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**Author(s)**: Concannon, Ruth M.

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The cannabinoid type-2 (CB₂) receptor as a target for anti-inflammatory disease modification in Parkinson’s disease

By

Ruth M. Concannon

B.Sc. M.Sc.

Supervisor: Dr. Eilís Dowd

Co-supervisor: Prof. David Finn

Pharmacology & Therapeutics

National University of Ireland, Galway

Doctor of Philosophy

August 2016
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.

Signed:………………………………………………………………………… Date:………………..
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First and foremost, I would like to thank my supervisor Dr. Eilís Dowd. To say your guidance was invaluable is an understatement. I consider myself extremely lucky and blessed to have had the opportunity to work with you. You always instilled a sense of pride and enthusiasm in our work, encouraging us professionally and personally and so importantly, you were approachable and kind. I can never thank you enough for all that you’ve done, you’ve gone above and beyond your duties as a supervisor and I just hope I’ve made you proud. I would also like to thank my co-supervisor, Prof. David Finn for his support, input and guidance over the last four years and for his unwavering enthusiasm for my work.

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“We never know how high we are ‘til we are caused to rise; and then, if we are true to plan, our statures touch the sky.”

Emily Dickinson

“The simple things are also the most extraordinary things, and only the wise can see them.”

Paolo Coelho, *The Alchemist*
Abstract

Parkinson’s disease is a multifaceted neurodegenerative disorder with no curative treatment. A key aspect in the pathogenesis of Parkinson’s disease is inflammation, which is initially triggered by dying neurons and which may form a self-sustaining cycle of neuroinflammation and neurodegeneration, ultimately propagating disease progression over time. As such, anti-inflammatory disease modification is an emerging field in the treatment of Parkinson’s disease. Because of its location on microglia and its immunosuppressant effect on these cells, the cannabinoid type-2 (CB$_2$) receptor has been hypothesised as a potential anti-inflammatory target for this condition. Thus, the overarching aim of this body of research was to investigate expression of the CB$_2$ receptor in preclinical models of Parkinson’s disease, and to determine if pharmacological targeting of this receptor could provide functional neuroprotection in these models.

We first assessed CB$_2$ receptor expression in a genetic model of Parkinson’s disease, using adeno-associated viral (AAV) delivery of human wild-type α-synuclein to the rat brain. Following on from this, we investigated CB$_2$ expression and changes in other elements of the endocannabinoid system in the neurotoxic, 6-hydroxydopamine, and the environmental, rotenone, models of Parkinson’s disease. We then characterised CB$_2$ receptor expression and the endocannabinoid system in ‘emerging’ inflammation-driven models induced by the bacterial mimetic, lipopolysaccharide (LPS), and the viral mimetic, polyinosinic-polycytidylic acid (Poly (I:C)). Finally, we investigated the potential of targeting the CB$_2$ receptor for anti-inflammatory disease modification in the inflammation-driven models. This
involved chronic administration of CB₂ agonists in the LPS and Poly (I:C) models, and assessing the impact of this treatment on motor impairment, neuroinflammation and nigrostriatal integrity.

We found dramatic differences in CB₂ receptor in the different models which was dependent on the nature of the model. The AAV-α-synuclein model, which was associated with mild neuroinflammation, was not associated with any changes in CB₂ receptor expression. In contrast, the neurotoxic, environmental and inflammatory models were all associated with pronounced neuroinflammation, with concomitant upregulation of CB₂ receptor expression, as well as other changes in the endocannabinoid system. Interestingly, the change in CB₂ receptor expression was particularly pronounced in the bacterial (LPS) and viral (Poly (I:C)) models indicating a potential key role for this receptor in inflammation-driven neurodegeneration. Subsequent pharmacological studies revealed that CB₂ agonists were capable of providing some functional neuroprotection in these inflammation-driven models.

To conclude, we have demonstrated that the CB₂ receptor is elevated in the brains of neurotoxic, environmental and inflammatory models of Parkinson’s disease, and pharmacological targeting of this receptor can provide some neuroprotection against inflammatory lesions. Therefore, this body of research indicates that the CB₂ receptor may indeed be a viable therapeutic option for anti-inflammatory disease-modification in Parkinson’s disease, and undoubtedly merits further investigation in this context.
Publications

Peer Reviewed Published Manuscripts


Peer Reviewed Published Abstracts

Other Research Dissemination

International Conferences Attended


2. **RM Concannon**, DP Finn, E Dowd. Pharmacological targeting of the cannabinoid type-2 (CB₂) receptor prevents inflammation-driven neurodegeneration in the lipopolysaccharide rat model of Parkinson’s disease. **Poster presentation** at the 7th European Workshop on Cannabinoid Research, Sestri Levante, Italy. September 2015.


National Conferences Attended


### List of commonly used abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Explanation</th>
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<tbody>
<tr>
<td>AEA</td>
<td>anandamide</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-arachidonylglycerol</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>anterioposterior</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CD11b</td>
<td>integrin alpha M/ CR3</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyl transferase</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenyl-acetaldehyde</td>
</tr>
<tr>
<td>DV</td>
<td>dorsoventral</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>Fig.</td>
<td>figure</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>GABA</td>
<td>gamma (γ)-aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>Gpe</td>
<td>globus pallidus externa</td>
</tr>
<tr>
<td>Gpi</td>
<td>globus pallidus interna</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HVA</td>
<td>homovanillic acid</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
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<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LRRK2</td>
<td>leucine-rich repeat kinase 2</td>
</tr>
<tr>
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<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>---------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>MFB</td>
<td>medial forebrain bundle</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
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<td>milligram per kilogram</td>
</tr>
<tr>
<td>min</td>
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</tr>
<tr>
<td>ML</td>
<td>mediolateral</td>
</tr>
<tr>
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<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
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<td>nanometre</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>OEA</td>
<td>Oleoylethanolamide</td>
</tr>
<tr>
<td>OX42</td>
<td>integrin alpha M</td>
</tr>
<tr>
<td>PEA</td>
<td>Palmitoylethanolamide</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
</tr>
<tr>
<td>Poly (I:C)</td>
<td>polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>standard error ± mean</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SNpc</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNpr</td>
<td>substantia nigra pars reticulata</td>
</tr>
<tr>
<td>STR</td>
<td>striatum</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>UPDRS</td>
<td>Unified Parkinson’s Disease Rating Scale</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
</tbody>
</table>

Most abbreviations, other than commonly used expressions, are also defined at the first point of occurrence in the text.
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Chapter 1: General introduction

Parkinson’s disease is a chronic, progressive neurodegenerative condition that is the second most prevalent neurodegenerative disease, after Alzheimer’s disease, worldwide affecting 1% of the population over 65 (Nussbaum and Ellis, 2003). The condition manifests primarily as a motor impairment which is characterised by postural instability, slowness of movement, rigidity and tremor. Non-motor symptoms also emerge affecting the autonomic and psychiatric systems. It is characterised pathologically by degeneration of the nigrostriatal dopaminergic pathway and the formation of intracellular inclusions termed Lewy bodies, incorporating aggregated α-synuclein protein (Spillantini et al., 1997). Sadly, by the time a patient is diagnosed, over 50% of their nigrostriatal neurons have already been lost, and current therapies are purely symptomatic and offer no neuroprotection to prevent further cell loss. Indeed, there are serious unmet clinical needs in the pharmacological treatment of this condition.

In addition to the classical neuropathological features of nigrostriatal neurodegeneration and Lewy body formation, Parkinson’s disease is also associated with chronic neuroinflammation. Indeed, disease progression is thought to be driven in part by inflammatory events which precede or succeed initial neuronal loss (Gao and Hong, 2008). Given this, the concept of anti-inflammatory therapy for Parkinson’s disease has emerged - slowing this inflammatory process may slow the progression of the disease and offer a viable disease-modifying therapy. The endocannabinoid system has recently emerged as a potential anti-inflammatory target to break the self-sustaining cycle of neuroinflammation and neurodegeneration.
Chapter 1: Introduction

Specifically, the cannabinoid type-2 (CB₂) receptor represents an attractive target as it is expressed on microglia, the resident immune surveillance cells in the brain, where it exerts pronounced anti-inflammatory effects.

To provide a background for the research presented in this thesis, this introductory chapter will provide an overview of Parkinson’s disease and the role of neuroinflammation in the pathogenesis of the condition. The endocannabinoid system will then be introduced, specifically highlighting the dysregulation of the endocannabinoid system that occurs in Parkinson’s disease, and the potential for targeting the CB₂ receptor to reduce inflammation-driven neurodegeneration in this condition.

1.1 PARKINSON’S DISEASE

1.1.1 AN HISTORICAL PERSPECTIVE

Parkinson’s disease was first described as a shaking palsy (paralysis agitans) in the early nineteenth century by James Parkinson in ‘An Essay on the Shaking Palsy” (Parkinson, 1817). Parkinson studied and observed impairment in motor function in six patients, becoming the first clinician to describe the classic Parkinsonian symptoms including resting tremor, postural instability, falls and altered gait. However it was many years later that Jean-Martin Charcot identified a distinguishable symptom, bradykinesia, which allowed for the condition to be separated from other neurological diseases such as multiple sclerosis (Charcot and Vulpian, 1861). In addition, Charcot characterised the tremorous and akinetic aspects, and described non-motor aspects such as autonomic dysregulation. Indeed, it
Chapter 1: Introduction

was he who suggested calling the condition ‘Parkinson’s disease’ as he noted not all patients displayed the typical shaking palsy or paralysis agitans as described by James Parkinson.

In the following years, many clinical descriptions of Parkinson’s disease began to emerge encapsulating the many facets of the disease we know today. William Gowers was the first to comment on the higher incidence of the disease in men in his ‘Manual of Diseases of the Nervous System’ (Gowers, 1887), and Babinski noted the characteristic motor fluctuations (Babinski et al., 1921). In the late nineteenth century, the anatomical origin of Parkinson’s disease was proposed by several researchers, initially by Brissaud et al (Brissaud, 1894) who postulated the substantia nigra was the location of neuronal damage, supported by observations by Blocq and Marinesco (1893) however it was a further 20 years definitive evidence was provided by Trétiakoff (1919). Classification of Parkinson’s disease progression followed in a seminal article by Hoehn and Yahr, where they described the Unified Parkinson’s Disease Rating Scale - rating stages based on unilateral and bilateral disease, a scale which is still used in the diagnosis of patients today (Hoehn and Yahr, 1967).

Unravelling the pathophysiology of Parkinson’s disease began in 1912 when Frederic Lewy found intracellular inclusions in neuronal populations in several brain regions post mortem (Lewy, 1912). A few short years later, Tretiakoff was the first to pinpoint the loss of neurons in the substantia nigra pars compacta (Trétiakoff, 1919). However it was not until the 1950s that the role of dopamine in Parkinson’s disease became apparent. The classification of dopamine as a neurotransmitter in 1957 by
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Swedish pharmacologist Arvid Carlsson, and the development of an assay to measure dopamine levels, paved the way for further investigation (Carlsson and Waldeck, 1958, Carlsson et al., 1957). Clinically, Hornykiewicz first reported reduced dopamine in the brains of Parkinson’s disease patients (Hornykiewicz, 1962) and subsequently it was identified that an approximate 80% reduction in striatal dopamine or a 60% reduction in nigrostriatal dopamine neurons was necessary in order to produce Parkinsonian symptoms (Bernheimer et al., 1973).

In subsequent work, Carlsson performed experiments with reserpine (which depletes dopamine) and found that this induced motor impairment in rabbits similar to symptoms observed clinically in Parkinson’s disease (Carlsson et al., 1957). He showed that administration of the dopamine precursor, L-DOPA, ameliorated the motor dysfunction induced by reserpine in these animals. Keen to determine if L-DOPA had any therapeutic potential, Horonykiewicz along with a clinician Birkmayer, trialled a low dose intra-venous L-DOPA regime which produced some beneficial effects (in conjunction with a monoamineoxidase (MAO) inhibitor) by reducing bradykinesia (Birkmayer and Hornykiewicz, 2001). However the effects were shortlived and only provided relief to some patients. Given the toxicity induced by high dose L-DOPA, several other groups worldwide tested similar doses and regimes of L-DOPA treatment with limited benefits. However, in 1967 an article by George Cotzias reported novel, successful L-DOPA treatment regime which allowed for a sustained oral L-DOPA administration, devoid of negative side-effects, that dramatically reduced Parkinsonian symptoms (Cotzias et al., 1967).
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1.1.2 **Etiology**

While the multi-faceted pathology of Parkinson’s disease has been well characterised since the first clinical description, the etiology has yet to be clarified. There are a vast number of factors associated with the condition which may predispose or contribute to its manifestation (Wirdefeldt et al., 2011). Despite the plethora of confounding factors, a certain few have emerged as key players in the causation of Parkinson’s disease, namely age, environmental factors and genetic background.

1.1.2.1 **Age**

Age is the biggest risk factor for developing Parkinson’s disease with a proportionate increase in the emergence of the condition with increasing age (Hindle, 2010). The mean age of onset of the disease is 70 years of age, and it affects 1% of the population over 65 (Nussbaum and Ellis, 2003). Ageing is a degenerative process in itself, affecting all bodily functions and systems. Dysfunction of ‘normal’ cellular repair mechanisms leads to an accumulation of cellular damage which perpetuates and, in some cases, may lead to the development of disease states (Kirkwood and Kirkwood, 2003). Neurons are particularly sensitive to the effects of ageing, one facet of which is mitochondrial dysfunction. Production of free radicals and oxidative stress can alter DNA, inducing mutations and shortening telomeres, which leads to cell death (Migliore and Coppedè, 2009). Several other events in the pathogenesis of Parkinson’s disease are associated with ageing – protein deposition, increased inflammatory profile, reduced efficacy of neurotrophic factors, deposition of iron, and loss of striatal dopamine and dopaminergic neurons (Tai and Schuman, 2008, Jellinger, 2004, Cherra 3rd and Chu, 2008, Zecca et al., 2004, VanItallie,
The mechanism of ageing has parallels with the pathogenesis of PD, however a distinct feature which may separate the two, is the failure of normal compensatory mechanisms in PD given the greater extent of neuronal loss, protein deposition and formation of Lewy bodies in vulnerable brain areas, which may be predisposed due to environmental and/or genetic influences.

1.1.2.2 Environmental factors

The role of the environment in the etiology of Parkinson’s disease has largely been informed by epidemiological studies which have identified potential risks to developing the condition later in life. The main sources of these include exposure to toxic chemicals and systemic infections which may sensitize brain regions, and predispose a person to developing Parkinson’s disease.

1.1.2.2.1 Chemical exposure

The association between exposure to toxic chemicals and the development of Parkinson’s disease first emerged following the report of Parkinsonian symptoms and nigrostriatal degeneration in intravenous drug users who accidentally injected themselves with MPTP (Langston et al., 1983). Given this evidence, it was hypothesized that exposure to neurotoxic substances may preclude or increase the susceptibility to the development of the condition. Exposure to hazardous chemicals including pesticides and herbicides from various sources (e.g. rural living, farming occupation, consuming well water) is associated with a significantly increased risk of developing the condition according to a number of epidemiological reports (Priyadarshi et al., 2000, Firestone et al., 2005, Petrovitch et al., 2002, Betarbet et al.,
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2000). Exposure to heavy metals has also been implicated as a potential etiological factor, as they can accumulate in brain regions, causing oxidative stress (Jankovic, 2005).

