mean ± SEM with n=6-10 animals per group and were analysed by one-way ANOVA.

6.4 DISCUSSION

The cannabinoid CB_2 receptor has been noted to be highly upregulated on microglia in the brain of Parkinson's disease patients (Gómez-Gálvez *et al.*, 2016). Activation of this receptor on microglial cells can result in a less active microglial phenotype, by reducing the release of pro-inflammatory cytokines (Facchinetti *et al.*, 2003; Ramirez *et al.*, 2005; Romero-Sandoval *et al.*, 2009) and modulating microglial migration (Offertáler *et al.*, 2003; Walter *et al.*, 2003). *In vivo* studies targeting the receptor have had promising results with regard to their effect on neuroinflammation and neuroprotection, but these results are inconsistent (García-Arencibia *et al.*, 2007; Price *et al.*, 2009; García *et al.*, 2011; Javed *et al.*, 2016). This variability can be attributed in part to the disparate mechanisms of action of the assorted toxins used to model Parkinson's disease, and the different affinities and efficacies of the particular CB_2 agonists used. Hence, much remains to be clarified regarding the potential of CB_2 agonism for therapeutic modulation of the disease.

Therefore, we endeavoured to address some of these unanswered questions about the role of the CB_2 receptor in Parkinson's disease by investigating if the highly potent CB_2 agonist JWH133 could alter neuroinflammation or neurodegeneration in the inflammatory Parkinson's disease model induced by the bacterial endotoxin, LPS. We used twice-daily injections in order to prolong the duration of time that the agonist was in the blood and thus was bioactive. Throughout the study, we assessed lateralised motor performance in a battery of behavioural tests before sacrificing the rats to carry out *post mortem* immunohistological analyses. We found that while LPS did induce alterations in microglial and astrocytic cell densities in the nigrostriatal pathway, JWH133 did not reverse this neuroinflammatory effect. Furthermore, while LPS induced degeneration of the nigral cell bodies, JWH133 did not provide appreciable neuroprotection to the dopaminergic cells.

From the times of the first studies which used a single intra-nigral LPS injection to model Parkinson's disease in rats, a profound microglial reaction was noted (Bing *et al.*, 1998; Castaño *et al.*, 1998). Further studies corroborated this effect (Herrera *et al.*, 2000; Kim *et al.*, 2000; Hoban *et al.*, 2013). The substantia nigra is one of the regions most densely populated by microglia in the brain (Lawson *et al.*, 1990), which likely contributes to the

sensitivity of these neurons to LPS-induced insults. One study that involved the injection of LPS into the hippocampus, cortex or substantia nigra of adult rats found that neurodegeneration was only induced in the substantia nigra, which could be due to the very high microglial density in this region (Kim *et al.*, 2000). Therefore, it is interesting that in this study, while there was a notable increase in microglial density in many of the rats that received the LPS lesion, other rats that received the intra-nigral injection of the toxin showed minimal or no alterations in microgliosis. One potential reasoning for this is the time of immunohistological assessment, as different studies have reported different peaks of microglial activation. In a study carried out by a previous member of our research group, LPS induced the greatest upregulation of CD11b and GFAP expression 14 days post-intra-nigral lesion surgery (Concannon *et al.*, 2015b). However, peaks of expression of the pro-inflammatory cytokines TNF-α and IL-6 have been observed much earlier, as soon as day 1 post-surgery (McCabe *et al.*, 2017). Furthermore, Castaño and colleagues reported some activated microglia cells from two days post-LPS injection, but the majority of microglia did not display this activated morphology until the day 9 time-point (Castaño *et al.*, 1998).

Another possible deduction is that the lack of consistent microglial activation was due to the serotype of LPS used. For this experiment, we purchased a new batch of a serotype of LPS used by a previous member of our group which induced contralateral motor dysfunction, nigrostriatal degeneration and neuroinflammation (Concannon, 2016). However, LPS is notorious for its variability in potency between strains (Miller *et al.*, 2005; Dehus *et al.*, 2006; Lam *et al.*, 2011) but also between batches of the same strain from the same manufacturer. These inconsistencies may account for the differences in microglial response seen in this study compared to the literature.

One potential reason for the lack of substantial anti-inflammatory or neuroprotective effects exerted by JWH133 may be the duration of action of the cannabinoid in the blood. The peak serum levels of JWH133 were reported to occur 2 hours following i.p. injection (Willecke *et al.*, 2011), and while the exact pharmacokinetics of this drug after subcutaneous injection have not yet been described, substances administered subcutaneously are often absorbed at a slower rate compared with other parenteral routes, providing a more sustained effect (Turner *et al.*, 2011). For this reason we decided to use the subcutaneous route, twice daily, to treat the animals. However, in the future using an

alternative route which would provide a more constant plasma level could be beneficial, such as via the animals' drinking water or using micro-osmotic pumps as has described previously (Murikinati *et al.*, 2010; Martín-Moreno *et al.*, 2012; Navarro-Dorado *et al.*, 2016).

It is also important to take into consideration that there is evidence of 'biased agonism' or 'functional selectivity' occurring amongst CB_2 agonists, in which all agonists do not induce the same response, and can induce different active conformations in the receptor and thus elicit receptor coupling to diverse downstream signalling effectors (Atwood *et al.*, 2012; Morales *et al.*, 2018). For example, evidence from studies investigating downstream signalling of CB_2 ligands suggests a role for JWH133 in adenylyl cyclase inhibition (Dhopeshwarkar and Mackie, 2016), but it fails to recruit β-arrestin, suggesting there should not be a tolerance effect of the drug if it does not induce internalisation (Soethoudt *et al.*, 2017). However, these studies were carried out using mouse CB_2 receptors, and it has been shown that ligands can cause disparate downstream signalling effects depending on the species. For example, with the CB_2 selective ligands HU-910 and HU-308, unbiased agonism was detected at the human CB_2 receptor, but biased agonism was revealed when testing on the mouse CB_2 receptor (Soethoudt *et al.*, 2017). To our knowledge, no research thus far has been carried out with regard to the downstream effects induced by the binding of JWH133 on the rat CB_2 receptor, so we do not know if it induces the initiation of the same signalling pathways as the mouse CB_2 receptor. Such investigations in the future could contribute greatly to our understanding of the lack of neuroprotection observed in this study. Furthermore, over the last decade, the concept of allosterism for G-protein coupled receptors has also emerged, in which the affinity for binding of a ligand is changed by the binding of another ligand away from the active site (Morales *et al.*, 2018). These results evidence the differences that could exist between the effects in a mouse vs. a rat model, in addition to the potential effects when administered to humans, so care must be taken when interpreting results.

Furthermore, there are also various limitations in extrapolating the results from *in vivo* rodent studies to the human condition, depending on the model used. Parkinson's disease has a very gradual onset, with cell death naturally occurring with age, initiating years or perhaps even decades before the first symptoms emerge (Ma *et al.*, 1999). However, many animal models follow a rapid onset, causing substantial neuronal death within just a few

days (reviewed in Tieu, 2011). The LPS model is one such model, swiftly causing neuronal death mediated by an inflammatory reaction. On the other hand, more slowly developing models, such as the AAV-α-synuclein model, may more accurately mimic the disease progression but they are often not economically feasible, due to the costs associated with housing animals long-term and the costs of a long treatment regime. Moreover, cannabinoid treatment in animal models of neurodegenerative disease is typically initiated on the same day or even before toxin administration, whereas such early intervention would not be possible in Parkinson's disease patients, as 50% of neurons have died before they even start exhibiting symptoms (Fearnley and Lees, 1991).

To summarise, while the CB_2 agonist JWH133 did not induce anti-neuroinflammatory or neuroprotective effects in this study, a role for CB_2 agonism as a potential therapeutic intervention in Parkinson's disease should not yet be ruled out. The outcomes of such pre-clinical studies depend on many factors, including the CB_2 agonist used, the mechanism of action of the modelling toxin and the speed of model progression. It is clear that much remains to be determined about the endocannabinoid system and its role in neuroimmunomodulation in the context of neurodegenerative disease.

Chapter 7: General Discussion

The work presented in this thesis sought to further elucidate the link between the cannabinoid CB_2 receptor and Parkinson's disease, and the potential of this receptor as a target for anti-inflammatory disease modification. A deeper comprehension of the link between this cannabinoid receptor and the pathological changes that are occurring in the Parkinsonian brain is vital in order for CB_2 receptor agonism to conceivably be developed as an anti-inflammatory therapy in the future. We thus attempted to contribute to the wider understanding of this by three avenues: 1) by examining alterations in the expression of components of the cannabinoid system in the highly relevant AAV-α-synuclein model of Parkinson's disease; 2) by attempting to improve the AAV-α-synuclein model by combination with an α-synuclein aggregator, to aid in future studies of the condition, and; 3) by investigating if agonism of the CB_2 receptor could reduce the neuroinflammatory response and confer functional neuroprotection in a viral priming model and in an inflammatory LPS model.

The main body of findings from this body of work are: 1) endocannabinoid and *N*-acylethanolamine levels were altered in a viral-mediated α-synuclein overexpression model of Parkinson's disease, but the expression of the CB_2 receptor was not changed; 2) the combination of AAV-α-synuclein and the α-synuclein aggregating molecule FN075 is a promising potential novel model of Parkinson's disease; 3) BCP did not induce any alterations in a truncated viral priming model study and; 4) the CB_2-selective agonist JWH133 did not produce anti-inflammatory or neuroprotective effects in an inflammatory LPS rat model.

There is an urgent requirement for innovative treatments for Parkinson's disease. The incidence of this condition has risen dramatically over the past decades (Dorsey *et al.*, 2018a), and with our ever-increasing elderly population, it will continue to climb (Dorsey *et al.*, 2018b). Levodopa, the current gold standard therapy, provides symptomatic relief from the motor symptoms of the disease, but its effectiveness decreases over time, leading to oscillations in motor functionality and to a re-emergence in Parkinsonian symptoms in the patient (Fox and Lang, 2008; López *et al.*, 2010). Other pharmacological treatments currently on the market, such as dopamine agonists and MAO-B inhibitors (Brooks, 2000; Dezsi and Vecsei, 2017), exert their effects by replacing or modulating the availability of

dopamine, but none of these dopamine-based therapies provide disease-modifying effects. Regenerative treatments that involve the intracerebral transplantation of stem cells are currently gaining much attention (Stoddard-Bennett and Pera, 2019; Parmar *et al.*, 2020), but there remains a clear requirement for therapeutic pharmacological regimes that could interfere with the disease progression.

Neuroinflammation was first suggested to play a role in neurodegenerative disease at the start of the 20th century (Fischer, 1907; Fischer, 1910), but this conjecture largely lost favour for many decades as it was widely assumed in the scientific community at the time that the central nervous system was an immune-privileged site. The discovery of microglia as the resident immune cells of the brain led to the breakdown of this conviction, and in recent years, research into this field has exploded exponentially. It is now generally accepted that neuroinflammation contributes to the progression of neurodegenerative diseases, including Parkinson's disease, by helping to drive a self-sustaining cycle of neurotoxicity and neuroinflammation (Frank-Cannon *et al.*, 2009; Tansey and Goldberg, 2010). Elevated numbers of microglial cells have been reported in the brains of Parkinson's disease patients at *post mortem* (McGeer *et al.*, 1988; Banati *et al.*, 1998; Imamura *et al.*, 2003), and several genetic variants in the *HLA* gene expressed by microglia have been associated with an increased risk of the disease (Hamza *et al.*, 2010; Hill-Burns *et al.*, 2011; Ahmed *et al.*, 2012; Wissemann *et al.*, 2013). Similarly, an increased incidence of Parkinson's disease has been linked to polymorphisms in the genes of pro-inflammatory cytokines such as *TNF-α*, *IL-1β* and *IL-6* (Krüger *et al.*, 2000; McGeer *et al.*, 2002; Håkansson *et al.*, 2005; Wahner *et al.*, 2007). Numerous epidemiological studies have attempted to determine if modulation of the neuroinflammatory processes has potential as an anti-inflammatory therapy, by investigating the relationship between chronic NSAID use and the risk of Parkinson's disease. Overall a strong conclusion could not be drawn, as some studies reported a reduced risk whereas others did not identify any relationship between chronic NSAID use and Parkinson's disease incidence (Chen *et al.*, 2003; Hernán *et al.*, 2006; Etminan *et al.*, 2008; Samii *et al.*, 2009; Manthripragada *et al.*, 2011). Due to this conflicting data, researchers are now investigating more selective targets for anti-inflammatory therapeutic modulation of Parkinson's disease. Numerous neuroprotective strategies have reached the

clinical trial phase, but as of yet, none have been approved (Sarkar *et al.*, 2016; Salamon *et al.*, 2019).