1.1.2.2.2 Infections and inflammation

The concept of an infectious etiology of Parkinson’s disease first emerged in the 1920s when patients with influenza virus-induced encephalitis lethargica developed Parkinsonism (Gamboa et al., 1974, Ravenholt and Foege, 1982). For some time it was thought that post-encephalitic Parkinsonism and Parkinson’s disease were the same condition, however retrospectively it was identified that several clinical features, including mean age of onset, differed between the conditions and that, although the pandemic induced Parkinsonism, it was clinically separate from Parkinson’s disease as characterised by James Parkinson (Duvoisin et al., 1963). This was further supported by the absence of influenza viral particles in the substantia nigra of patients with Parkinson’s disease (Schwartz and Elizan, 1979, Wetmur et al., 1979). More recently, however, influenza A virus has been identified in macrophages within the substantia nigra of idiopathic Parkinson’s disease patients (Rohn and Catlin, 2011) and several animal studies have also drawn a link between influenza viral infection and the development of Parkinson’s disease pathology (Jang et al., 2009, Jang et al., 2012).

There is also evidence to suggest that other viruses, and not just influenza, may induce post-encephalitic Parkinsonism, and given the sensitivity of midbrain regions to viruses due to the high density of microglia in this region (Lawson et al., 1990), it stands to reason that viral infection is considered a potential etiological factor in
Parkinson’s disease. Clinical evidence to support this however, is lacking, with conflicting reports on the associated risk between many viruses (herpes simplex, diptheria, measles), and development of the condition (Marttila et al., 1977, Elizan et al., 1979). The most recent data from a case-controlled study in British Columbia, indicated an increased Parkinson’s disease risk with influenza virus infection, and a reduced risk associated with red measles infections, which is consistent with previous reports (Harris et al., 2012). The hepatitis C virus (HCV) is neurotropic, and a recent study reported an association between HCV infection and Parkinson’s disease, and also identified a significant dopaminergic cell loss in midbrain neural cells infected with HCV (Wu et al., 2015). A case-controlled Swedish study specifically addressed ‘severe’ CNS infection and found these were associated with a future high risk for developing Parkinson’s disease (Fang et al., 2012). Overall, viral infection in the etiology of Parkinson’s disease requires further clinical and preclinical investigation to decipher its role.

Inflammation is a key contributor to the progression of PD and it has been hypothesized that environmental toxin and/or infection-induced inflammation may also play a role in the etiology of the condition. Thus, chronic microglial activation, and other immune responses, may be initiated by environmental toxins or infectious agents. However, the question of whether inflammation may precede neurodegeneration is unanswerable in the context of the human post-mortem evidence as it is also a pathological feature (Liu et al., 2003). In examining the causal role of inflammation in Parkinson’s disease, epidemiological evidence has provided some interesting insights. In particular, the use of NSAIDs has been linked to
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reduced risk of developing the disease, although much debate still reigns. As detailed in section 1.1.2.3.1, the data is inconclusive on the potentially neuroprotective effects of NSAIDs and although there is a strong argument for their use, it remains unclear how this information, conflicting as it may be, informs any etiological role of inflammation in Parkinson’s disease. Pre-existing autoimmune conditions do not appear to contribute to the risk of developing Parkinson’s disease (Rugbjerg et al., 2009), however elevated levels of IL-6 is found in the serum of patients, approximately 5 years prior to diagnosis (Chen et al., 2008). Given that there is a considerable proportion of nigrostriatal degeneration at diagnosis, it is arguable that this observation may be more directly related to the pathology rather than etiology however it is interesting that levels of other peripheral inflammatory biomarkers are not elevated at this stage.

1.1.2.3 Genetic predisposition

Parkinson’s disease was initially characterised as a sporadic disease, however epidemiological evidence indicates that ~10% of cases have a familial link. The first concrete evidence for autosomal dominant Parkinson’s disease was presented by Polymeropoulos and colleagues, identifying an association between this form of the disease and a mutation in the gene encoding α-synuclein (Polymeropoulos et al., 1997), which is the main constituent of pathogenic intracellular inclusions known as Lewy bodies (Spillantini et al., 1997). Since the discovery of α-synuclein as a genetic ‘driving force’ in PD, many more genes have been identified as being potentially etiological (Table 1.2). Deficiencies or mutations in these genes have been identified as the principal driving force in early-onset familial Parkinson’s disease, and
although this only accounts for ~10% of cases, understanding the genetic etiology has provided a window into factors that may contribute to an increased risk of developing the more common, idiopathic form of Parkinson’s disease (Verstraeten et al., 2015, Lesage and Brice, 2009).

Identification of PD ‘risk’ genes has been achieved through various genetic association studies, including gene mapping and candidate gene approaches. Studies using linkage analysis and genome-wide association studies (GWAS) first identified the five main causal PD genes – SNCA (α-synuclein), LRRK2, Parkin (E3 ubiquitin protein ligase), PINK1 and DJ-1 (protein deglycase) (Nuytemans et al., 2010). Candidate gene association studies looks specifically at genes implicated in Parkinson’s disease on the assumption that genetic variants in these candidate genes are associated with an increased or decreased risk of developing a condition (Westerlund et al., 2010). Common variation has been identified in SNCA, LRRK2 and GBA (glucosidase B acid), with both ‘protective’ and ‘destructive’ haplotypes associated with Parkinson’s disease (Sidransky and Lopez, 2012, Ross et al., 2011). The most recent GWAS data, a meta-analysis of previous studies, identified 26 independent loci with a significant association with PD (Nalls et al., 2014), thus the contribution of genetics to the etiology of Parkinson’s disease may be wide-ranging and heterogenous. Interestingly, the genes/proteins that have been highlighted as being associated with Parkinson’s disease are present in all neurons and other cell types, not specifically dopaminergic neurons, which reiterates the particular susceptibility or sensitivity of dopamine neurons to stress-induced damage.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Inheritance</th>
<th>Gene product and known function(s)</th>
<th>Onset &amp; progression</th>
<th>Pathology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNCA</td>
<td>Autosomal dominant</td>
<td>Presynaptic protein, regulation of synapse vesicle trafficking</td>
<td>Early onset, aggressive course</td>
<td>Nigrostriatal degeneration &amp; Lewy bodies</td>
<td>(Polymeropoulos et al., 1997)</td>
</tr>
<tr>
<td>LRRK2 (PARK8)</td>
<td>Autosomal dominant</td>
<td>Protein kinase</td>
<td>Late onset, typical course</td>
<td>Nigrostriatal degeneration &amp; Lewy bodies</td>
<td>(Paisan-Ruiz et al., 2004)</td>
</tr>
<tr>
<td>GBA</td>
<td>Autosomal dominant</td>
<td>Lysosomal enzyme, lipid metabolism</td>
<td>Late onset, typical course</td>
<td>Nigrostriatal degeneration &amp; Lewy bodies</td>
<td>(Neudorfer et al., 1996)</td>
</tr>
<tr>
<td>VPS35</td>
<td>Autosomal recessive</td>
<td>Retromer component, endosomal transport</td>
<td>Late onset, typical course</td>
<td>Unknown</td>
<td>(Zimprich et al., 2011, Vilarino-Guell et al., 2011)</td>
</tr>
<tr>
<td>Parkin (PARK2)</td>
<td>Autosomal recessive</td>
<td>Ubiquitin ligase</td>
<td>Early-onset, slow course</td>
<td>Nigrostriatal degeneration variable synucleinopathy</td>
<td>(Kitada et al., 1998)</td>
</tr>
<tr>
<td>PINK1</td>
<td>Autosomal recessive</td>
<td>Mitochondrial kinase, protects against stress-induced dysfunction</td>
<td>Early onset, slow course</td>
<td>Nigrostriatal degeneration &amp; Lewy bodies</td>
<td>(Valente et al., 2004)</td>
</tr>
<tr>
<td>DJ-1 (PARK7)</td>
<td>Autosomal recessive</td>
<td>Redox-sensitive protein, sensor for oxidative stress, anti-oxidant activity</td>
<td>Early onset, slow course</td>
<td>Unknown</td>
<td>(Bonifati et al., 2003)</td>
</tr>
<tr>
<td>ATP13A2</td>
<td>Autosomal recessive</td>
<td>Cation-transporting ATPase</td>
<td>Juvenile onset, atypical</td>
<td>Unknown</td>
<td>(Ramirez et al., 2006)</td>
</tr>
<tr>
<td>PLA2G6</td>
<td>Autosomal recessive</td>
<td>Phospholipase, multiple cellular functions</td>
<td>Juvenile onset, atypical</td>
<td>Nigrostriatal degeneration &amp; Lewy bodies, iron accumulation</td>
<td>(Paisan-Ruiz et al., 2009)</td>
</tr>
<tr>
<td>FBX07</td>
<td>Autosomal recessive</td>
<td>Ubiquitin ligases</td>
<td>Juvenile onset, atypical</td>
<td>Unknown</td>
<td>(Shojaei et al., 2008, Di Fonzo et al., 2009)</td>
</tr>
<tr>
<td>SYNJ1</td>
<td>Autosomal recessive</td>
<td>Phosphoinoside phosphatase, regulation of synapse vesicle trafficking</td>
<td>Juvenile onset, atypical</td>
<td>Unknown</td>
<td>(Krebs et al., 2013, Quadri et al., 2013)</td>
</tr>
<tr>
<td>DNAJC6</td>
<td>Autosomal recessive</td>
<td>Heat shock protein, molecular chaperone stimulating ATPase activity</td>
<td>Juvenile onset, atypical</td>
<td>Unknown</td>
<td>(Edvardson et al., 2012)</td>
</tr>
</tbody>
</table>

Table 1.1. Genes implicated in the etiology of Parkinson's disease. Modified from (Verstraeten et al., 2015, Bonifati, 2014, Shulman et al., 2011). SNCA, α-synuclein; LRRK2, leucine-rich repeat-containing kinase; GBA, Lysosomal glucocerebrosidase; VPS35; Parkin, parkin E3 ubiquitin protein ligase; PINK1, PTEN-induced mitochondrial serine/threonine kinase; DJ-1, Protein deglycase DJ-1; ATP13A2, ATPase 13A2; PLA2G6, Phospholipase A2 group VI; FBX07, F-box protein 7; SYNJ1, synaptojanin 1; DNAJC6, DNAJ heat shock protein family (Hsp40) member C6;
1.1.3 PATHOPHYSIOLOGY

1.1.3.1 Nigrostriatal Degeneration

1.1.3.1.1 The basal ganglia

Arguably the most recognisable and debilitating symptoms of the disease are those affecting the motor system. Motor function and coordination is intricately wired in a circuitry known as the basal ganglia (Fig. 1.1). It is essentially composed of four structures – the striatum, globus pallidus, substantia nigra and the subthalamic nucleus, which, through various stimulatory and inhibitory pathways, converge on the cerebral cortex forming a cortico-striato-pallidal-thalamo loop (Parent and Hazrati, 1995).

Glutamatergic input from the motor cortex and thalamus and dopaminergic input from nigrostriatal neurons converge at the level of the striatum, engaging with neuronal cell types including cholinergic interneurons and GABAergic medium spiny neurons (MSNs). These MSNs are densely expressed in the striatum and project to two distinct output nuclei, which are act antagonistically to facilitate movement. Striatonigral MSNs, expressing excitatory dopamine D_1 receptors, project to the substantia nigra pars reticulata (SNr) and the globus pallidus pars interna (GPi), termed the ‘direct pathway’. The ‘indirect’ pathway is comprised of striatopallidal MSNs expressing inhibitory dopamine D_2 receptors which project to the globus pallidus pars externa (GPe). These pathways are physiologically antagonistic and as such control movement. The opposing actions of D_1 and D_2...
receptors on MSNs is due to the different signalling mechanisms associate with activation of these G protein-coupled receptors and downstream adenylyl cyclase activity and cellular excitation (Kebabian and Greengard, 1971). GABAergic MSNs in the direct pathway, project to the SNr and GPi where they inhibit GABAergic projections to the thalamus, thereby ultimately stimulating excitatory thalmocortical projections and activating cortical neurons to induce movement. Conversely, in the indirect pathway, MSNs, inhibit the GABAergic GPe neurons thereby simulating excitatory glutamatergic neurons in the subthalmic nucleus. These excitatory neurons form synapses at the GPi and SNr thereby activating inhibitory GABAergic output neurons resulting in inhibition of thalamocortical projections and an inhibition of movement (reviewed in Kreitzer and Malenka, 2008).

Overall, motor control is executed via a delicate balance of excitatory and inhibitory signalling, and although initial cortical glutamatergic input is excitatory, the role of striatal dopamine in regulation of the basal ganglia is crucial, as it facilitates neuronal firing in the striatum via D$_1$ receptors and inhibits firing via D$_2$ receptors, both of which serve to facilitate normal movement. This not surprising then, that the primary clinical manifestation of Parkinson’s disease is motor impairment, given the depletion of striatal dopamine that typifies the condition (Hornykiewicz, 1962).

In addition to a role in motor co-ordination there are several other basal ganglia circuits integrated in these structures that are affected by dopamine depletion and responsible for the manifestation of non-motor symptoms in Parkinson’s disease. Cognitive manifestations of Parkinson’s disease are attributable to dysregulation of
the associative circuit which receives input from the prefrontal cortex, and psychiatric dysfunction manifests due to disregulation of the limbic circuit which receives input from the anterior cingulate cortex (Alexander et al., 1990). These non-motor symptoms are often overshadowed by the predominant motor features observed however they represent important clinical features which are very debilitating for patients (reviewed in Rodriguez-Oroz et al., 2009). Indeed it has been proposed that these symptoms may precede motor dysfunction and represent a predicator for the development of Parkinson’s disease and this has the potential for earlier intervention and potentially improve treatment benefits (Lang, 2011, Morley et al., 2014).
Fig. 1.1: Basal ganglia circuitry in the normal and Parkinson’s disease brain. In the normal brain (A), the direct and indirect pathways of the basal ganglia exert opposing functions in order to facilitate movement. Dopamine released from nigrostriatal neurons (SNC-Putamen) activates the direct pathway (Putamen-GPi) via D_{1} receptors and inhibits the indirect pathway (Putamen-GPe-STN-) via D_{2} receptors. Both of these actions ultimately lead to disinhibition of the thalamocortical neurons and facilitation of motor function. In the Parkinsonian state (B), deficiency of dopamine leads to an increase in indirect pathway activity, and STN hyperactivity and direct pathway hypoactivity drives output inhibition of the VL nucleus of the thalamus which reduces activation of cortical and brainstem motor regions. Image adapted from (Rodriguez-Oroz et al., 2009). GPe, globus pallidus pars externa; GPi, globus pallidus pars interna; VL, ventrolateral nucleus; STN, subthalamic nucleus; SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulata.
1.1.3.1.2 Loss of dopaminergic nigrostriatal neurons

The characteristic motor symptoms of Parkinson’s disease can be attributed to the loss of dopaminergic neurons in the substantia nigra and striatum. Although Parkinson’s disease pathology is extensive, it is the loss of nigrostriatal dopamine neurons and the consequent reduction in striatal dopamine that produces the motor impairment that typifies the condition. Degeneration of nigrostriatal dopamine neurons induces compensatory responses in intact dopaminergic cells which leads to increased dopaminergic turnover, and a maintenance of dopamine levels for quite some time which may explain why the majority of patients do not become symptomatic until a significant percentage (up to 50%) of dopaminergic cells are lost (Zigmond et al., 1990, Sossi et al., 2002). Indeed, evidence suggests that a combination of this increased dopamine synthesis and compensatory neuronal sprouting temporarily preserves the functionality of the nigrostriatal system (Whone et al., 2003). Increases in nigropallidal signalling maintains a balanced output from the motor thalamus in early Parkinson’s disease, however progressive nigrostriatal degeneration in advanced Parkinson’s disease incurs a loss of such compensation and the motor symptoms of the disease manifest (Moore, 2003).