The endocannabinoid system has emerged in recent decades as a potential target for anti-inflammatory therapy in Parkinson's disease, due to its role in immunomodulation (reviewed in Kelly *et al.*, 2020) and in the basal ganglia circuitry (Herkenham *et al.*, 1991; Mailleux and Vanderhaeghen, 1992). Dysregulation of multiple components of the endocannabinoid system have been reported in Parkinson's disease patients, including alterations in the levels of the endocannabinoid anandamide (Pisani *et al.*, 2005; Pisani *et al.*, 2010), the enzyme MAGL (Navarrete *et al.*, 2018) and in the expression of the CB_1 (Hurley *et al.*, 2003; Van Laere *et al.*, 2012; Navarrete *et al.*, 2018) and CB_2 receptors (García *et al.*, 2015; Gómez-Gálvez *et al.*, 2016; Navarrete *et al.*, 2018). With regard to prospective anti-neuroinflammatory therapies, particular attention is being paid to the CB_2 receptor due to its presence on activated microglial cells (Carlisle *et al.*, 2002; Maresz *et al.*, 2005), and as it is thought to be devoid of psychoactivity due to its extremely restricted expression on neuronal cells in the CNS (Atwood and Mackie, 2010).

However, still very little is known about the CB_2 receptor and its link to the neuroinflammatory response and to other pathology changes that are occurring in neurodegenerative diseases. An upregulation in CB_2 receptor expression has been reported clinically and in pre-clinical models of numerous neurodegenerative diseases, including Alzheimer's disease (Benito *et al.*, 2003; Solas *et al.*, 2013), Huntington's disease (Palazuelos *et al.*, 2009; Sagredo *et al.*, 2009), multiple sclerosis (Maresz *et al.*, 2005; Yiangou *et al.*, 2006), and Parkinson's disease (García *et al.*, 2011; Concannon *et al.*, 2015b; Concannon *et al.*, 2016; Gómez-Gálvez *et al.*, 2016). In animal models of Parkinson's disease, pharmacological targeting of the CB_2 receptor has resulted in neuroprotective effects, but this is inconsistent, producing different results with various models and disparate CB_2 agonists (García-Arencibia *et al.*, 2007; Price *et al.*, 2009; García *et al.*, 2011; Javed *et al.*, 2016). Considerably more research is required regarding the CB_2 receptor and its links to the pathology of neurodegenerative disease in order for targeting of the receptor to conceivably become a valid therapeutic option in the future.

Therefore in Chapter 3, we investigated alterations in components of the endocannabinoid system in the AAV-α-synuclein model of Parkinson's disease. This model induces

gradual degeneration of dopaminergic neurons and incorporates α-synuclein pathology, unlike many other Parkinsonian models. Upregulations in CB_2 receptor expression have been reported previously in neurotoxic models of Parkinson's disease (García *et al.*, 2011; Concannon *et al.*, 2015b; Concannon *et al.*, 2016), but these models all exert dopaminergic degeneration by different molecular mechanisms, which may affect the extent of the alterations in CB_2 receptor expression. A better understanding of the links between CB_2 receptor expression and the pathological progression of this very pertinent disease model would contribute greatly to our overall comprehension of the disease. In this study, we did not detect any alterations in CB_2 receptor expression or in the gene expression of other components of the endocannabinoid system. However, AAV-α-synuclein administration did induce a reduction in the levels of the lipid mediators 2-AG and OEA at Week 12 compared to controls, indicating that there is a dysregulation of the endocannabinoid system in this model. However, it is unclear if this was a true decrease or a prevention of an increase, as there was a gradual increase in the levels of these cannabinoids over time in the controls. This may have been induced by aging, or by the overexpression of the GFP protein, and in the future the use of sham animals could help elucidate this link. Interestingly, there was an overall decrease in the markers of microglial and astrocytic expression in the substantia nigra compared to controls. This is in contrast to data from clinical studies, in which elevated numbers of microglia and astrocytes have been reported in the substantia nigra of Parkinson's disease patients at *post mortem* (McGeer *et al.*, 1988; Damier *et al.*, 1993). However, it is unclear whether the decrease in microglial and astrocytic markers that we detected reflects a decrease in the numbers of the corresponding cells, as this would require proper quantitative histological analyses, which was outside the scope of this study. As the upregulation of the CB_2 receptor in Parkinson's disease models has been largely attributed to their increased presence on microglia, without considerable microgliosis it was foreseeable that we would not detect alterations in CB_2 receptor expression. However, the results from this study are still interesting in the study of the prodromal condition, indicating that CB_2 receptor upregulation may not precede cell death.

The lack of a rodent animal model that incorporates all the pathophysiological and motor features of the disease is a major challenge to the field. As previously mentioned, the AAV-α-synuclein rat model has its advantages, but ultimately is hindered by its high

degree of variability. The primate MPTP model is the paragon for the testing of pre-clinical strategies, as this model produces nigrostriatal degeneration and many of the characteristic motor impairments of the disease. However, due to the ethical and other issues associated with using primates, using this model would usually not be possible without prior testing of the intervention in other smaller animals such as rodents. There are now several toxins that can be used to induce nigrostriatal pathology in rats and/or mice, which are undeniably very valuable in the study of the disease (outlined in section 1.4). However, none are currently available which consistently induce slow, progressive nigrostriatal degeneration with α-synuclein pathology and the characteristic motor features of the disease. This is a major issue, particularly with regard to the rapidity at which cell death is caused, when researchers are investigating potential anti-inflammatory therapies or other interventions that aim to slow the progression of the disease. Attempts are currently being made to produce novel models and in the coming years as more genetic variants and environmental factors that contribute to the etiology of the disease are revealed, this may permit the development of animal models that will incorporate more facets of the disease pathology. Until more comprehensive models of the disease are produced, researchers should take care to select the most appropriate model to address their particular research question.

Since the AAV-α-synuclein model we used did not induce discernible dopaminergic degeneration, we attempted to enhance the pathological features of the AAV-α-synuclein model by combining AAV-mediated α-synuclein overexpression with FN075-mediated α-synuclein aggregation (Chapter 4). We carried out two studies, one that used a viral vector that expressed the wild-type version of the human α-synuclein protein, and the second that used a vector that expressed human α-synuclein protein with the A53T mutation that is associated with an autosomal dominant form of Parkinson's disease (Polymeropoulos *et al.*, 1997). We found that although both AAV-α-synuclein vectors induced widespread expression of human α-synuclein along the nigrostriatal pathway, this was not sufficient to result in dopaminergic degeneration. However, the combination of AAV-α-synuclein and FN075 resulted in a significant increase in phosphorylated α-synuclein, as measured by both optical density analyses and by counts of pS129-α-synuclein-positive accumulations. This promising result verifies that FN075 does indeed aggregate α-synuclein, and this potential model warrants further investigation. Novel

models of Parkinson's disease are urgently required, as the animal models that are currently available to study Parkinson's disease do not fully recapitulate the disease condition. The AAV-α-synuclein model is one of the best currently available, but it is limited by its variability and its slowly developing pathology. Researchers have previously attempted to improve the AAV-α-synuclein model, using fibrils or neurotoxins, with varying levels of success (Mulcahy *et al.*, 2012; Naughton *et al.*, 2017; Thakur *et al.*, 2017). However, research into this field must continue, as we need more and disparate models in which to study the disease. The differences in the natures of the toxins used to model the disease and the degree of dopaminergic degeneration and inflammation these models induce will greatly influence the outcome of investigative studies.

As we did not detect appreciable dopaminergic degeneration in the combined AAV-α-synuclein and FN075 model, we regressed to using inflammatory models in subsequent chapters to address whether the CB_2 receptor represents a viable target for anti-inflammatory disease modification in Parkinson's disease. In Chapter 5, we investigated the potential of the CB_2 agonist, BCP, to limit the neuroinflammatory response and provide neuroprotection in a viral priming Parkinsonian model. In the wake of the current global viral pandemic, a Parkinson's epidemic may likely follow (Beauchamp *et al.*, 2020), and thus it has never been more paramount that we investigate if we could prevent the development of post-viral infection Parkinson's disease cases. In this study, an unforeseen adverse effect of the cannabinoid drug, likely due to a contaminant from the manufacturing process, resulted in a curtailment of the experiment 5 weeks early. Thus the model was not induced, and we could not determine if the drug treatment suppressed the exacerbated neurodegeneration and microgliosis observed previously by the sequential administration of poly I:C and FN075 (Olsen *et al.*, 2019). In the forthcoming decades, we will likely see an onslaught of Parkinsonian cases as a result of the COVID-19 epidemic, akin to after the Spanish flu pandemic (Ravenholt and Foege, 1982; Hoffman and Vilensky, 2017), but very little research has been carried out to determine the cellular mechanisms that underlie this 'priming' effect, or if this response could be pharmacologically negated. It is thus imperative that this field is investigated without delay. This study was well designed and if it was repeated using a purified form or a different source of BCP, it could provide valuable insights to researchers. If the effects of

several cannabinoid agonists were tested in this model, and/or in an alternative viral priming model, such as the one involving the combination of poly I:C and the herbicide paraquat Parkinsonian model designed by Bobyn and colleagues (Bobyn *et al.*, 2012), a wealth of information would be gained that could potentially be used to prevent the probable surge in the incidence of Parkinson's disease induced in the coming years as a result of COVID-19 infections.

In Chapter 6, we further explored the viability of the CB_2 receptor as a target for anti-inflammatory disease modification, by investigating the capability of the CB_2-selective agonist JWH133 to reduce neuroinflammation and confer functional neuroprotection in an LPS rat model. LPS is a bacterial endotoxin and has been shown to induce a robust inflammatory response following intracerebral administration (Bing *et al.*, 1998; Castaño *et al.*, 1998; Herrera *et al.*, 2000). Therefore we believed this was a fitting model in which to scrutinize the potential immunomodulatory and anti-inflammatory effects of JWH133. We found that although LPS induced significant microgliosis in the substantia nigra compared to controls, this was not seen in the LPS & JWH133 group, suggesting perhaps a mild anti-neuroinflammatory effect of the CB_2 agonist. Furthermore, LPS induced degeneration of nigral cell bodies, but JWH133 did not provide appreciable neuroprotection to these cells. This is in contrast to previously published studies, which indicated that JWH133 provided beneficial effects in Alzheimer's disease models in addition to an MPTP mouse Parkinson's disease model (Martín-Moreno *et al.*, 2012; Chung *et al.*, 2016; Çakır *et al.*, 2019). However, the model that is used to examine the effects of CB_2 activation is highly relevant and can affect the outcome of a study. There are numerous models of Parkinson's disease, induced by different toxins which bring about dopaminergic degeneration by disparate mechanisms of action. Therefore a beneficial effect that is produced by an agonist in one model may not be necessarily replicated in another, and since none of these models fully recapitulate the disease condition, care must be taken in extrapolating the results of these studies. Furthermore, the mouse and rat protein sequences of the CB_2 receptor have been found to differ quite substantially in the C-terminus domain, with the rat sequence being 63 amino acids longer than the mouse protein (Brown *et al.*, 2002). Therefore, it is not unanticipated that although JWH133 provided beneficial effects in a mouse MPTP model (Chung *et al.*, 2016), that no profound anti-inflammatory effects were observed in this rat LPS model.