1.1.3.2 Lewy Bodies and α-synuclein

At a cellular level, proteinaceous inclusions termed Lewy bodies occur in the surviving neurons of the nigrostriatal pathway (Lewy, 1912). These are formed of a protein called α-synuclein, which is ubiquitiously expressed in neurons, and which was identified as the primary component of Lewy bodies (Spillantini et al., 1997). Several mutations in the α-synuclein gene (SNCA) are associated with Lewy body
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formation, with the most common and well-studied being the A53T mutation which gives rise to autosomal-dominant familial Parkinson’s disease (Polymeropoulos et al., 1997) however several others have been identified in recent times (Krüger et al., 1998, Zarranz et al., 2004, Kiely et al., 2013, Appel-Cresswell et al., 2013, Proukakis et al., 2013). In addition to SNCA mutations, multiple copies of the gene, including duplications and triplications, have been associated with both early and late onset, autosomal-dominant Parkinson’s disease (Singleton et al., 2003, Muenter et al., 1998, Miller et al., 2004, Ibanez et al., 2004, Chartier-Harlin et al., 2004). As well as being a prominent pathophysiological feature of genetic forms of Parkinson’s disease, α-synuclein inclusions are also reported in the sporadic, idiopathic disease state (Spillantini et al., 1997).

The normal physiological role of α-synuclein remains to be elucidated, although its location in presynaptic terminals, and results from animal studies, suggest it may play a role in synaptic vesicle trafficking and dopamine signalling (Cabin et al., 2002, Chandra et al., 2004). The conformation of α-synuclein appears to be crucial for its pathogenicity as it has a propensity to aggregate when triggered by various stimuli (e.g. oxidative stress (Hashimoto et al., 1999)), forming oligomeric or fibrillar precursor forms which further transition to aggregated neurotoxic α-synuclein (Winner et al., 2011, Conway et al., 2000). This is supported by clinical studies which have shown altered aggregation profiles caused by Parkinson’s disease-associated SNCA mutations (Ghosh et al., 2013, Narhi et al., 1999, Li et al., 2001) and the predominant presence of fibrillar and aggregated α-synuclein in Lewy bodies (Baba et al., 1998, Dickson et al., 1989).
The selective susceptibility of dopaminergic neurons to α-synuclein-induced degeneration is yet to be explained, although clinical and preclinical evidence has pointed to cytosolic dopamine oxidation and calcium-induced mitochondrial dysfunction as potential causative mechanisms (Moussa et al., 2008, Fahn and Group, 2005, Surmeier and Schumacker, 2013, Becker et al., 2008). Overall, much remains to be elucidated regarding the role of α-synuclein in Parkinson’s disease and whether its presence in the form of Lewy bodies is a pathological hallmark or a secondary feature of PD.

1.1.3.3 Neuroinflammation

The immune system plays an integral role in protecting the brain from infection or injury and in normal cell homeostasis. In ‘normal’, non-pathological conditions the innate immune system, led by resident brain immune cells called microglia, perform multiple neurprotective actions including secretion of anti-inflammatory cytokines and neurotrophic factors, promoting neuron survival and proliferation in its quiescent state (reviewed in Chen and Trapp, 2016). Upon stimulation by a pathogen or physical injury, microglia become activated to eradicate the source of damage, triggering downstream immune signalling and in normal conditions this is a ‘self-limiting’ response to prevent excessive tissue damage (Glass et al., 2010). However, in neurodegenerative diseases, chronic activation of the innate immune system has been suggested to contribute to disease progression via a so-called ‘self-sustaining cycle” of neurodegeneration and neuroinflammation (Tansey and Goldberg, 2010). Thus, neuroinflammation is an important pathophysiological feature of Parkinson’s disease (Fig 1.2).
1.1.3.3.1 Inflammatory pathology in Parkinson’s disease

Parkinson’s disease manifests due to the degeneration of nigrostriatal dopamine neurons and although it was once believed that disease progression was due to loss of a single neuronal cell type it is now well accepted that this is not the case- the clinical picture encompasses many pathological events and inflammation is an integral component (Block and Hong, 2007, McGeer and McGeer, 2008). There is accumulating evidence that inflammation drives neuronal loss in Parkinson’s disease, specifically non-neuronal cells such as microglia release pro-inflammatory mediators such as ROS, NO, TNFα and IL-1β (reviewed in Hirsch and Hunot, 2009). One of the seminal findings in the area of neuroinflammation in Parkinson’s disease was the identification of Nurr1, an orphan nuclear receptor with a regulatory role in dopaminergic cell maintenance, as an inhibitory regulator of NFκB in microglia and astrocytes (Saijo et al., 2009). This identified a novel target that could confer neuroprotection to dopaminergic neurons from inflammation-induced degeneration and this has been supported by subsequent pharmacological studies in animal models of Parkinson’s disease (Smith et al., 2015, Kim et al., 2015). These findings are supported by (and also informed by) clinical evidence and observations from experimental models of Parkinson’s disease which are discussed in the following sections.
1.1.3.3.2 Clinical evidence for inflammatory involvement in Parkinson’s disease

It is now appreciated that inflammation is an important pathological hallmark of neurodegenerative disease; a phenomenon which has been well established in Parkinson’s disease (Fig. 1.2). Reactive microglia have been identified in the substantia nigra of patients post mortem (McGeer et al., 1988). This was determined by positive immunohistochemical staining for the MHC class II cell surface receptor, human leucocyte antigen DR (HLA-DR), which was supported by subsequent studies which identified other microglial markers on cells in the substantia nigra (Banati et al., 1998, Mirza et al., 1999, Imamura et al., 2003). Reactive astrocytes have also been identified in the substantia nigra of patients (Damier et al., 1993), and may represent a neuroprotective mechanism via free radical scavenging and/or production of glial cell line-derived neurotrophic factor (GDNF) although the exact role of these cells in the pathogenesis remains to be elucidated. Other indications of innate immune response include the upregulation of Toll-like receptors (TLRs) which are present on macrophages and dendritic cells (Drouin-Ouellet et al., 2015).
Fig.1.2. **Inflammatory responses in Parkinson’s disease.** In Parkinson’s disease we observe the characteristic degeneration of dopaminergic neurons due to the formation of intracellular α-synuclein aggregates called Lewy bodies. Degeneration and subsequent extracellular α-synuclein activate microglia and astrocytes leading to activation of NF-κB pathway, release of proinflammatory cytokines (e.g. IL-1β, TNF-α) and activation of NADPH oxidase and the generation of ROS. This in turn drives further dopaminergic degeneration and potentiates microglial activation forming a cycle of neurodegeneration and neuroinflammation. FASL, a pro-apoptotic protein associated with the TNF family is also released from activated astrocytes and contributes to dopaminergic cell death. Nurr1, is a transcription factor that acts to suppress microglial via inhibiton of NF-κB (taken from Glass et al., 2010). NF-κB, nuclear factor kappa B; ROS, reactive oxygen species., FASL, FAS ligand, Nurr1, nuclear receptor-related protein 1.
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The response of glial cells to injury represents an innate immune response, however evidence suggests the adaptive immune system may also play a role in disease pathology. In addition to the identification of activated microglia, McGeer and colleagues identified CD8+ T lymphocytes in the substantia nigra (McGeer et al., 1988), a finding which was corroborated by more recent evidence in a Parkinson’s disease mouse model where Brochard and colleagues noted the presence of CD4+/CD8+ T lymphocytes in the murine brain (Brochard et al., 2009). Indeed in this work, microglial activation and T cell proliferation was noted prior to neuronal damage which supports the role of inflammation in driving a ‘self-sustaining’ cycle of neurodegeneration and neuroinflammation (Tansey and Goldberg, 2010). Brochard’s work identified T lymphocytes in highly vascularised areas suggesting that infiltration of peripheral cells can occur due to a weakening of the blood brain barrier - indeed the microvasculature is altered in the substantia nigra in Parkinson’s disease brains (Faucheux et al., 1999, Farkas et al., 2000). Despite this, evidence from animal models does not support lymphocyte infiltration via blood brain barrier leakage (Brochard et al., 2009), and therefore the contribution of the adaptive immune response to the clinical condition remains to be seen.

Elevated levels of pro-inflammatory cytokines and increased cytokine receptor expression has been identified in the striatum and substantia nigra of Parkinson’s disease patients (Mogi et al., 1994b, Mogi et al., 1994a, Mogi et al., 1995a, Mogi et al., 1995b, Mogi et al., 1996, Hunot et al., 1999). Pro-inflammatory cytokines exert direct effects via receptor binding inducing various ‘pro-inflammatory’ pathways, and can also induce indirect cytotoxicity by increasing levels of inducible nitric
oxido synthase (iNOS) which catalyse the formation of reactive oxygen (ROS) and nitrogen species (RNS) (see section 1.1.2.4) and increasing cyclooxygenase-2 (COX-2) activity (Boje and Arora, 1992, Chao et al., 1992, Vane et al., 1998). In addition to the post mortem data indicating an immune-related component to the pathogenesis of Parkinson’s disease, clinical studies also indicate a strong presence for immune system dysregulation in the course of the condition. Pro-inflammatory cytokines and chemokines such as tumour necrosis factor alpha (TNF-α), interleukin-2 (IL-2), IL-6 and chemokine ligand 2 (CCL2) (Dobbs et al., 1999, Stypula et al., 1996, Grozdanov et al., 2014), CCL5 levels (Rentzos et al., 2007, Tang et al., 2014), antibodies to dopaminergic proteins (McRae-Degueurce et al., 1988, Rowe et al., 1998), ‘primed’ peripheral monocytes (Grozdanov et al., 2014) and activated T lymphocytes (Fiszer et al., 1994, Bas et al., 2001) are increased in blood samples of Parkinson’s disease patients. Notably, the profile of T lymphocytes is altered in the disease, with a shift towards a pro-inflammatory phenotype that is similar to that observed following viral infection (Hisanaga et al., 2001, Blum-Degena et al., 1995) which is particularly interesting as viral infections have been suggested to contribute to pathogenesis in Parkinson’s disease. A study which examined a small group of subjects reported men with elevated Il-6 levels in their plasma have an increased risk of developing the condition (Chen et al., 2008) and interestingly elevated Il-6 may contribute to mortality in patients with Parkinson’s disease (Dufek et al., 2015). Genetic studies have identified a host of polymorphisms and genetic aberrations in cytokine genes – TNF-α (Krüger et al., 2000, Wu et al., 2007), interferon-γ (Mizuta et al., 2001), Il-1β (McGeer et al., 2002, Mattila et al., 2002, Schulte et al., 2002, Möller et al., 2004,
Wahner et al., 2007), \( IL-6 \) (Håkansson et al., 2005) in addition to alterations in \( CD14 \) monocyte gene structure in women with Parkinson’s disease (Lin et al., 2006).

Clearly, there is overwhelming evidence for chronic immune activation in Parkinson’s disease; however whether or not neuroinflammation represents a target for disease-modifying therapy is the most pertinent question. Indeed, epidemiological evidence would suggest that immune suppression may confer a degree of protection against the development of Parkinson’s disease. The chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) has been linked with a lower incidence of Parkinson’s disease. Initially, patients taking non-aspirin NSAIDs were identified to be at a reduced risk of developing Parkinson’s disease (Chen et al., 2003). A subsequent study identified a reduced risk in people taking ibuprofen but not other NSAIDs or paracetemol (Chen et al., 2005). Other studies confirmed non-aspirin NSAIDs are associated with lower risk, however this was only observed in men (Hernán et al., 2006), while a trend for non-aspirin NSAIDs and steroidal anti-inflammatory use with concomitant reduction in risk was noted by Bower and colleagues (Bower et al., 2006). However contradictory evidence also exists - Ton and colleagues did not observe any association between NSAID use and the incidence of Parkinson’s disease (Ton et al., 2006), and more recently, Canadian and Danish studies found no evidence for reduced risk with aspirin or non-aspirin NSAIDs, paracetemol and ibuprofen (Etminan et al., 2008, Manthripragada et al., 2011). This conflicting data may indicate that anti-inflammatory therapy could be a viable neuroprotective strategy, however more selective targets need to be identified.
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1.1.3.3 Neuroinflammation in Parkinson’s disease models

Preclinically, most animal models of Parkinson’s disease all exhibit a strong neuroinflammatory profile (Table 1.1). The classic toxin-induced models are induced by neurotoxins including the Complex I inhibitor, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Liberatore et al., 1999, Hurley et al., 2003b, McGeer et al., 2003, Barcia et al., 2004), the organic pesticide, rotenone (Sherer et al., 2003a, Gao et al., 2002a), and the catecholaminergic toxin, 6-hydroxydopamine (Akiyama and McGeer, 1989, Depino et al., 2003) which all induce marked microglial activation. Mice overexpressing α-synuclein also exhibit increased microglial levels (Su et al., 2008) and oligomeric α-synuclein that is released from neurons can activate microglia via TLR2 (Kim et al., 2013), providing a strong indication for inflammation-driven neurodegeneration. Indeed, inflammmagens such as lipopolysaccharide (LPS), isolated from Gram negative bacteria, can induce selective dopaminergic degeneration and motor dysfunction typical of Parkinson’s disease animal models (Choi et al., 2009, Hoban et al., 2013a). Viral infection, mimicked using synthetic compounds such as polyinosinic:polycytidylic acid (Poly (I:C)), has an additive effect when combined with other neurotoxins including 6-hydroxydopamine (Deleidi et al., 2010) and paraquat (Bobyn et al., 2012). Neuroinflammation has also been observed in genetic animal models of Parkinson’s disease. Activated microglia and astrocytosis has been identified in both transgenic models and models that induce the over-expression of wild-type and mutant α-synuclein (Dawson et al., 2010, Watson et al., 2012), although microglial activation in particular is more consistently reported in over-expression models than transgenic α-synuclein models (Sekiyma et al., 2012, Theodore et al., 2008). Elevated pro-
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Inflammatory cytokines, TLR expression and B and T lymphocytes have all been reported in α-synuclein models (Watson et al., 2012, Su et al., 2008, Theodore et al., 2008), and may increase susceptibility to further neuronal damage in response to subsequent inflammatory or toxic stimuli (Sekiyama et al., 2012). Other genetic models which induce the knockout or mutation of familial PD-associated genes also display a neuroinflammatory phenotype although a complete profile is yet to be ascertained. Models carrying mutations in leucine-rich repeat kinase 2 (LRRK 2), Parkin and PTEN-induced putative kinase 1 (PINK1) all display innate immune activation. Overexpression of LRRK2 induces microglial activation (Lee et al., 2010), while knockout of Parkin results in increased microglial activation, reduced astrocyte numbers, and elevation in proinflammatory mediators in aged mice (Solano et al., 2008, Tran et al., 2011).

Overall, neuroinflammation is a pathological feature in clinical and experimental PD. The causality and contribution of inflammation to the occurrence and progression of the disease is unclear and needs further exploration, however greater understanding of the mechanisms underlying inflammatory responses in the disease state may contribute to our knowledge of the etiology of Parkinson’s disease and identify disease-modifying targets.
### Table 1.2. Inflammatory profile observed in Parkinson’s disease animal models

Activation or increased density of innate immune cells (indicated by an upwards arrow) was observed in the nigrostriatal pathway in these animal models. In addition increased levels (protein and mRNA) of pro-inflammatory mediators were found in plasma and CSF*.