Although we did not find that clear evidence that CB_2 agonism would provide disease-modifying effects as we had hypothesised, these results are still important because if cannabinoid-based anti-inflammatory therapies do reach the clinic for Parkinson's disease, it will likely not be via a 'one size fits all' approach. Disease heterogeneity has been long recognised by clinicians dealing with Parkinson's disease patients (reviewed in Ryden and Lewis, 2018), and studies that investigate the effect of a cannabinoid agonist in a particular animal model could assist in the future in understanding differential effects amongst patients. However, translation to the clinic has been a major issue in this field for numerous reasons. Firstly, it is challenging to extrapolate the results of animal studies to patient outcomes due to the lack of Parkinson's disease animal models that encompass all the features of the human condition. Secondly, the endocannabinoid system is a relatively newly discovered signalling system, and is yet poorly comprehended. A more thorough understanding of this system will be necessary to accurately predict the consequential effects of attempting to modulate the receptors, enzymes or other components of this system. Moreover, as subtypes of the two cannabinoid receptors have not yet been discovered, there will likely be off-target effects associated with systemic administration of modulators of these receptors. Clearly, much research remains to be carried out regarding cannabinoids and their potential as Parkinson's disease therapies.

To summarise, the studies presented in this thesis have provided interesting insights into the link between the CB_2 receptor and Parkinson's disease, and the potential of targeting this receptor for anti-inflammatory disease modification. Furthermore, we formulated a novel Parkinson's disease model that could aid in the future study of the disease.

Future directions

Future studies investigating alterations of the CB_2 receptor in Parkinson's disease would benefit greatly from the development of a specific monoclonal CB_2 antibody. Currently, the lack of specificity of the commercially available antibodies is a severe limitation (Baek *et al.*, 2013; Marchalant *et al.*, 2014), as the cellular localisation of the CB_2 receptor cannot be properly investigated. Many of the studies verifying the upregulation of the CB_2 receptor in the brain of neurodegenerative disease patients at *post mortem* utilise these antibodies (Benito *et al.*, 2003; Gómez-Gálvez *et al.*, 2016). While qRT-PCR

analyses are beneficial and can determine if there is an alteration in CB_2 receptor expression at the gene level, they cannot determine if these alterations are occurring on the microglia or on other cells of the central nervous system. *In situ* hybridisation or RNAscope® technologies do allow the cellular localisation of DNA or RNA, but not of protein. A specific antibody would permit the localisation of protein in tissue sections and thus enable research in this field to greatly progress.

Our attempt to develop an enhanced model by the combination of AAV-α-synuclein and FN075 had some promising results and necessitates further attention. FN075 is a novel molecule, and very few *in vivo* studies have been published to date (Chermenina *et al.*, 2015; Olsen *et al.*, 2019). In the future, a combined study using an AAV-α-synuclein vector that is capable of independently inducing dopaminergic degeneration would allow us to comprehensively assess the additive effect of FN075. However, modifications to the timing of the surgeries or of the doses used may be necessary to optimise the model to allow it to be of use pre-clinically. Furthermore, it would be interesting to investigate the potential of a novel model involving FN075 in combination with other methods of α-synuclein overexpression, such as a transgenic α-synuclein model, and to determine if this would result in an exacerbation of the model phenotype.

While we did not observe neuroprotective effects in the two CB_2 agonist studies in this thesis, this does not imply that targeting of this receptor is not a viable research avenue for disease modification in Parkinson's disease. Several studies have indicated that the agonism of this receptor has anti-inflammatory effects, amongst other disease-modifying effects (García-Arencibia *et al.*, 2007; Price *et al.*, 2009; García *et al.*, 2011; Chung *et al.*, 2012). However, these effects vary depending on the specific agonist used, as well as the mechanism of action of the animal model. In order to gain a comprehensive understanding of the therapeutic potential of targeting this receptor, an extensive array of studies utilising different agonists and disparate animal models are required. Initially, inflammatory models are a good starting place to determine if the agonists induce anti-inflammatory effects. Ideally, research will progress to incorporate models that integrate multiple pathological features, but currently the long time frame of some such models (e.g. viral-induced models) poses financial difficulties to researchers, as such long-term agonist treatment can be very costly. However, ever-increasing numbers of CB_2 agonists are being developed which will assist in reducing the price and aiding future research.

Concluding remarks

In conclusion, we found evidence to suggest that dysregulation of the cannabinoid system may precede cell death in Parkinson's disease, as we detected alterations in the levels of the endocannabinoids 2-AG and OEA following viral vector-mediated α-synuclein overexpression. Moreover, anti-inflammatory therapy for disease modification in Parkinson's disease remains a viable option, and targeting the CB_2 receptor for this purpose is still feasible. Numerous lines of *in vitro* and *in vivo* evidence suggest that the agonism of this receptor could provide beneficial therapeutic effects (Price *et al.*, 2009; García *et al.*, 2011; Mecha *et al.*, 2016; Kelly *et al.*, 2020). While this body of research did not provide irrefutable evidence to support this hypothesis, it nevertheless must continue to be investigated. Furthermore, the work presented here did provide evidence of a novel potential animal model of Parkinson's disease, which can be used in the future to deepen our understanding of the progression of the pathological changes that are occurring in the brains of Parkinson's disease patients and can be used to test hypothetical therapeutic therapies.

For many years, Parkinson's disease therapy has focused almost entirely on dopamine replacement strategies, which are purely symptomatic and reduce in effectiveness over time. Therapeutic interventions that interfere with the progression of the disease, such as anti-inflammatory treatment regimes, will probably not be sufficient in isolation to halt the progression of the disease, but by alleviating the inflammatory response they could be of major benefit as an adjunct therapy to current dopaminergic or emerging restorative treatments. Thus, novel anti-inflammatory therapies could greatly improve the quality of life of Parkinson's disease patients.

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Appendix I: Immunohistochemistry

Solutions for Immunohistochemistry

0.9% Saline	Sodium Chloride	9 g
	Distilled water	1 L
Quench	Methanol (98%)	5 ml
	Hydrogen peroxide (30%)	5 ml
	Distilled Water	40 ml
TBS	Trizma Base	12 g
	Sodium Chloride	9 g
	Distilled water	
	Adjust to pH 7.4 with conc. HCl	Make up to 1 L
TBS azide	Trizma Base	12 g
	Sodium Chloride	9 g
	Sodium azide	1 g
	Distilled water	
	Adjust to pH 7.4 with conc. HCl	Make up to 1 L
TXTBS	TBS	250 ml
	Triton X-100	500 µl
ABC	DAKO Streptavidin Kit	
	TBS with 1% serum	1 ml
	Solution A	5 µl
	Solution B	5 µl
TNS	Prepare fresh prior to use	
	Trizma base	6 g
	Distilled water	
	Adjust to pH 7.4 with conc. HCl	Make up to 1 L
DAB stock	DAB	1 g
	TNS	100 ml
	Aliquot into 2 ml aliquots and store at -20°C	20 mg in 2 ml aliquot
DAB working	DAB stock	2 ml
	TNS (fresh)	40 ml
	Hydrogen peroxide (30%)	12 µl
	This solution may be diluted to 1 in 5 with TNS if the reaction proceeds too quickly.	

General Immunohistochemistry Protocol

Suitable for 30 µm free-floating sections. Cut from tissue that has been perfused with phosphate buffer, fixed in 4% buffered paraformaldehyde, and then equilibrated with 30% buffered sucrose.

Free-floating sections are processed in "Greiner pots" on a rotating mixer. The lids of the pots are cut away partly and a gauze square is fitted between the lid and the pot in a way such as to retain the sections but allow the liquid to be tipped away and more added.

Step by step process

Day 1

1. *Wash 1*5 min with TBS.*

2. Quench for 5 min.
   - Methanol 5 ml
   - 30% H_2O_2 5 ml
   - Distilled H_2O 40 ml

3. *Wash 3*5 min with TBS.*

4. Incubate sections in 3% NHS or NGS* for 60 min.
   - 30 µl/ml NHS or NGS in TXTBS *(freshly made-up)*
   -*serum dependent on 2° host

5. Draw off excess and incubate in primary in 1% NHS or NGS at room temperature overnight.

Day 2

6. *Wash 3*10 min with TBS.*

7. Incubate in biotinylated secondary in 1% NHS or NGS for 3 hours.

8. *Wash 3*10min with TBS.* Make ABC Complex when you start these washes.
   Must be made 30 min before incubating the tissue in it. Cover the tubes with tin foil.
   - 5 µl of solution A and 5 µl of solution B per ml in 10 µl/ml NHS or NGS in TBS.

9. Incubate in ABC Complex for 2 hours. ***Keep the pots covered with tin foil.***

10. *Wash 3*10 min with TBS.*

11. Wash with TNS (freshly made-up) and leave overnight @ 4°C.

DAB INCUBATION can also be done the same day after step 11.

Day 3

12. In the fume hood, incubate in H_2O_2/DAB solution until colour develops (3 to 5 min. No more than 5 min). First, use only one pot per immuno to determine the time and darkness of staining. Check the tissue then proceed with the rest of the pots.

    - TNS 40 ml
    - DAB 20 mg (frozen in 2ml aliquots).
    - 30% H_2O_2 12 µl

13. *In the fume hood wash 3*5 min with TNS.*

14. Mount (in TBS with a little TXTBS) on gelatin-coated slides, and allow to air dry overnight.

15. Dehydrate the slides in an ascending series of alcohols: 5 min in 50% alcohol, 5 min in 70% alcohol, 5 min in 100% alcohol (container 1 then repeat in container 2), 5 min in xylene (container 1 then repeat in container 2).

16. Coverslip using DPX mountant.

Slide Subbing

Materials

-Gelatin (10 g/L)
-Chromic Potassium Sulphate (500 mg/L)
-Distilled H_20
-Slides

Step by step process

1. Heat dH_20 to +40°C and add gelatin slowly allowing it to dissolve before adding more
2. Add chromic potassium sulphate
3. Subbing Medium is then cooled to ~30°C
4. Slides placed in slide holders and dipped into subbing medium for ~ 1 min
5. Remove slides and allow to dry for ~ 1 week.

Appendix II: Solutions for Perfusions

1. Heparinised saline

1 ml of heparin is added per 1 L saline (5000 units/litre). Each small vial of heparin contains 25000/ 5 ml i.e. 1 ml of heparin has 5000 units.

2. 30% w/v sucrose solution (1 L)

1. Dissolve 5 PBS tablets in ~500 ml dH_2O (1 PBS tablet per 200 ml water)
2. Add 300 g of sucrose
3. Stir until dissolved, apply heat if necessary
4. Make up to 1 L with dH_2O

3. 30% w/v sucrose solution (1 L) with 0.1% sodium azide

1. Dissolve 5 PBS tablets in ~500 ml dH_2O (1 PBS tablet per 200 ml water final)
2. Add 300 g of sucrose
3. Add 1 g sodium azide
4. Stir until dissolved, apply heat if necessary
5. Make up to 1 L with dH_2O

Appendix III: Quantitative Real-Time PCR (qRT-PCR)

Steps:

1. Tissue collection and storage
2. RNA isolation from *post mortem* tissues
3. RNA quantification
4. RNA normalisation
5. cDNA synthesis
6. Quantitative real-time polymerase chain reaction (qRT-PCR)
7. Analysis

1. Tissue collection and storage

- *Post mortem* tissues from animals will be rapidly dissected following the experiment and snap-frozen immediately on dry ice to prevent RNA degradation.
- Store samples at -80°C until required for further analysis.

2. RNA isolation

- All work surfaces must be cleaned using RNAse removing agents (e.g. RNaseZAP) before commencing RNA extraction procedures.
- Gloves must be worn <u>*at all times*</u> when extracting RNA.
- Use commercially available kit for RNA isolation – **Macherey-Nagel Nucleospin RNA kit**. Perform RNA isolation according to the manufacturer's instructions
- Do not allow tissues to thaw before processing them. Once processed, keep all samples chilled on ice.

3. RNA quantification

- Use Nanodrop Spectrophotometer (NS) for RNA quantification.
- NS provides <u>**three**</u> useful pieces of information:
  a) *RNA concentration (**Yield**):* measured in **ng/µL**.
  b) *RNA integrity (RIN)*: indicated by λ **260/280 ratio**
     - Normal range: **1.8-2.0** for good quality RNA (values slightly outside this range may still be acceptable).
     - Extreme RIN values indicate the presence of high levels of protein, genomic DNA contamination or degraded RNA which may affect downstream work.
  c) *RNA purity:* determined by λ **260/230 ratio**.
     - Acceptable range: **1.8-2.0**.
     - As with (a) above, ratios lying slightly outside this range may be acceptable, whereas extreme outliers could indicate the presence of a significant amount of chemical contamination, which could

interfere/inhibit downstream qRT-PCR reactions (seek advice from somebody more experienced before proceeding).