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Innate cellular response</th>
<th>Cytokine/Chemokine response</th>
<th>Other inflammatory responses</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPTP</td>
<td>↑ microglia</td>
<td>↑ II-1β, IL-6, IL-2, IL-10, IL-11, IL-12, IL-13, IFN-γ, TNF</td>
<td>↑ T lymphocytes</td>
<td>(Członkowska et al., 1996, Kurkowska-Jastrzębska et al., 1999, McGeer et al., 2003, Liberatore et al., 1999, Grünblatt et al., 2001, Shimoji et al., 2009, Yasuda et al., 2008, Ohnuki et al., 2010, Barcia et al., 2004, Barcia et al., 2011, Vázquez-Claverie et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>↑ astrocytes</td>
<td>↑ CXCR4, CXCL13, CXCL4, CXCL12-α, MCP-1, NFkB</td>
<td>↑ PF4, C1S, C4B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ IL-1R, IL-3R, IL-4R, IL-10-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑ astrocytes</td>
<td>↓ II-1β, IL-6*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotenone</td>
<td>↑ microglia</td>
<td>↑ TNF-α</td>
<td>↑ COX-2</td>
<td>(Sherer et al., 2003a, Sherer et al., 2003b, Gao et al., 2002a, Zhou et al., 2007)</td>
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<tr>
<td></td>
<td>↑ astrocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paraquat</td>
<td>↑ microglia</td>
<td>↑ TNF-α, II-1β, NFkB</td>
<td>↑ iNOS, ROS</td>
<td>(Purisai et al., 2007, Yadav et al., 2012, Cicchetti et al., 2005, Gupta et al., 2010, Mitra et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>↑ astrocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>↑ microglia</td>
<td>↑ TNF-α, II-1β, II-1α</td>
<td>↑ iNOS, ROS, COX-2</td>
<td>(Choi et al., 2009, Hoban et al., 2013a, Concannon et al., 2015, Castano et al., 1998, Qin et al., 2007, Lima et al., 2006, Qin et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>↑ astrocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly (I:C)</td>
<td>↑ microglia</td>
<td>↑ II-1β, IL-6, TNF-α, MCP-1, TGF-β1</td>
<td>↑ perivascular &amp; parenchymal macrophages</td>
<td>(Deleidi et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>↑ astrocytes</td>
<td>↑ CX3CR1</td>
<td>↑ TLR3</td>
<td></td>
</tr>
<tr>
<td>α-synuclein</td>
<td>↑ microglia</td>
<td>↑ TNF-α, II-4, II-13</td>
<td>↑ TLR1, TLR4, TLR8</td>
<td>(Watson et al., 2012, Su et al., 2008, Theodore et al., 2008, Sanchez-Guajardo et al., 2010, Barkholt et al., 2012, Wilms et al., 2009, Kirik et al., 2002b)</td>
</tr>
<tr>
<td></td>
<td>↑ astrocytes</td>
<td></td>
<td>↑ B &amp; T lymphocytes</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ arginase 1</td>
<td></td>
</tr>
<tr>
<td>LRRK2</td>
<td>↑ microglia</td>
<td></td>
<td></td>
<td>(Lee et al., 2010)</td>
</tr>
<tr>
<td>Parkin</td>
<td>↑ microglia</td>
<td>↑ TNF-α, II-1α</td>
<td>↑ iNOS</td>
<td>(Solano et al., 2008, Tran et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>↑ astrocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PINK1</td>
<td>No data</td>
<td>↑ II-1β</td>
<td></td>
<td>(Lee et al., 2012)</td>
</tr>
</tbody>
</table>
1.1.3.4 Oxidative stress

In addition to or as a consequence of inflammation, oxidative stress is another facet in the pathophysiology of Parkinson’s disease. Oxidative stress may manifest as a result of increased production of ROS and/or dysfunction of normal oxidative stress responses. In the Parkinson’s disease, this may manifest as a result of two phenomena – increased cytosolic dopamine metabolism and mitochondrial dysfunction. Excess cytosolic dopamine in degenerating neurons may undergo oxidation to cytotoxic ROS which drives dopaminergic death further (Zucca et al., 2014). Animal studies have shown that increased cytosolic dopamine coupled with impaired activity of the vesicular monoamine transporter 2 (VMAT2) induces dopaminergic cell loss (Caudle et al., 2007), and formation of toxic dopamine quinones by auto-oxidation of dopamine can induce inactivation of the dopamine transporter (DAT) and tyrosine hydroxylase as well as alterations in PD-related proteins (e.g. α-synuclein, parkin, DJ-1) (da Silva et al., 2013, LaVoie et al., 2005, Girotto et al., 2012) . Clinically this is supported by evidence of accumulated neuromelanin, which can be generated via quinone oxidation, in the substantia nigra pars compacta of Parkinson’s disease patients (Ohtsuka et al., 2014, Ohtsuka et al., 2013) and experimentally, injection of dopamine into the striatum induced dopamine oxidation which was directly proportionate to degeneration of terminals in the striatum (Rabinovic et al., 2000). Interestingly, cytosolic dopamine oxidation may drive cellular stress and mutation of α-synuclein, although this would suggest that L-DOPA treatment should then exacerbate disease progression which is unproven (Xu et al., 2002, Moussa et al., 2008).
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In addition to ‘direct’ oxidative stress via dopamine, mitochondrial stress plays an important role in the generation of ROS. Mitochondria are organelles involved in a number of key cellular processes including energy metabolism, oxidative stress, calcium homeostasis and production of ROS (rev. in Camilleri and Vassallo, 2014). As neurons have a high metabolic activity and maintaining ion gradients across synapses is integral for their function, they are especially susceptible to the effects of oxidative stress. Generation of ROS drives oxidative stress and is a result of altered mitochondrial complex signalling (Liu et al., 2002).

Deficiencies in this system have been identified clinically in patients with Parkinson’s disease. Reduced activity of the one of the four mitochondrial complexes, mitochondrial Complex 1, has been identified in the substantia nigra pars compacta of patients, as well is in blood and fibroblasts, indicating that this may contribute to the formation of ROS and/or apoptosis of neurons, ultimately contributing to disease progression (Schapira et al., 1990, Kroemer et al., 2007, Bindoff et al., 1989, Parker et al., 1989). Although evidence exists for mitochondrial dysfunction in clinical Parkinson’s disease, its contribution to the etiology is not certain as some patients do not present with mitochondrial Complex I deficiency. The implications for mitochondrial dysfunction in the pathogenesis of Parkinson’s disease emerged in the 1970s when a 23-year old man was accidently exposed to a toxic by-product of recreational drug desmethylprodine, namely MPTP, which acts as a potent Complex I inhibitor and produced an acute Parkinsonism which could be alleviated with L-DOPA treatment (Davis et al., 1979). Subsequently, systemic exposure to MPTP was shown to induce neuropathological hallmarks typical of
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Parkinson’s disease in primates and rodents, including degeneration of dopamine neurons in the nigrostriatal pathway and inclusion body formation (Burns et al., 1983, Rojo et al., 2006, Sherer et al., 2007). The mechanism of toxicity involves selective uptake of the MPTP metabolite, 1-methyl-4-phenylpyridinium (MPP+), by dopamine neurons which becomes concentrated in mitochondria and inhibits Complex I activity (Ramsay et al., 1986, Mizuno et al., 1988, Bezard et al., 1999).

MPTP is not the only compound known to induce clinical and experimental Parkinsonism by way of oxidative stress-driven neurodegeneration. Herbicides such as paraquat, which is structurally similar to MPP+, is also a potent generator of ROS and acts as a Complex I inhibitor albeit not as strongly. Environmental exposure to paraquat is associated with a higher incidence of Parkinson’s disease (Liou et al., 1997) and experimental Parkinsonism induced by this toxin is also characterised by oxidative dopaminergic degeneration and aggregation of α-synuclein (Brown et al., 2006). Rotenone, an organic pesticide, has also been linked to an increased incidence of Parkinson’s disease and has been used to model the condition in vivo (Tanner et al., 2011, Betarbet et al., 2000, Sherer et al., 2003b). Genetic models of Parkinson’s disease also exhibit strong oxidative stress profiles which provide strong evidence for a role for oxidative stress in disease etiology. For example, overexpression of the most common form of mutated α-synuclein (A53T) induces mitochondrial DNA damage prior to neurodegeneration and the emergence of motor symptoms (Martin et al., 2006, Norris et al., 2007). Crucially, other genes commonly mutated in Parkinson’s disease such as LRRK2 and PINK-1, are all involved in mitochondrial function, and in vitro and in vivo evidence shows concomitant mitochondrial
dysfunction, oxidative damage and neurodegeneration in these genetic models (reviewed in Camilleri and Vassallo, 2014). This further highlights the importance of mitochondrial dysfunction in Parkinson’s disease.

Mitochondria are a key convergence point for many of the pathological observations and etiological factors that are well established in Parkinson’s disease. As mentioned previously, the profile of genes commonly mutated or altered in Parkinson’s disease are associated with mitochondrial function and signalling. α-synuclein, which primarily displays cytosolic and vesicular localisation, can be ‘imported’ into the mitochondria where it inhibits complex I activity (Devi et al., 2008). PINK1 is thought to be localised to the inner mitochondrial membrane and attenuates apoptotic cell death in normal conditions (Petit et al., 2005), thus dysfunction of the mutated kinase impairs this normal regulatory function. Parkin plays an important role in proteosome function, however it has also been localised in mitochondria and can modulate mitochondrial DNA transcription and replication (Kuroda et al., 2006), which when inhibited causes respiratory chain deficiency and progressive degeneration of the nigrostriatal pathway (Ekstrand et al., 2007) and indeed, several other Parkinson’s disease-associated genes are similarly implicated in mitochondrial dysfunction (reviewed in Vila et al., 2008). Another key relationship between mitochondria and the emergence of Parkinson’s disease is age. As discussed in the etiology section of this thesis, age is the most significant risk factor to the development of Parkinson’s disease, and indeed mitochondrial dysfunction prevails with increasing age (Wallace, 2005).
Thus, in addition to an increased susceptibility to the development of Parkinson’s disease following exposure to oxidative stressors, oxidative stress driven largely by mitochondrial dysfunction strongly contributes to the pathogenesis of the condition, and mounting evidence makes a convincing argument for a role in the etiology of the disease in some cases.

1.2 CURRENT TREATMENTS FOR PARKINSON’S DISEASE

Parkinson’s disease has many therapeutic requirements, the forefront of which is alleviating the debilitating motor systems associated with the condition. However, current treatments only address the symptomatic condition, and no curative or neuroprotective therapies have been brought to the clinic. This section will summarise the current therapies, their pharmacological action, and limitations as long-term treatment strategies.

1.2.1 L-DOPA

There are several strategies employed in addressing motor symptoms in Parkinson’s disease. The most effective strategy has been to replace striatal dopamine in the form of the dopamine precursor L-DOPA. Developed in 1967 by Cotzias and colleagues, L-DOPA has been the ‘gold standard’ treatment for the motor symptoms in Parkinson’s disease for over 50 years (reviewed in Fahn, 2015). It can readily traverse the blood brain barrier, and replaces the striatal dopamine lost as a result of dopaminergic degeneration. L-DOPA is converted to active dopamine by the enzyme dopa-decarboxylase (Cotzias et al., 1967). It is usually co-administered with benserazide or carbidopa, peripherally-restricted dopa decarboxylase inhibitors, which prevent peripheral L-DOPA metabolism, thus maximising central dopamine
levels and avoiding cardiovascular and gastrointestinal side-effects associated with elevated peripheral catecholamines (Markham et al., 1974, Robertson et al., 1989). L-DOPA is a very effective treatment for the motor symptoms in Parkinson’s disease, and greatly improves quality of life for patients as a result.

1.2.2 DOPAMINE AGONISTS

Dopamine agonists were developed as an alternative to L-DOPA due to the negative side effects associated with L-DOPA’s prolonged use (see section 1.2.5). They work by targeting postsynaptic dopamine receptors directly thereby bypassing degenerating dopamine neurons (Montastruc et al., 1999). These drugs have shown anti-Parkinsonian efficacy (Uitti and Ahlskog, 1996) without inducing motor complications (Stacy and Galbreath, 2008) or toxic metabolic by-products (Brooks, 2000). Although they are somewhat inferior to L-DOPA in terms of efficacy, they are often the first-line choice of drug in treating a newly diagnosed Parkinson’s disease patient as the risk of developing L-DOPA-induced motor complications increases with longer duration of L-DOPA treatment (Worth, 2013). Some examples of dopamine agonists commonly prescribed to treat motor symptoms are bromocriptine, cabergoline, apomorphine, piribedil, pramipexole, ropinrole and rotigotine (Kalia and Lang, 2015).

1.2.3 MAO-B/COMT INHIBITORS

In addition to replacing striatal dopamine (using L-DOPA) or directly stimulating dopamine receptors (via dopamine receptor agonists), a third strategy for treating
motor symptoms is using enzyme inhibitors to reduce dopamine metabolism. Two key enzymes involved in dopamine metabolism are monoamine oxidase B (MAO-B) and catechol-O-methyl transferase (COMT), in addition to aldehyde dehydrogenase (ALDH) which produced dopamine metabolites including 3,4-dihydroxyphenyl-acetaldehyde (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT) (Elsworth and Roth, 1997). Therefore, MAO–B inhibitors, such as selegiline and rasagiline, and COMT inhibitors, such as entacapone, inhibit dopamine metabolism and thus L-DOPA metabolism to increase the levels of dopamine in the brain. They are usually prescribed as an adjuvant to L-DOPA, and co-treatment with a COMT inhibitor is particularly effective at increasing the L-DOPA ‘on’ time in patients with advanced disease (Bonifati and Meco, 1999). MAO-B/COMT inhibitor monotherapy is rarely used, although MAO-B inhibitors are effective as a monotherapy but only in early, mild stages of Parkinson’s disease (Worth, 2013).

1.2.4 OTHER PHARMACOLOGICAL TREATMENTS

Other pharmacological approaches that are frequently used to treat Parkinson’s disease include non-dopaminergic drugs that have anti-Parkinsonian effects. Anticholinergics are one such class of compounds which are often prescribed, usually to treat patients with tremor-dominant disease (Worth, 2013). Their exact mechanism of anti-Parkinsonian action is unknown, however it is hypothesized that they counteract the dopamine-opposing effects of acetylcholine released from striatal interneurons, thereby restoring balance in the basal ganglia (Schapira, 2007). Amantadine, a glutamatergic NMDA antagonist, is often used to alleviate L-DOPA-induced dyskinesias in advanced disease. Alterations in glutamatergic neurotransmission is
observed in Parkinson’s disease, as a consequence of dysregulated basal ganglia
circuitry due to dopamine depletion, and has been hypothesized as a mechanism for
the development of these dyskinesias (Calon et al., 2003, Ouattara et al., 2011,
Ahmed et al., 2011).
Fig 1.3. Sites of action of antiparkinsonian drugs in the striatum. Drugs used to treat the motor symptoms of Parkinson’s disease are illustrated here. Levodopa is converted to dopamine in nigrostriatal dopamine neurons, is released into the synapse and binds to post-synaptic dopamine receptors on medium spiny neurons. Dopamine agonists bind directly to these post-synaptic dopamine neurons. MAOI drugs inhibit breakdown of dopamine following reuptake (COMT inhibitors act similarly, not shown). Non-dopaminergic drugs act by reducing excessive action of other neurotransmitters- anticholinergic drugs block post-synaptic acetylcholine receptors and amantadine blocks NMDA receptors which stimulate the release of glutamate from corticostriatal projection neurons. Image modified from (Connolly and Lang, 2014).
1.2.5 **Deep Brain Stimulation**

Deep brain stimulation (DBS) is a non-pharmacological treatment for Parkinson’s disease and involves the surgical implantation of electrodes into the subthalmic nucleus, globus pallidus interna or ventrolateral nucleus of the thalamus which then receive electrical impulses from an input device, positioned externally behind the ear (Olanow et al., 2000, Yu and Neimat, 2008). DBS is selected as a treatment option in patients who exhibit symptoms which are not sufficiently addressed by pharmacological treatment, particularly when there is an emergence of “wearing off” effect of medication and drug-induced dyskinesias (Chang and Chou, 2006). The location for electrode implantation is dependent on the clinical presentation and thus varies widely between patients, for example electrode implantation into the subtalaric nucleus is employed in advanced Parkinson’s disease and implantation in the globus pallidus interna alleviates generalised dystonias (Yu and Neimat, 2008). Similar to pharmacological treatments, DBS does not offer any long-term, neuoprotective or neurorestorative treatment and is a highly invasive surgical procedure which is not a viable treatment option for all Parkinson’s disease patients (Limousin and Martinez-Torres, 2008).

1.2.6 **Limitations of Current Treatments**

The pharmacological strategies discussed above have varying levels of efficacy, and depending on the patient and the course of the condition, these drugs alone or in combination can provide enormous clinical benefit to patients. That being said, each is not without their limitations.
Despite the success of L-DOPA, it has several weaknesses, namely its efficacy reduces over time and, due to some of the side effects it produces, the long-term therapeutic potential of L-DOPA falls short. Although the benefits of the treatment is significant in its early stages, many patients experience issues with “off” states (unpredictable re-emergence of motor symptoms) as a result of inefficient delivery of the compound to the brain - this is mainly associated with oral intake as the human gastrointestinal system is not well suited to uptake of L-DOPA (LeWitt, 2015). L-DOPA also has a short half-life which causes problematic fluctuations in plasma concentrations leading to the ‘wearing off’ phenomenon (end-of-dose re-emergence of motor symptoms) as well as contributing to the development of the severe motor fluctuations known as L-DOPA-induced dyskinesias. In addition, as L-DOPA has no neuroprotective effects on nigrostriatal dopamine neurons, ongoing degeneration of the pathway may impede the long-term efficacy of the drug (Fox and Lang, 2008).

As mentioned dopamine agonist monotherapy is often recommended in the early stages following diagnosis to reduce the possibility of the emergence of L-DOPA-induced dyskinesias later in the course of treatment, however this may also precipitate impulse control disorders (Voon et al., 2011). MAO-B inhibitors do not have any significant side effects however their efficacy is limited in comparison to L-DOPA and dopamine agonists. COMT inhibitors enhance dopaminergic effects and can induce complications including nausea, hallucinations and confusion in addition to diarrhoea (Reichmann et al., 2005). Anti-cholinergics are also associated considerable side-effects including dry eyes and mouth, cognitive disturbance and
urinary retention (Worth, 2013). Amantadine is also associated with negative side effects such as diarrhoea and confusion.

The key and overriding limitation of all current treatments is that none have any neuroprotective potential; no treatment addresses the degenerating nigrostriatal pathway and thus this limits the efficacy of these drugs long-term. There is a serious unmet clinical need in the treatment of Parkinson’s disease due to the symptomatic nature of all current treatments. Neuroprotective therapies are needed, and in order to develop these we must consider the clinical picture of Parkinson’s disease. At diagnosis, a significant percentage of nigrostriatal dopamine neurons are already lost - 50% loss of dopaminergic neurons has been consistently reported (Ross et al., 2004), although as high as 70% has been observed (Dauer and Przedborski, 2003). More recently, several studies using both regression analysis and quantitative morphological approaches have pinpointed an approximate 30% loss of nigral dopaminergic neurons at the onset of motor symptoms (Fearnley and Lees, 1991, Ma et al., 1997, Greffard et al., 2006). If a therapy could be developed that could slow, halt or even reverse the progression of the disease, then this would have major consequences for the quality of life of Parkinson’s disease patients.

1.2.7 PROSPECTIVE TREATMENT THERAPIES: STEM CELL TRANSPLANTATION

Given the limited effectiveness of current treatments in Parkinson’s disease, the focus of preclinical and clinical research is on improving or developing new treatment approaches (reviewed in Lotia and Jankovic, 2016). Although pharmacological strategies to replace dopamine remains the most prevalent approach
in clinical trials, the next logical approach has been to stimulate regeneration of the ‘endogenous’ dopamine pathway, by reinnervating the nigrostriatal pathway using cell transplantation. There are many cell types which are under investigation, including genetically modified mesenchymal stem cells (reviewed in Glavaski-Joksimovic and Bohn, 2013), induced pluripotent stem cells differentiated to a neuronal, dopaminergic lineage (reviewed in Li et al., 2015b) and fetal ventral mesencephalic neurons, the later of which has displayed the most promise for clinical translation (reviewed in Barker et al., 2015). Since the first clinical trial almost 30 years ago, which transplanted human fetal ventral mesencephalic cell grafts into the striatum of Parkinson’s disease patients (Lindvall et al., 1989), the field has grown thanks to encouraging results of graft survival in patients post mortem, 20 years post-transplantation (Kordower et al., 1995, Kefalopoulou et al., 2014) and evidence of graft dopamine release (Piccini et al., 1999). Despite a negative outcome in an NIH-funded clinical trial in the early 2000’s which was largely attributed to broad patient inclusion criteria (Olanow et al., 2003), the ongoing TRANSEURO clinical trial has reported positive outcomes (TRANSEURO, 2014), indicating that human fetal ventral mesencephalic cell transplantation may be a viable restorative therapy in Parkinson’s disease although this is not without ethical concerns given the source of the tissue (Master et al., 2007)

Although restoration or modulation of the nigrostriatal pathway is the most obvious target for disease treatment, an understanding of the clinical picture is key in order to ascertain and tailor therapies to best address the condition. As detailed in a previous section, the contribution of inflammation to the progression of neurodegenerative
disease is hypothesized to be a key driving force in the pathogenesis of Parkinson’s disease, forming a ‘self-sustaining’ cycle of neuroinflammation and neurodegeneration (Tansey and Goldberg, 2010). Given that ~50% of nigral neurons are lost at diagnosis, immune system activation and inflammatory responses are already at play, potentially contributing to disease progression, and thus, hampering the efficacy of any therapies. Anti-inflammatory therapy for disease modification in Parkinson’s disease is an emerging field, and potential anti-inflammatory targets are of keen interest. One such potential target is the endocannabinoid system, which is intricately involved in basal ganglia circuitry, and may also play a role in central immune responses.

1.3 THE ENDOCANNABINOID SYSTEM

The endogenous cannabinoid, or endocannabinoid, system is so-named as it is the biological system responsible for the action of cannabinoid compounds in the Cannabis sativa plant. Although the field of endocannabinoid research is barely 50 years old, evidence of cannabis therapeutic use can be seen as early as 2600 BC by Chinese emperor Huang Ti (reviewed in Mechoulam and Hanu, 2001). It was used to treat pain, inflammation and various neurological diseases for millennia, however attempts to clinically examine its efficacy were without result due to the impure nature of the plant extracts. In 1964, Mechoulam and colleagues isolated Δ⁹-tetrahyrdocannabinol (THC), the principal psychoactive component of cannabis, and thus the era of modern cannabinoid research was born (Mechoulam and Gaoni, 1965). In the half century since then, many natural and synthetic cannabinoid drugs have been identified or developed, and our understanding of the endogenous cannabinoid system has grown, not just in terms of cannabinoid drug action, but also
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in terms of the importance of endogenous cannabinoid systems in normal brain function and disease.

1.3.1 CANNABINOID RECEPTOR PHARMACOLOGY

1.3.1.1 CB₁ & CB₂

Following the isolation of pure Δ⁹-THC, the search for its site of action gained much interest, and in the late 1980s the first cannabinoid receptor, CB₁, was chemically defined (Devane et al., 1988, Matsuda et al., 1990). The second cannabinoid receptor, CB₂, was identified some years later in 1993 by Monroe and colleagues (Munro et al., 1993). The CB₁ receptor is widely expressed in the central nervous system, especially in the basal ganglia (Matyas et al., 2006), as well as the hippocampus, cerebellum and cortex (Galiègue et al., 1995). It is positioned on presynaptic terminals and engages in retrograde signalling to inhibit neurotransmitter release and is also present on glial cells (Di Marzo and Petrosino, 2007). Interestingly, CB₁ receptors have been identified intracellularly on mitochondria, which is of particular interest given the prominence of mitochondrial dysfunction in Parkinson’s disease (Benard et al., 2012). The CB₂ receptor is highly expressed in peripheral immune tissues, with evidence to suggest low levels of CB₂ receptor expression in the CNS, possibly localised to activated microglia (Ehrhart et al., 2005, Núñez et al., 2004, Maresz et al., 2005), and it is believed to mediate central and peripheral immune responses. The presence of CB₂ receptors on neurons remains questionable (Atwood and Mackie, 2010), however recent studies have identified CB₂ receptors on ventral tegmental area (VTA) dopamine neurons in mice (Zhang et al., 2014).
The CB₁ and CB₂ receptors are G-protein coupled receptors (GPCRs), coupled to Gᵢ and Gₒ classes (Fig. 1.4). The activation of these receptors inhibits adenylate cyclase, certain voltage-gated calcium-channels, inwardly rectifying potassium channels, and they can also activate mitogen-activated protein kinase (MAPK) pathways (Howlett et al., 2002).

**Fig. 1.4. Signalling pathways involved in cannabinoid receptor activation.** Activation of cannabinoid receptors stimulates Gᵢ/o proteins which is coupled to inhibition of adenylate cyclase and inactivation of the protein kinase A (PKA) phosphorylation pathway or stimulation of mitogen-activated protein kinase (MAPK) pathway which induces gene expression. CB₁ receptor stimulation also induces inhibition of Ca²⁺ channels and stimulates inwardly rectifying K⁺ channels in neurons (taken from Di Marzo et al., 2004).
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1.3.2 ENDOGENOUS CANNABINOID LIGANDS

1.3.2.1 Anandamide & 2-AG

The characterisation of the CB₁ receptor led to the hypothesis that endogenous cannabinoid ligands may exist. This was proven in 1992 when Will Devane discovered N-arachidonoyl ethanolamine or anandamide, a compound which bound to the CB₁ receptor eliciting effects similar to Δ⁹-THC (Devane et al., 1992). A second compound with cannabinoid activity was discovered in 1995, 2-arachydonoyl-glycerol (2-AG) (Mechoulam et al., 1995, Sugiura et al., 1995). These compounds are lipophilic fatty acids and have varying levels of affinity and efficacy for cannabinoid receptors – anandamide has high affinity for CB₁, with approximately 60% of the maximal effect, whereas 2-AG binds CB₁ with lower affinity but very high efficacy (Hillard, 2000). Anandamide and 2-AG bind to CB₂ with similar affinity, 2-AG acts as a full agonist and anandamide acts as weak partial agonist (Gonsiorek et al., 2000). Endocannabinoids may also act at non-cannabinoid receptors. Anandamide can bind to the transient receptor potential, vanilloid subtype 1 (TRPV1), a receptor which is upregulated in pathological conditions (Starowicz et al., 2007). Additionally, both endocannabinoids may activate GPR55, an orphan G-protein-coupled-receptor (reviewed in Balenga et al., 2011) and evidence suggests anandamide and 2-AG interact with peroxisome proliferator-activating receptors (PPARs) α and γ (O'Sullivan and Kendall, 2010).

Some other endogenous cannabinoid compounds have been isolated, 2-arachidonyl glyceryl ether (noladin ether) (Hanuš et al., 2001), O-arachidonoyl-ethanolamin
(virodhamine) (Porter et al., 2002) and N-arachidonoyl dopamine (NADA) (Huang et al., 2002) although their physiological function is not fully understood.

1.3.2.2 Endocannabinoid biosynthesis & metabolism

The synthesis and metabolism of endocannabinoids, as with all biological signals, involves a specific set of anabolic and catabolic reactions which regulates their production, and as a result, their physiological function. There are multiple pathways responsible for the synthesis of anandamide and 2-AG (Fig. 1.5). Anandamide is an N-acylethanolamine and is primarily synthesised from the precursor N-arachidonoyl-phosphatidylethanolamide, via four possible pathways, although the most direct and well characterised pathway is catalysed by N-acyl-phosphatidylethanolamine-selective phosphodiesterase (NAPE-PLD). 2-AG is a monoacylglycerol and synthesized via 2 pathways, however the one relevant to 2-AG’s endocannabinoid activity involves hydrolysis of diacylglycerols (DAGs) by DAG lipases (DAGLs) α and β, with DAGL-α expressed in adult nervous tissue and DAGL-β expressed in developing nervous tissue (Di Marzo and Petrosino, 2007).

Cellular reuptake of endocannabinoids has not been fully elucidated although it occurs via a putative ‘endocannabinoid membrane transporter (EMT)’. Anandamide is degraded to arachidonate and ethanolamine by fatty acid amide hydrolase (FAAH) and 2-AG is degraded to arachidonate and glycerol, primarily by monoacylglycerol lipase (MAGL) although other enzymes can also catalyse its hydrolysis namely, the αβ-hydrolases 6 and 12 (ABHD6, ABHD12) (Blankman et al., 2007). These metabolites are inactive at cannabinoid receptors, although under certain conditions
the by-products of endocannabinoid degradation can form prostaglandin ethanolamide (anandamide) and prostaglandin glycerol esters (2-AG) by COX-2 which may act at non-cannabinoid binding sites (Di Marzo, 2008).

Fig. 1.5. Synthesis activation and metabolism of anandamide and 2-AG. Biosynthetic pathways are in blue, metabolic pathways in red. Anandamide and 2-AG are synthesized from NArPE via NAPE-PLD and diacylglycerols via DAG lipases, respectively. Degradation of anandamide and 2-AG is catalysed by FAAH and MAGL, respectively (taken from Di Marzo, 2008). 2-AG, 2-arachidonoylglycerol; NArPE, N-arachidonoyl-phosphatidyl-ethanolamine; NAPE-PLD, N-acyl-phosphatidylethanolamine-selective phosphodiesterase; DAG, diacylglycerol; FAAH, fatty acid amid hydrolase; MAGL, monoacylglycerol lipase.

1.3.2.3 Endocannabinoid-like compounds

There is another class of compounds that are not considered endocannabinoid but are ‘endocannabinoid-like’ compounds due to their structural similarity to anandamide.
Palmitoylethanolamine or N-palmitoylethanolamide (PEA) and oleoylethanolamine or N-oleoylethanolamide (OEA) are two such compounds which share the same degradative mechanisms as endocannabinoids. They play an important role in the endocannabinoid system as they can potentiate endocannabinoid effects by competitively inhibiting their hydrolysis or via allosteric modulations of receptors, a so-called ‘entourage effect’ (Ho et al., 2008). They do not activate cannabinoid receptors, but they do bind PPARα receptors and facilitate endocannabinoid action at cannabinoid and TRPV1 receptors (reviewed in Fezza et al., 2014). PEA has a well-characterised anti-inflammatory action (Skaper and Facci, 2012) whereas OEA is predominantly involved in appetite suppression and nociception (Fezza et al., 2014).

1.3.3 THE ENDOCANNABINOID SYSTEM IN BASAL GANGLIA FUNCTION

The endocannabinoid system, in addition to dopaminergic neurotransmission, GABAergic and glutamatergic signalling, is intricately involved in the basal ganglia circuitry, which is the network responsible for motor control. Normal, coordinated movement manifests as a result of a controlled balance of direct striatopallidal and indirect striatopallidal GABAergic signalling. This balance is facilitated by excitatory D1 receptors in the direct pathway and inhibitory D2 receptors in the indirect pathway, in a corticostriatopallidal loop (reviewed in Grillner et al., 2005). An imbalance in this circuitry occurs in Parkinson’s disease, due to a loss of striatal dopamine, causing increased inhibition of the motor thalamus, leading to development of typical Parkinsonian symptoms.
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CB₂ receptors located on microglia and astrocytes do not appear to play a role in modulating the corticostriatopallidal loop. Rather these are up-regulated primarily in response to neuroinflammatory and cytotoxic injury (Ternianov et al., 2012). The CB₁ receptor, however, is highly expressed in the basal ganglia, and is in fact, most dense in the substantia nigra, a finding which indicated a likely role in motor control (Mailleux and Vanderhaeghen, 1992, Herkenham et al., 1991). Although the nigrostriatal neurons themselves do not express CB₁ receptors, they do express vanilloid TRPV1 receptors which are activated by the endocannabinoid anandamide (Mezey et al., 2000). The dense localisation of CB₁ receptors in the substantia nigra is due to their expression on the presynaptic terminals of medium spiny GABAergic neurons in the striatum that project to the substantia nigra pars reticulata and the globus pallidus (Hohmann and Herkenham, 2000). CB₁ receptors are also present at other levels of the ‘motor loop’, including on corticostriatal glutamatergic terminals in the striatum and on GABAergic projections from the globus pallidus to the subthalamic nucleus (Fig 1.6) (Brotchie, 2003, Benarroch, 2007).
Fig 1.6 Localisation of CB₁ and TRPV1 receptors in the basal ganglia motor circuit. CB₁ receptors are co–expressed with D₁ and D₂ receptors on corticostriatal afferents and are also located on presynaptic terminals of GABAergic MSNs of the ‘direct’ and ‘indirect’ pathways and on GABAergic projections from the subthalamic nucleus. TRPV1 receptors are located on nigrostriatal dopamine neurons. The neuronal presence of CB₂ receptors in this circuit remains under investigation (taken from Morera-Herreras et al., 2012).