- RNA measurement:
    1. Follow instructions on instrument initialization and BLANK setting as appropriate (this may be instrument-specific).
    2. Carefully, without touching the sample pedestal with the pipette tip, apply 1 µL of RNA sample onto the sample pedestal of the Nanodrop. Erratic or inconsistent readings can be corrected by increasing the volume of RNA to 1.5 µL or 2 µL or eliminating bubbles from the sample.
    3. Take duplicate readings for each RNA sample and use the average to calculate the amount of RNA to be normalised for reverse transcription (see next section).
    4. Thoroughly clean the sample pedestal and side of the arm in contact with the sample using a soft cleaning tissue before and after every sample assayed.

4. RNA normalisation:

- All RNA samples should be normalised/equalised to a fixed concentration after quantification to ensure that the amount of RNA reverse-transcribed is the same for all samples under study.
- It is ideal to normalise all samples to the lowest concentration obtained in a given batch of samples
- A sufficient volume of normalised RNA samples e.g. 100 µL or another appropriate volume should be prepared using RNase-free water. The remainder of RNA that is not normalised should be returned for storage at -80°C.
- The normalised concentration also determines the maximum amount of RNA that can be reverse-transcribed.
- Once normalised, store all samples at -80°C until required for the next stage of the process.

5. Complementary DNA (cDNA) synthesis:

- Reagents needed for cDNA synthesis:
  - Enzyme for reverse transcription: SuperScript III Reverse Transcriptase
  - RNase OUT Recombinant ribonuclease inhibitor
  - Stock random primers (3 µg/µl): prepare a working concentration of 50-250 ng/µl (typically 250 ng/µl) from stock using RNase free water.
  - DNTP set (100 mM, 4x25 µmol): prepare a working concentration of 10 mM DNTP mix from stock DNTPs using RNase free water.

- **Procedure for cDNA synthesis**
  - Make up the following 2 master mixes in RNase-free Eppendorf tubes and keep them on ice. (Note: Prepare enough to cover all your samples and a couple extra)

Master Mix 1:

REAGENTS	PER SAMPLE
Random primers (250 ng/µl)	1 µL
10 mM dNTP mix	1 µL
TOTAL	**2 µL**

Master Mix 2:

REAGENTS	PER SAMPLE
5X First strand buffer	4 µL
0.1 M DTT	2 µL
RNase OUT	1 µL
TOTAL	**7 µl**

- Procedure:

1. Pipette 10 µL of normalised RNA from each sample into newly labelled PCR tubes (nuclease-free microcentrifuge tube- 200 µL or 500 µL may be used)
2. Add 2 µL of master mix 1 to each tube.

3. Heat the mixture to 65°C for 5 minutes in the thermocycler and quickly chill on ice.
4. Collect the contents of the tube by brief centrifugation (< 10 sec).
5. Add 7 µL of master mix 2 to each tube.
6. Mix contents of tube gently and incubate at 37°C for 2 minutes on the thermocycler.
7. Collect the contents of the tube by brief centrifugation (< 10 sec).
8. Add 1 µL (200 units) of superscript III reverse transcriptase to the bottom of the tube and mix by pipetting gently up and down (or by stirring).
9. If using random primers, incubate tubes at 25°C for 10 minutes (room temperature).
10. Again, load the tubes on the thermocycler, incubate for 50 minutes at 50°C and inactivate the reaction by heating at 70°C for 15 minutes.
11. Dilute (1:4) cDNA samples in RNAase-free water and store at -20°C for qRT PCR assays.

6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Prepare the following master mix in an RNase-free Eppendorf tube and keep it on ice:

- 7.5 µL is per sample. Need this in duplicate and then some spare!

REAGENTS	**PER WELL**
Taqman reagent (2X)	5 µL
Target primer (20X) (e.g for CB_1)	0.5 µL
Reference/endogenous control gene: Beta-actin primer (20X)	0.5 µL
RNAase-free water	1.5 µL
TOTAL	**7.5 µL**

- Before plating, have tubes set up in the correct order (pseudo-randomised plating).
- Add 2.5 µL of cDNA samples into the wells of the plate first (samples are to be plated in duplicate). The Blank is 2.5µL of RNase-free water.
- Add 7.5 µL of the Taqman master mix into the wells (total reaction volume per well = 10 µL).
- Cover the plate using sealing film (make sure the wells are sealed properly). Centrifuge the plate briefly (~ 10 sec)
- Wrap the plate in tin foil and store at -20°C until ready to run it on the StepOne Plus instrument.

7. Analysis

- Calculate the difference (ΔCT) in Cycle thresholds (C_T) values between the target gene (e.g CB_1) and internal reference gene (e.g GAPDH) as follows:

$\Delta CT_{(TARGET-GAPDH)} = C_{T\ Target} - C_{T\ GAPDH}$

for each sample.

- Express **ΔCT** as the negative exponent of **2** ie ($2^{-(\Delta CT)}$) for each sample.
- Express the ($2^{-\Delta CT}$) values of each sample as a fraction or percentage (%) of the **mean** ($2^{-\Delta CT}$) values for the control (e.g vehicle-treated) group of your data set.
- Use these figures to construct a histogram and perform statistical analysis as appropriate.
- If desired an independent sample may be included in the batch of runs as a **CALIBRATOR** sample. The $\Delta CT_{(TARGET-GAPDH)}$ of this sample can be used to further normalise $\Delta CT_{(GAPDH-TARGET)}$ above as follows:

  $\Delta\Delta CT = \Delta CT\ sample_{(TARGET-GAPDH)} - \Delta CT\ cal_{(TARGET-GAPDH)}$

- Express the ΔΔCT as the negative exponent of 2 i.e. $2^{-(\Delta\Delta CT)}$ and proceed with analysis as described above

Appendix IV: Liquid chromatography mass spectrometry

STOCK PREPARATION

10X stock D0 solution containing PEA, OEA and AEA at a final concentration of 5 µg/ml and 2-AG at 50 µg/ml.