CB₁ receptors have a functional role in the modulation of the basal ganglia motor circuit and locomotor suppression is one of the classic tetrad of behavioural effects of CB₁ receptor agonists in rodents (Smith et al., 1994). In the striatum, CB₁ receptors are co-expressed with D₁ and D₂ receptors on GABAergic medium spiny neurons.
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(Hermann et al., 2002), and CB₁ receptor stimulation inhibits D₁ receptors, whereas, D₂ receptor activation stimulates anandamide release (Hermann et al., 2002, Giuffrida et al., 1999). The importance of CB₁ receptor signalling is illustrated by reduced basal motor activity and striatal dopamine content observed in CB₁ knockout mice (Zimmer et al., 1999, Li et al., 2009a). The CB₁ receptor is also involved in regulation of glutamatergic input into the striatum. The CB₁ receptors expressed on corticostriatal glutamatergic terminals modulate a form of synaptic plasticity at these synapses (Gerdeman and Lovinger, 2001, Kreitzer and Malenka, 2007). The mechanism of action remains to be fully elucidated, however evidence suggests that release of endocannabinoids in the striatum following D₂ receptor stimulation activates CB₁ receptors on the corticostriatal glutamatergic terminals to induce long-term depression of glutamatergic activity in the striatum (Gerdeman and Lovinger, 2001, Kreitzer and Malenka, 2007). The possible role of CB₂ in this circuitry is unknown, largely due to the unanswered question of its cellular location, however the low basal levels of CB₂ in the ‘normal’ brain suggests any role it may have is related to the disease state. Stimulation of CB₂ induces a range of actions, including downstream MAPK signalling (Howlett et al., 2002) which can result in transcription of NFκB and the production of proinflammatory cytokines, which may then alter neuronal firing or induce neuronal death (reviewed in Hirsch et al., 2003) If CB₂ is primarily expressed on activated microglia, stimulation of CB₂ may alter immune responses and there is evidence for CB₂ mediation of neuronal-glia interactions (Luongo et al., 2014), although extensive further investigation is needed to substantiate these claims. Indeed genetic ablation of the CB₁ and CB₂ receptor has resulted in increased motor deficits in several mouse models of Huntington’s disease.
and Parkinson’s disease (Bouchard et al., 2012, Mievis et al., 2011, Pérez-Rial et al., 2011). Thus, there is considerable evidence that the endocannabinoid system plays a crucial role in the normal functioning of the basal ganglia motor circuitry, and that its dysregulation is implicated in the motor dysfunction observed in conditions such as Parkinson’s disease.

1.4 THE ENDOCANNABINOID SYSTEM AND PARKINSON’S DISEASE

1.4.1 ENDOCANNABINOID SYSTEM DYSREGULATION IN PARKINSON’S DISEASE

Several studies have investigated changes in cannabinoid receptors and their endogenous ligands in basal ganglia structures of Parkinson’s disease patients (Table 1.3). The first such study demonstrated an increase in CB₁ receptor binding in the caudate nucleus and putamen in post mortem Parkinson’s disease brains (Lastres-Becker et al., 2001). Subsequent post mortem studies were somewhat contradictory to this, however, and revealed either a decrease in CB₁ receptor mRNA levels in the caudate nucleus, anterior dorsal putamen and external segment of the globus pallidus (Hurley et al., 2003a) or no change in CB₁ receptor density in these structures (Farkas et al., 2012). These studies, however, are confounded by variability in the disease course and drug treatment regimes. Only one study thus far has attempted to address these limitations by using MRI and PET imaging to detect CB₁ receptor availability in PD patients at various disease stages undergoing different L-DOPA treatment regimens (Van Laere et al., 2012). This study revealed a reduction in CB₁ receptors in the substantia nigra which was unaffected by L-DOPA treatment. Although largely unexplored in clinical studies, there is an indication that the CB₂ receptor may also be altered in the Parkinson’s disease. CB₂
receptor changes have been identified in the disease state - decreased CB$_2$ receptor mRNA expression in the cerebellum and hippocampus of both early and late Parkinson’s disease (Grünblatt et al., 2007), and more recently elevated CB$_2$ receptor expression was observed on microglia and neurons in a small cohort of Parkinson’s disease patients (Garcia et al., 2015, Gómez-Gálvez et al., 2015).

Generally, findings from clinical studies indicate that the nigrostriatal neurodegeneration associated with Parkinson’s disease is accompanied by an increase in endocannabinoid levels (Pisani et al., 2005, Pisani et al., 2011, Pisani et al., 2010). A study investigating anandamide levels in newly-diagnosed, non-medicated patients, and patients with mild or advanced diseases following medication wash-out, showed that levels of anandamide were increased two-fold in patients regardless of clinical progression (Pisani et al., 2005). A subsequent study by the same group found that anandamide levels in untreated patients were more than double that of controls, and chronic dopaminergic replacement therapy was capable of restoring anandamide to control levels (Pisani et al., 2010). Interestingly, there is also clinical evidence to suggest that the endocannabinoid system may play a role in the non-motor symptoms of Parkinson’s disease. For example, the risk of depression (which affects up to 50% of patients (Costa et al., 2012)) has been found to be reduced in Parkinson’s disease patients with specific genetic polymorphisms in the $CB_1$ receptor gene (Barrero et al., 2005), while polymorphisms in the FAAH gene have been associated with disease-related musculoskeletal pain (Greenbaum et al., 2012). The phytocannabinoid, cannabidiol, has also demonstrated some efficacy in reducing psychotic symptoms in Parkinson’s disease patients (Zuardi et al., 2009).
<table>
<thead>
<tr>
<th>Measure</th>
<th>Patient groups</th>
<th>Drug treatment</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB₁ receptor density and activity in the basal ganglia by [3H]CP-55 940 binding assay</td>
<td>Idiopathic PD (n=7) Age-matched controls (n=5)</td>
<td>All PD patients were receiving L-DOPA</td>
<td>Increased CB₁ receptor density and downstream GTP-binding in caudate nucleus and putamen in PD patients. No change in lateral globus pallidus or substantia nigra</td>
<td>Lastres-Becker et al. (2001)</td>
</tr>
<tr>
<td>CB₁ mRNA levels post mortem in the basal ganglia by RT-qPCR</td>
<td>Idiopathic PD (n=10) Age-matched controls (n=8)</td>
<td>All PD patients were receiving L-DOPA and/or direct dopamine agonists</td>
<td>Decreased CB₁ receptor expression in the caudate nucleus, anterior dorsal putamen and external globus pallidus</td>
<td>Hurley et al. (2003)</td>
</tr>
<tr>
<td>CB₁, D₂/D₃ receptor density post mortem in the basal ganglia by autoradiography</td>
<td>Idiopathic PD (n=8) Age-matched controls (n=5)</td>
<td>All chronic L-DOPA</td>
<td>CB₁ receptor density unaltered in PD patients D₂/D₃ receptor density reduced in the caudate nucleus and putamen</td>
<td>Farkas et al. (2012)</td>
</tr>
<tr>
<td>CB₁ receptor availability in the substantia nigra by MRI and PET</td>
<td>Idiopathic PD (n=29) Age-matched controls (n=12)</td>
<td>All PD patients were receiving L-DOPA treatment regimes</td>
<td>Decreased CB₁ receptor availability in the substantia nigra which was unaffected by L-DOPA treatment</td>
<td>Van Laere et al. (2012)</td>
</tr>
<tr>
<td>CB₂ mRNA levels post mortem in hippocampus and cerebellum by GeneChip hybridization array and RT-PCR</td>
<td>Idiopathic PD (n=8-9)</td>
<td>No information provided on patient treatment regimes</td>
<td>Decreased CB₂ receptor expression in the cerebellum and hippocampus of PD patients</td>
<td>Grünblatt et al. (2007)</td>
</tr>
</tbody>
</table>
### Table 1.3 Clinical reports of changes in the endocannabinoid system in Parkinson’s disease patients

Dysregulation of the endocannabinoid system has been reported in several clinical and post mortem studies. Abbreviations: UPDRS: Unified Parkinson’s Disease Rating Scale.

<table>
<thead>
<tr>
<th>Endocannabinoid System</th>
<th>PD Patients</th>
<th>Controls</th>
<th>Summary</th>
<th>Reference</th>
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<tbody>
<tr>
<td>CB2 receptor density in the substantia nigra using immunohistochemistry</td>
<td>Idiopathic PD (n=5) Age-matched controls (n=5)</td>
<td>Patients were receiving various dopamine replacement therapy prior to death</td>
<td>Reduced CB2 receptor expression in dopaminergic neurons in PD compared to controls.</td>
<td>Garcia et al. (2015)</td>
</tr>
<tr>
<td>CB2 receptor density in the substantia nigra using immunohistochemistry</td>
<td>Idiopathic PD (n=5) Age-matched controls (n=5)</td>
<td>Patients were receiving various dopamine replacement therapy prior to death</td>
<td>Elevated CB2 receptor expression on Iba1-positive microglial cells in PD, compared to controls</td>
<td>Goméz-Gálvez et al. (2015)</td>
</tr>
<tr>
<td>Anandamide levels in the CSF by HPLC</td>
<td>Idiopathic PD (n=16) Age-matched controls (n=14)</td>
<td>Patients underwent drug washout</td>
<td>Increased anandamide compared to controls Increase independent of disease stage or UPDRS</td>
<td>Pisani et al. (2005)</td>
</tr>
<tr>
<td>Anandamide levels in the CSF by HPLC</td>
<td>Idiopathic PD (n=56) Age-matched controls (n=37)</td>
<td>Patients underwent drug washout</td>
<td>Increased anandamide in de novo &amp; L-DOPA ‘washout’ patients compared to controls</td>
<td>Pisani et al. (2010)</td>
</tr>
</tbody>
</table>
The studies described above suggest dysregulation of the endocannabinoid system in the brains of patients with Parkinson’s disease which may play a role in both the motor and non-motor symptoms of this condition. Interestingly, these clinical findings were preceded by, or paralleled by, a small number of preliminary clinical studies investigating cannabinoid drug use in Parkinson’s disease patients which will be discussed in the following section.

1.4.2 Clinical trials of cannabinoid drugs in Parkinson’s disease

The few clinical reports of cannabinoid drug use in Parkinson’s disease patients have focused on CB₁ receptor agonists as modulators of motor symptoms and/or as adjuncts to L-DOPA therapy to reduce L-DOPA-induced dyskinesia (Table 1.4). The location of CB₁ receptors in the basal ganglia circuit, on MSNs of both the ‘direct’ and ‘indirect’ pathways, make them an attractive candidate for pharmacological manipulation of motor function, however given the complex and uncertain pathophysiology of motor symptoms and L-DOPA-induced dykinesias (reviewed in Huot et al., 2013), activation and/or blockade of CB₁ receptors have produced some positive effects. The first study to investigate the effect of cannabinoid drugs on L-DOPA-induced dyskinesias was quite promising, with the CB₁/CB₂ receptor agonist, nabilone, reducing this debilitating side effect of dopamine replacement therapy in a randomised, double-blind, placebo-controlled crossover trial in a small number of patients (Sieradzan et al., 2001). Subsequent randomised, controlled trials did not provide encouraging results, with neither cannabis (Carroll et al., 2004) nor the CB₁ receptor antagonist/inverse agonist, SR141716A (Mesnage et al., 2004), providing relief from motor symptoms or L-DOPA-induced dyskinesias. Despite these disappointing results from randomised, controlled trials, other studies have indicated
positive anti-Parkinsonian or anti-dyskinetic effects of cannabinoid receptor agonists in patient surveys (Venderová et al., 2004) or open-label trials (Zuardi et al., 2009, Chagas et al., 2014, Lotan et al., 2014). Notably, despite some positive results in these pilot studies, almost none of these cannabinoid drugs have been examined in large-scale studies with Parkinson’s disease patients, however cannabidiol treatment for tremor in Parkinson’s disease is currently in the recruitment stage of a Phase I clinical trial (ClinicalTrials.gov Identifier: NCT02818777).
<table>
<thead>
<tr>
<th>Measure &amp; Patients</th>
<th>Trial Design</th>
<th>Drug Treatment</th>
<th>Findings</th>
<th>Reference</th>
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<tbody>
<tr>
<td>L-DOPA-induced dyskinesias in idiopathic PD patients (n=7) all receiving chronic L-DOPA</td>
<td>Randomised, double-blind, placebo-controlled, crossover trial Pilot study</td>
<td>Nabilone (0.03 mg/kg, split dose at 12 hr and 1 hr prior to L-DOPA)</td>
<td>The cannabinoid agonist, nabilone, reduced L-DOPA-induced dyskinesia</td>
<td>(Sieradzan et al., 2001)</td>
</tr>
<tr>
<td>L-DOPA-induced dyskinesias in idiopathic PD patients (n=19) all receiving chronic L-DOPA</td>
<td>Randomised, double-blind, placebo-controlled, crossover trial Pilot study</td>
<td>Cannabis extracts (&lt;0.25 mg Δ9-THC/0.125 mg cannabidiol per day for 4 weeks)</td>
<td>Cannabis extract did not reduce L-DOPA-induced dyskinesia</td>
<td>(Carroll et al., 2004)</td>
</tr>
<tr>
<td>Motor disability &amp; L-DOPA-induced dyskinesias in idiopathic PD patients (n=24) all receiving chronic L-DOPA</td>
<td>Randomized, double-blind, placebo-controlled trial Exploratory study</td>
<td>SR141716A (20mg/day 1hr prior to L-DOPA for 16 days)</td>
<td>The CB1 receptor antagonist, SR141716A, did not improve motor ability or L-DOPA-induced dyskinesia</td>
<td>(Mesnage et al., 2004)</td>
</tr>
<tr>
<td>Motor disability &amp; L-DOPA-induced dyskinesias in PD patients (n=339)</td>
<td>Self-reported patient survey</td>
<td>Chronic oral cannabis consumption (Cannabis users (n=85), non users (n=254))</td>
<td>Cannabis improved motor symptoms and L-DOPA-induced dyskinesia</td>
<td>(Venderová et al., 2004)</td>
</tr>
<tr>
<td>Motor disability in idiopathic PD patients (n=6)</td>
<td>Open-label pilot study</td>
<td>Cannabidiol (Flexible dose (started at 150 mg/day) for 4 weeks)</td>
<td>The phytocannabinoid cannabidiol improved motor ability</td>
<td>(Zuardi et al., 2009)</td>
</tr>
<tr>
<td>Motor and non-motor symptoms in PD patients (n=22)</td>
<td>Open-label observational study</td>
<td>Smoked cannabis (UPDRS measured at baseline and 30mins post-smoking)</td>
<td>Patient smoking cannabis had reduced tremor, rigidity and bradykinesia Patients also had improved sleep and pain scores</td>
<td>(Lotan et al., 2014)</td>
</tr>
<tr>
<td>Motor symptoms, non-motor symptoms, well-being, neuroprotective effects (n=21)</td>
<td>Exploratory double-blind trial</td>
<td>Cannabidiol (75mg, 300mg or placebo once daily for 6 weeks)</td>
<td>Patients taking 300mg/day had a higher score in well being and quality of life. No effects on motor symptoms or neuroprotection</td>
<td>(Chagas et al., 2014)</td>
</tr>
</tbody>
</table>

Table 1.4. Clinical reports of cannabinoid drugs in Parkinson’s disease patients.
1.4.3 THE ENDOCANNABINOID SYSTEM AS A DISEASE-MODIFYING TARGET IN PARKINSON’S DISEASE

In addition to its potential role in the development of, and therapeutic relief from, Parkinsonian motor symptoms and L-DOPA-induced dyskinesias, the endocannabinoid system also has been shown to have considerable potential as a disease-modifying therapeutic target for PD. Indeed, there is evidence that cannabinoid drugs may be capable of reducing Lewy body formation, slowing nigrostriatal neurodegeneration and suppressing neuroinflammation.

1.4.3.1 Neuroprotective effects of cannabinoid drugs in preclinical models of Parkinson’s disease

The neuroprotective effects of cannabinoid drugs in preclinical Parkinson’s disease have been illustrated in many studies across various animal models (Table 1.5). Initial indications from a study in 2005 by Lastres-Becker and colleagues were that pharmacological targeting of cannabinoid receptors using Δ⁹-THC and cannabidiol, resulted in neuroprotection as evidenced by a reduction in 6-OHDA-induced nigrostriatal degeneration (Lastres-Becker et al., 2005). Other cannabinoid receptor agonists, including HU210 and WIN55,212-2, have also been shown to exhibit neuroprotective properties in various Parkinson’s disease models, with evidence of direct protection of nigrostriatal neurons, restoration of striatal dopamine and reduction of oxidative stress (Price et al., 2009, Chung et al., 2011, Chung et al., 2012), similar neuroprotection has been observed by enhancement of endogenous cannabinoids (Fernandez-Suarez et al., 2014). The mechanism by which cannabinoid drugs, specifically CB₁ receptor agonists, work to exert these neuroprotective effects
are most likely not restricted to CB1 receptor interactions. AM251, a selective CB1 receptor antagonist, did not alter WIN55,212-2-mediated neuroprotection in MPTP-treated mice and WIN55,212-2 conferred neuroprotection in CB1-null mice (Price et al., 2009). ∆9-THCV, a CB1 receptor inverse agonist/antagonist (which may also act as a partial CB2 receptor agonist (Pertwee, 2008)), attenuates nigrostriatal damage in and 6-OHDA-treated mice and rats, respectively (García et al., 2011). The phytocannabinoid, cannabidiol (a CB1 indirect agonist with CB2 inverse agonist properties), acts similarly to ∆9-THCV in this model, however to a much greater extent, indicating non-CB1 interactions (García et al., 2011). These findings, coupled with the inability of CB1 receptor antagonists to block neuroprotective effects of non-selective cannabinoid drugs, indicate alternative signalling mechanisms are involved.

In vitro studies corroborate in vivo evidence and suggest that the mechanism of cannabinoid-mediated neuroprotection involves anti-oxidative action, as observed in MPP+ treated mesencephalic cultures and PC12 cells (Moldzio et al., 2012, Iuvone et al., 2007). Interestingly, one study in 6-OHDA-treated PC12 cells showed that anandamide pre-treatment significantly attenuated cell death; however, CB1, CB2 and TRPV1 receptor antagonists failed to block these effects, implying non-cannabinoid receptor mediation of the effects of administered anandamide (Mnich et al., 2010).

Further to this, recent evidence has shown that administration of the endogenous the N-acylethanolamines may also have neuroprotective potential. Systemic administration of OEA, an analogue of anandamide with poor affinity for CB1 and CB2 receptors, partially inhibited nigrostriatal degeneration and oxidative stress in the 6-OHDA rat model and attenuated behavioural deficits, effects which were...
almost entirely reversed by a peroxisome proliferator-activated receptor (PPAR)-α antagonist (Gonzalez-Aparicio et al., 2013). Similarly, PEA, another PPAR-α receptor agonist, is neuroprotective following MPTP administration to mice, reducing nigrostriatal damage and reversing MPTP-induced motor deficits (Esposito et al., 2012). Both PEA and OEA are known to modulate anandamide activity via an ‘entourage effect’ (reviewed in Hansen, 2010) thus the neuroprotective effects elicited by exogenous administration of PEA and OEA may occur via cannabinoid and non-cannabinoid modulation. More recently, administration of 2-AG has been shown to protect nigral dopaminergic cells from MPTP-induced toxicity, which was potentiated by inhibition of COX-2 enzyme (Mounsey et al., 2015). It would appear, given the evidence discussed above, that cannabinoid drugs exert neuroprotective effects in animal models of Parkinson’s disease. The modulation of endogenous cannabinoid signalling, and interplay from non-cannabinoid compounds and targets, may also represent viable neuroprotective avenues for continued investigation in the search for a disease-modifying, neuroprotective treatment for PD.

1.4.3.2 Anti-inflammatory effects of cannabinoid drugs in preclinical models of Parkinson’s disease

The first indication that the endocannabinoid system might have anti-inflammatory potential relevant to PD came when Lastres-Becker et al. (2005) showed that the neuroprotective effect of the non-selective cannabinoid receptor agonist, HU-210, against the Parkinsonian neurotoxin, 6-OHDA, was largely dependent on its ability to modify glial cells. More recent studies have further corroborated the anti-inflammatory potential of targeting the cannabinoid receptors in animal models of
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PD. The non-selective receptor agonists, WIN55,212-2 and HU210 respectively, were found to have potent anti-inflammatory effects (reduced microglial activation, reduced expression of pro-inflammatory cytokines, reduced NADPH oxidase and reactive oxygen species) in both the MPTP mouse (Chung et al., 2011) and the LPS-treated rat (Chung et al., 2012) models of PD. Moreover, these anti-inflammatory effects were associated with protection of nigrostriatal neurons indicating that reducing neuroinflammation can have consequent neuroprotective actions in these models. Chung et al (2011) identified a specific role for CB₁-mediated effects as selective CB₁ receptor antagonists blocked these effects.
<table>
<thead>
<tr>
<th>Species &amp; Model</th>
<th>Cannabinoid Drug(s)</th>
<th>Neuroprotective/Anti-inflammatory Effect of Cannabinoid Drug(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats Intra-MFB 6-OHDA</td>
<td>Δ⁹-THC (3 mg/kg) Cannabidiol (3 mg/kg)</td>
<td>Cannabinoid agonist, Δ⁹-THC and CB₁/CB₂ receptor inverse agonist, cannabidiol, protected against neurodegeneration</td>
<td>(Lastres-Becker et al., 2005)</td>
</tr>
<tr>
<td>Rats ICV 6-OHDA</td>
<td>Rimonabant (0.1, 1 mg/kg)</td>
<td>Rimonabant, CB₁ antagonist/inverse agonist enhanced striatal glutamate levels, at doses previously shown to alleviate motor symptoms</td>
<td>(García-Arencibia et al., 2008)</td>
</tr>
<tr>
<td>Mice MPTP CB₁ KO mice No model</td>
<td>WIN55, 212-2 (4 mg/kg) AM251 (4 mg/kg) JWH015 (4 mg/kg)</td>
<td>WIN55,212-2, a non-selective cannabinoid receptor agonist attenuated nigrostriatal damage, increased dopamine in ventral midbrain and reduced microglial activation WIN55,212-2 also displayed similar effects in CB₁ knockout mice AM251 had no effect on WIN55,212-2-mediated neuroprotection WIN55,212-2 was neuroprotective to a similar extent in WT and CB₁ KO mice WIN55,212-2 and JWH015, a CB₂ receptor agonist, reduced MPTP-induced microglial activation</td>
<td>(Price et al., 2009)</td>
</tr>
<tr>
<td>Mice Intra-striatal LPS</td>
<td>Δ⁹-THCV (2 mg/kg) Cannabidiol (3 mg/kg)</td>
<td>Δ⁹-THCV, a CB₁ receptor antagonist/ CB₂ agonist, attenuated nigrostriatal dopaminergic loss Cannabidiol attenuated dopaminergic loss also, but to a greater extent than Δ⁹-THCV. Δ⁹-THCV protected nigrostriatal degeneration induced by the inflammmagen, LPS</td>
<td>(García et al., 2011)</td>
</tr>
<tr>
<td>Rats Intra-MFB 6-OHDA</td>
<td>HU308 (5 mg/kg)</td>
<td>HU-308, a CB₂ receptor agonist, preserved dopaminergic neuron survival in LPS-lesioned mice</td>
<td></td>
</tr>
<tr>
<td>Mouse MPTP</td>
<td>WIN55,212-2 (10 µg/kg) HU210 (10 µg/kg)</td>
<td>Both non-selective cannabinoid receptor agonist, WIN55, 212-2 and HU210, produced a significant increase in nigrostriatal dopamine neuron survival and increased striatal dopamine levels Both drugs attenuated MPTP-induced oxidative damage and ROS production and reduced inflammatory markers and neurodegeneration, which was reversed by AM251 and SR141716A</td>
<td>(Chung et al., 2011)</td>
</tr>
</tbody>
</table>
### Table 1.5: Preclinical reports of the neuroprotective and/or anti-inflammatory effects of cannabinoid drugs in PD models

<table>
<thead>
<tr>
<th>Species</th>
<th>Condition</th>
<th>Drug(s)</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>Intra-nigral LPS</td>
<td>WIN55,212-2 (5 µg, i.c.v) HU-210 (5 µg, i.c.v)</td>
<td>Both drugs increased survival of nigral dopamine neurons in addition to inhibiting ROS production. Both agonists reduced levels of proinflammatory cytokines TNF-α (WIN55,212-2, HU210), and IL-1β (WIN55,212-2 only). (Chung et al., 2012)</td>
</tr>
<tr>
<td>Rats</td>
<td>Intra-MFB 6-OHDA</td>
<td>WIN55,212-2 (31.25–250 µg/kg) Δ⁹-THC (250–2,000 µg/kg), AEA (50–150 µg, i.c.v)</td>
<td>All agonists inhibited subthalamic hyperactivity associated with the Parkinsonian state. AEA exerted a more intense inhibitory effect than other agonists. (Morera-Herreras et al., 2011)</td>
</tr>
<tr>
<td>Mice</td>
<td>MPTP</td>
<td>PEA (10mg/kg)</td>
<td>PEA, a weak cannabinoid agonist capable of enhancing AEA, protected nigrostriatal dopamine neurons from MPTP-induced neurotoxicity. PEA also reduced alterations indicative of inflammation, including microglial and astrocytic activation. (Esposito et al., 2012)</td>
</tr>
<tr>
<td>Rats</td>
<td>Intra- striatal 6-OHDA</td>
<td>OEA (5mg/kg)</td>
<td>OEA partially protected against nigral dopaminergic degeneration and reduced oxidative markers, which was blocked by PPARα receptor antagonism. (Gonzalez-Aparicio et al., 2013)</td>
</tr>
<tr>
<td>Mice</td>
<td>MPTP</td>
<td>JZL184 (8mg/kg)</td>
<td>MAGL inhibitor, JZL184, prevented MPTP-induced motor impairment, altered astrocytic and microglial phenotypes. JZL184 increased TGF-β, GDNF and β-catenin. (Fernandez-Suarez et al., 2014)</td>
</tr>
<tr>
<td>Mice</td>
<td>MPTP</td>
<td>2-AG (3, 5mg/kg)</td>
<td>Administration of 2-AG protected nigral TH+ neurons from MPTP-induced neurodegeneration, but did not significantly affect striatal dopamine levels. (Mounsey et al., 2015)</td>
</tr>
</tbody>
</table>

**Abbreviations:** LPS: lipopolysaccharide; AEA: anandamide; PEA: palmitoylethanolamide.; TGF-β: transforming growth factor β; GDNF: glial-derived neurotrophic factor.
Although several studies exist that support the anti-inflammatory potential of activating the CB₁ receptor, the psychoactive side effects associated with targeting this receptor may limit its therapeutic potential as an anti-inflammatory target. There is a substantial body of evidence to suggest that the CB₂ receptor may be the more promising anti-inflammatory target. The CB₂ receptor is widely accepted to be expressed on microglia, the resident immune surveillance cells in the brain, and its expression is strongly up-regulated when these cells are activated (reviewed in Benito et al., 2008). Moreover, numerous in vitro studies have revealed that activation of microglial CB₂ receptors suppresses their release of pro-inflammatory cytokines, enhances their release of anti-inflammatory cytokines, and reduces their neurotoxicity (reviewed in Little et al., 2011). These data suggest that pharmacological activation of the CB₂ receptor may be a promising approach for anti-inflammatory intervention in PD.

1.4.4 THE CB₂ RECEPTOR AS AN ANTI-INFLAMMATORY TARGET IN NEURODEGENERATIVE DISEASE

Post mortem evidence has identified increased expression of the CB₂ receptor in a host of neurodegenerative conditions, including Parkinson’s disease, further reiterating its potential role in inflammation and neurodegeneration (Garcia et al., 2015, Gómez-Gálvez et al., 2015, Benito et al., 2007, Yiangou et al., 2006, Palazuelos et al., 2009, Dowie et al., 2014, Rodriguez-Cueto et al., 2014). The physiological function of the CB₂ receptor is focused on immune signalling, which stands to reason given its high peripheral expression in key immune organs such as
Chapter 1: Introduction

the spleen and thymus (Munro et al., 1993, Galiègue et al., 1995). CB2 receptor mRNA and protein expression has also been identified in human leukocytes in including macrophages, microglia, B cells and T cells (Klegeris et al., 2003, Oka et al., 2004, Castaneda et al., 2013). As such it plays a role in several leukocyte functions, including regulation of T and B cell differentiation, maintenance of Th1/Th2 cytokine balance, and in macrophages, stimulation of CB2 induces release of pro-inflammatory mediators, inhibits phagocytosis and reduces signaling to T cells (Ziring et al., 2006, Chuchawankul et al., 2004). Its expression pattern in the nervous system remains unclear, due to issues with non-specificity of commercially available antibodies and positive staining observed in CB2-null mice (Atwood and Mackie, 2010, Marchalant et al., 2014). Nevertheless, it is widely accepted that CB2 receptors are present on microglia, although their presence on neurons remains contentious.

Although CB2 knockout animals do not display any developmental or morphological differences to wild-type animals, they do have low bone mass, and delayed or impaired maturation of T cell and B cell subsets (Ofek et al., 2006, Ziring et al., 2006). CB2−/− mice do not develop any autoimmune conditions despite these deficits, however evidence suggests they are more susceptible to inflammatory challenges (reviewed in Malfitano et al., 2014).

1.4.4.1 The CB2 receptor in models of neurodegeneration

There is considerable evidence indicating the CB2 receptor may have multiple roles in modifying or mediating the immune response both in vitro and in vivo (reviewed in Ashton and Glass, 2007). Characterisation of gene expression and genetic
Chapter 1: Introduction

manipulation of the CB2 receptor in animal models of neurodegeneration have illustrated its potential as a therapeutic target. Upregulation of the CB2 receptor has been identified in experimental models of Alzheimer’s disease (Wu et al., 2013), Huntington’s disease (Fernandez-Ruiz et al., 2007, Sagredo et al., 2009, Bouchard et al., 2012), multiple sclerosis (Lou et al., 2011), amyotrophic lateral sclerosis (Shoemaker et al., 2007) and indeed, Parkinson’s disease (García et al., 2011) although the majority of these studies have utilized CB2 receptor antibodies for their analyses.