Reagents:

-Unlabelled/non-deuterated (D0) endocannabinoids from Cayman chemicals (see table 1 below)
-100% Acetonitrile (ACN)

CAY62160	2-AG	1 mg	Supplied in 1 ml of acetonitrile, dilute further in ACN to a final conc. 0.5 mg/ml and store at -20°C
CAY90050	AEA	5 mg	Supplied in 100 µL of EtOH, dilute to a final conc. 2.5 mg/ml in ACN and store at -20°C
CAY90350	PEA	5 mg	Supplied as a powder, insoluble in ethanol. Dissolve to a final conc. of 2.5 mg/ml in ACN and store at -20°C
CAY90265	OEA	5 mg	Supplied as a powder, dissolve to a final conc. of 2.5 mg/ml in ACN and store at -20°C

Table 1 Details of non-deuterated Endocannabinoids

i) Add 800 µl acetonitrile to 1.5 ml Eppendorf tubes by positive displacement. Label tubes as 10X D0 calibrations.
ii) Add 100 µl D0 2-AG (0.5 mg/ml)
iii) Add 2µl each of PEA, OEA, AEA (2.5 mg/ml) using a 10 µl glass syringe for accuracy or alternatively any suitable, well-calibrated pipette and aspirate up/down in the acetonitrile to totally deliver calibrations into the acetonitrile. Be careful to wash out the syringe with 100% acetonitrile/change pipette tips between each calibration transferred to avoid contaminations!
iv) Add 94 µl acetonitrile to the above mix to give a final volume of 1000 µl and vortex and aliquot (100 µl each).
v) Each 10X aliquot now contains all calibrations at 5 µg/ml except 2-AG which is 50 µg/ml and should be stored for later.

Working (1X) solution containing PEA, OEA and AEA (0.5 µg/ml) and 2-AG (5 µg/ml) solution.

i) To 100 µl (10X stock solution), add 900 µl of 100% acetonitrile using positive displacement pipettes. Vortex and ‘pulse’ centrifuge for 10 seconds i.e. just to

get the liquid down from the sides and prepare 100 µl aliquot for storage at -80°C.

ii) Each aliquot contains PEA, OEA and AEA (0.5 µg/ml) and 2-AG (5 µg/ml) and can be used for preparing your calibration curve.

Deuterated endocannabinoid stock containing 2.5 ng (PEA, OEA, AEA) and 50 ng 2-AG in 200 µl deuterated mixture added to sample or calibration curve as internal calibration.

Reagents:

i) Stock deuterated concentrations for all (PEA, OEA, AEA and 2-AG) is 100 µg/ml (see table 2 below)

Procedure:

(i) Prepare enough for 800 samples as follows:

(ii) To a measuring cylinder (preferably glass cylinder if available), add:
   a. 159.54 ml Acetonitrile; followed by
   b. 400 µl stock (*100 µg/ml*) deuterated 2-AG; and finally
   c. 20 µl each of stock (*100 µg/ml*) deuterated PEA, OEA, AEA

(iii) Stir/mix well. You should now have 160 ml stock containing all deuterated compounds at 2.5 ng (PEA, OEA and AEA) and 50 ng (2-AG) per 200 µl mixture.

(iv) Prepare 40 ml aliquots and store at -80°C until required for use

CAY 362160	2-AG(d8)	100 µg/ml	Supplied in 100 µl ACN, make up to 1 ml in ACN and store at -20°C
CAY390050	AEA(d8)	100 µg/ml	Supplied in 100 µl ethyl acetate solution, make up to 1 ml in EtOH and store at -20°C
CAY10007824	PEA(d4)	100 µg/ml	Supplied in 100 µl EtOH, make up to 1 ml in EtOH and store at -20°C
CAY10007823	OEA(d4)	100 µg/ml	Supplied in 100 µl EtOH, make up to 1 ml and store at -20°C

Table 2 Details of Deuterated endocannabinoids

2.0 PREPARATION OF CALIBRATION CURVES

i) Add 75 µl acetonitrile to 10 Eppendorf tubes labelled #1- #10

ii) Next, add 25 µl of 1X stock to tube #10, vortex then take out 25 µl and transfer to the next tube (#9) containing 75 µl acetonitrile. Vortex, and aspirate 25 µl into the next tube (#8) etc. Repeat the process until tube #1 using the same pipette tip. Discard 25 µl from tube #1 to ensure the correct volume. All the

10 tubes should now have 75 µl of a mixture of PEA, OEA, AEA and 2-AG. See the table below (Table 3) detailing the concentrations related to this method.

iii) Spike tubes (#1-#10) by adding 200 µl of deuterated endocannabinoid mixture which acts as an internal standard. Each tube should now have a total volume of 275 µl.

iv) Transfer up to 40 µl of the mixture into a plastic cap HPLC vial for measurement (We typically inject 2-6 µL per sample).

	2-AG	AEA	OEA	PEA
1X stock (25 µl)	125 ng	12.5 ng	12.5 ng	12.5 ng
Tube #10 Final Conc. (75 µl)	93.75 ng	9.375 ng	9.375 ng	9.375 ng
Deuterated Mixture (200 µl)	50 ng	2.5 ng	2.5 ng	2.5 ng

Table 3 Concentrations related to 1/4 Calibration Curve for endocannabinoids

3.0 TISSUE SAMPLE PREPARATION:

i) Using a positive displacement pipette, add 200 µl of deuterated endocannabinoid mixture.

ii) Make up the total volume by adding 75 µl 100% ACN.

iii) Gently sonicate samples. When carrying out sonication, ensure that you do not overheat the samples and also that the probe is immersed in solution throughout (approx. 5 seconds). Keep homogenates on ice to chill after sonication while you process the rest of your samples. Quickly clean the sonicator between samples to remove any tissue debris and avoid cross-contamination.

iv) Place homogenates in a refrigerated centrifuge (4°C) and spin for 15 minutes @ 14,000 rpm.

v) Transfer up to 40 µl of the supernatant into a plastic capped HPLC vial for measurement. Transfer the remainder of the supernatant into fresh HPLC vials, label and store at -80°C for future use if required.