Genetic manipulation of the CB2 receptor in neurodegenerative animal models suggests it plays a key role in the pathogenesis of many disease phenotypes. In transgenic Alzheimer’s disease mice, crossed with CB2-deficient mice, there is an exacerbated disease phenotype including elevated levels of soluble Aβ40/42 and Aβ deposition (Aso et al., 2016, Koppel et al., 2013), although the converse has also been reported (Schmole et al., 2015b). Clinically, CB2 receptor expression is significantly correlated with Aβ42 levels and senile plaque score (Solàs et al., 2013).

Genetic ablation of CB2 receptors accelerates the onset of motor symptoms in Huntington’s disease BACHD mice (Bouchard et al., 2012), and in the R6/2 mouse model, which produces a more severe disease phenotype, CB2 receptor deficiency exacerbated disease severity and was coupled with enhanced microglial activation and reduced lifespan (Palazuelos et al., 2009). In animal models of Parkinson’s disease, genetic deletion of CB2 accelerates death in an MPTP mouse model (Price et al., 2009) and CB2−/− animals are more vulnerable to LPS-induced nigrostriatal damage (García et al., 2011). In contrast, mice overexpressing CB2 receptors present
with significantly less motor impairment following intra-caudate 6-hydroxydopamine lesion, in addition to reduced microglial and astrocytic activation, and markers of oxidative stress (Ternianov et al., 2012)

1.4.4.2 Pharmacological targeting of the CB$_2$ receptor in models of neurodegeneration

1.4.4.2.1 CB$_2$-mediated effects of non-selective cannabinoids

There are a number of non-selective cannabinoid drugs which have shown neuroprotective activity in animal models of neurodegeneration and in some cases these effects are partially or almost entirely mediated via CB$_2$ receptor activation. Table 1.5 summarises cannabinoid drugs which have some anti-inflammatory or neuroprotective efficacy in animal models of Parkinson’s disease. Two studies identified a specific role for CB$_2$ in the neuroprotective effects observed – Price et al. (2009) found that the non-selective cannabinoid, WIN55, 212-2, reduced expression of MAC-1, a microglial/macrophage marker, by 40% in the ventral midbrain which was blocked by the CB$_2$ antagonist, JTE907. Administration of CB$_1$ antagonist/CB$_2$ partial agonist, Δ9-THCV, also protected tyrosine hydroxylase positive neurons following intra-nigral LPS lesion (García et al., 2011), although these effects may attributable to Δ9-THCV-mediated enhancement of endocannabinoid tone (McPartland et al., 2015).

1.4.4.2.2 CB$_2$-selective drug studies

Given the evidence for CB$_2$-mediated effects of cannabinoid drugs, selective targeting of the CB$_2$ receptor has emerged as an attractive therapeutic option and
recent data is very encouraging (Table 1.6). Two studies have examined the potential of selective CB2 receptor targeting in Parkinson’s disease models and identified anti-inflammatory and neuroprotective effects, although this was not the focus of these reports (Price et al., 2009, García et al., 2011). In Alzheimer’s disease models, CB2 agonism has been shown to ameliorate memory impairment, enhance glucose uptake and reduce inflammatory and oxidative markers ultimately improving the disease phenotype (Jayant et al., 2016, Kofalvi et al., 2016, Cheng et al., 2014, Wu et al., 2013, Aso et al., 2013, Tolon et al., 2009). In one particular study, the CB2 agonist, JWH133, normalised cognitive deficits in APP mice, which was not observed in WIN55, 212-2-treated APP mice (Martin-Moreno et al., 2012). In Huntington’s disease transgenic and toxin models, CB2 agonists extended lifespan, improved motor deficits, protected neurons and reduced inflammation (Sagredo et al., 2009, Palazuelos et al., 2009), although there is evidence to suggest the central effects of CB2 agonism may be peripherally mediated (Bouchard et al., 2012). Multiple sclerosis is an autoimmune disease and as such has a strong, persistent inflammatory profile which, in animal models, is alleviated following treatment with CB2 agonists (Fu and Taylor, 2015, Kong et al., 2014), and exacerbated by CB2 antagonism (Lou et al., 2011). Although not well-explored, CB2 receptor agonism also increases survival rate in a model of amyotrophic lateral sclerosis (Shoemaker et al., 2007). Clearly, there is potential for the CB2 receptor as a viable anti-inflammatory target in neurodegenerative conditions however much is still unknown about its role in disease pathogenesis and as such, the effects of targeting this receptor in vivo.
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<table>
<thead>
<tr>
<th>Drug</th>
<th>Disease model</th>
<th>Neuroprotective effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JWH133</td>
<td>Tg APP mice (AD)</td>
<td>Normalised cognitive deficits, reduced microglial density, COX-2 and TNFα</td>
<td>(Martin-Moreno et al., 2012, Aso et al., 2013, Kofalvi et al., 2016)</td>
</tr>
<tr>
<td>CB2 Ki = 3.4 nM</td>
<td>AβPP/PS1 mice (AD)</td>
<td>Ameliorated cognitive deficits, reduced microglia, II-1β, II-6, TNF-α, IFNγ, oxidative markers Enhanced glucose transport and uptake in hippocampal slices</td>
<td></td>
</tr>
<tr>
<td>JWH015</td>
<td>MPTP mice (PD)</td>
<td>Reduced MAC-1 protein expression in the ventral midbrain</td>
<td>(Price et al., 2009, Tolon et al., 2009)</td>
</tr>
<tr>
<td>CB2 Ki = 14 nM</td>
<td>Human AD tissue</td>
<td>Induced clearance of amyloid-β in tissue and Thp-1 macrophages</td>
<td></td>
</tr>
<tr>
<td>HU308</td>
<td>Quinolinic acid (HD)</td>
<td>Reduced microglial and astrocytic activation, striatal neuronal loss, ameliorated motor symptoms.</td>
<td>(Palazuelos et al., 2009, Sagredo et al., 2009, García et al., 2011)</td>
</tr>
<tr>
<td>CB2 Ki = 22.7 nM</td>
<td>Malonate (HD)</td>
<td>Reduced microglial and astrocytic activation, striatal neuronal loss, ameliorated motor</td>
<td></td>
</tr>
<tr>
<td>LPS (PD)</td>
<td>Protected striatal neurons, reduced TNF- α, reduced GABA, dopamine &amp; DOPAC striatal loss</td>
<td>(2009, García et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>MDA7</td>
<td>i.c.v. Aβ fibrils (AD)</td>
<td>Reduced microglial and astrocytic activation, reduced II-1β, increased Aβ clearance, aided restoration of synaptic plasticity, memory and cognition</td>
<td>(Wu et al., 2013)</td>
</tr>
<tr>
<td>CB2 Ki = 422 nM</td>
<td>Increased survival rate of animals by 56%</td>
<td>(Shoemaker et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>AM1241</td>
<td>G93A-SOD1 (ALS)</td>
<td>Increased survival rate of animals by 56%</td>
<td></td>
</tr>
<tr>
<td>CB2 Ki = 7.1 nM</td>
<td>R6/2 mice (HD)</td>
<td>Extends life span, improves motor deficits, synaptic loss, reduced microglia (Iba-1) in brain, reduced II-6 and II-1β</td>
<td>(Bouchard et al., 2012)</td>
</tr>
<tr>
<td>GW405833</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB2 Ki = 3.92 nM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Compound</th>
<th>Model</th>
<th>Effect</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-caryophyllene</td>
<td>APP/PS1 mice (AD)</td>
<td>Reduced astrocytic and microglial activation, reduced COX-2, TNF-α, IL-1β in cerebral cortex</td>
<td>(Cheng et al., 2014)</td>
</tr>
<tr>
<td>CB₂ Ki = 155 nM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP1a</td>
<td>Tg APP mice (AD)</td>
<td>Enhanced glucose uptake in hippocampal slice, stimulates glucose transport</td>
<td>(Kofalvi et al., 2016, Kong et al., 2014)</td>
</tr>
<tr>
<td>CB₂ Ki = 0.037 nM</td>
<td>EAE (MS)</td>
<td>Reduced clinical scores, demyelination and axonal loss, reduced accumulation of pathogenic immune cells in CNS</td>
<td>Kong et al., 2014</td>
</tr>
<tr>
<td>1-phenylisatin</td>
<td>AlCl₃+d-galactose, STZ (AD)</td>
<td>Ameliorated memory impairment, reduced markers of oxidative and nitritative stress, reduced inflammatory markers, reduced plaque load</td>
<td>(Jayant et al., 2016)</td>
</tr>
</tbody>
</table>

Table 1.6. CB₂ receptor-selective agonists and their anti-inflammatory/neuroprotective effects. Abbreviations: Ki: Human CB₂ receptor binding affinity, STZ, streptozocin; aluminum trichloride (AlCl₃)+d-galactose; ICV, intracerebroventricular; EAE, experimental autoimmune encephalomyelitis; ALS, amyotrophic lateral sclerosis.
1.5 HYPOTHESIS

Given the accumulating evidence supporting a potential role for the CB$_2$ receptor in neuroinflammation and neurodegeneration, we hypothesised that Parkinson’s disease is associated elevated microglial CB$_2$ receptor expression and pharmacological activation of the CB$_2$ receptor will suppress neuroinflammation in animal models of Parkinson’s disease and protect nigrostiatal integrity and improve motor function in these animals.

1.6 AIM OF THIS STUDY

In order to test our hypothesis we embarked on several lines of investigation which are detailed below:

- In order to discern if CB$_2$ receptor expression is altered in Parkinson’s disease we assessed the temporal profile of CB$_2$ receptor expression in several preclinical animal models of Parkinson’s disease including genetic (α-synuclein), neurotoxic (6-hydroxydopamine and rotenone), and inflammatory (LPS and Poly (I:C)) stimuli which is detailed in Chapter 3-5.
- Following this, we attempted to examine if pharmacological activation of the CB$_2$ receptor could suppress neuroinflammation and lesion-induced motor impairment in these animals, which is detailed in Chapter 6.
Chapter 2: Materials & Methods

2.1 ETHICAL STATEMENT

All procedures were carried out in accordance with the European Union Directive 2010/73/EU and S.I. No. 543 of 2012, were completed under a CAA license issued to Dr. Eilís Dowd (B100/3827) by the Irish Department of Health and Children and a Personal Authorisation issued to Ms. Ruth Concannon (AE19125/I048) by the Irish Health Products Regulatory Authority, and were approved by The Animal Care and Ethics Committee of the National University of Ireland, Galway. 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 determine if pharmacological targeting of the CB$_2$ receptor can suppress neuroinflammation in animal models of Parkinson’s disease, and to determine if this anti-inflammatory effect is sufficient to protect nigrostriatal integrity and motor function in these animals. As part of this, the main objectives of the project were to determine and compare the temporal relationship between neuroinflammation, neurodegeneration and the expression of the CB$_2$ receptor in animal models of Parkinson’s disease and to determine if pharmacological targeting of the CB$_2$ receptor can suppress neuroinflammation and confer functional neuroprotection in following PD-related insults in these models. In order to reliably assess CB$_2$ receptor expression, we employed qRT-PCT as the main quantitative analytical technique due to
the known issues with non-specificity of commercially available antibodies (Atwood and Mackie, 2010, Baek et al., 2013, Marchalant et al., 2014).

In the first results chapter (Chapter 3) we assessed temporal changes in CB$_2$ receptor expression, as well as other changes in the endocannabinoid system, in a genetic model of Parkinson’s disease, using AAV delivery of human wild-type α-synuclein to the rat brain. In the second results chapter (Chapter 4), we sought to further characterise CB$_2$ receptor expression and changes in other elements of the endocannabinoid system in the neurotoxic, 6-hydroxydopamine, and the environmental, rotenone, models of Parkinson’s disease. In the third results chapter (Chapter 5), we then characterised CB$_2$ receptor expression and the endocannabinoid system in ‘emerging’ inflammation-driven models induced by the bacterial mimetic, LPS, and the viral mimetic, Poly (I:C). Finally, in Chapter 6, we investigated the potential of targeting the CB$_2$ receptor for anti-inflammatory disease modification in the inflammation-driven models. This involved chronic administration of CB$_2$ agonists in the LPS and Poly (I:C) models, and assessing the impact of this treatment on motor impairment, neuroinflammation and nigrostriatal integrity.

This chapter will detail the methodologies and techniques employed in order to conduct this research, and specific experimental designs will be provided in subsequent results chapters.
2.3 ANIMAL HUSBANDRY

A total of 260 male Sprague Dawley rats were used in this research. Animals were sourced from Charles River, UK. Unless indicated otherwise, all animals were housed four 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. Animals were allowed to free-feed with access to food and water \textit{ad libitum} unless undergoing food restriction as part of behavioural assessment, in which case animals were provided with sufficient food in order to maintain 85-90\% of their free-feeding body weight (as determined by a weight curve provided by Charles River). Animals were housed on a 12:12 hour light:dark cycle, under a regulated temperature and humidity of 19-23°C and 40-70\% respectively. All behavioural testing, tissue processing and analyses were performed blind to the treatment of the animals.

2.4 DRUGS USED IN THIS STUDY

The drugs used in the neuroprotection studies in Chapter 6 are detailed in Table 2.1.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Vehicle</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>JWH133 (Tocris, UK)</td>
<td>Highly selective CB$_2$ receptor agonist</td>
<td>EtOH:cremaphor:saline (1:1:18)</td>
<td>1 mg/kg i.p.</td>
</tr>
<tr>
<td>β-caryophyllene (TCI Chemicals, UK)</td>
<td>Non-selective CB$_2$ receptor agonist</td>
<td>EtOH:cremaphor:saline (1:1:18)</td>
<td>10 mg/kg i.p.</td>
</tr>
<tr>
<td>AM630 (Tocris, UK)</td>
<td>CB$_2$ receptor antagonist</td>
<td>EtOH:cremaphor:saline (1:1:18)</td>
<td>1 mg/kg i.p.</td>
</tr>
<tr>
<td>GW9662 (Tocris, UK)</td>
<td>PPARγ antagonist</td>
<td>EtOH:cremaphor:saline (1:1:18)</td>
<td>1 mg/kg i.p.</td>
</tr>
</tbody>
</table>
Chapter 3: Characterisation of temporal CB$_2$ receptor expression in a genetic model of Parkinson’s disease

Table 2.1 Drugs used in neuroprotection studies in Chapter 6. PPAR$\gamma$, peroxisome proliferator-activated receptor.

2.5 AAV VIRUS PRODUCTION

The viral vectors used in Chapter 3 are AAV$_{2/5}$-GFP and AAV$_{2/5}$-α-synuclein viruses, kindly synthesised and provided by Dr. Teresa Moloney and Ms. Carol Naughton, members of the Dowd research group. Transgenes (GFP and human wild-type α-synuclein) under the synapsin promoter were packaged in a pseudotyped AAV$_{2/5}$ viral vector, which has a particular tropism for neuronal cells, as described previously (Mulcahy et al., 2012). The titres of the viruses were 8 x 10$^7$ vector genomes/µl (vg/µl) for AAV$_{2/5}$-GFP and 1 x 10$^{10}$ vg/µl for AAV$_{2/5}$-α-syn. For the purposes of clarity, these viruses will be referred to as AAV-α-synuclein and AAV-GFP in the relevant results chapter.

2.6 SURGERY

All surgery was performed under gaseous anaesthesia (5% in O$_2$ for induction, 2% in O$_2$ for induction) in a stereotaxic frame with the nose bar set at -2.3 mm, as described previously (Hoban et al., 2013a, Hoban et al., 2013b). The animal’s head was shaved at the site of surgery and their heads were secured in a stereotaxic frame using the ear bars. An incision was made along the skin at the top of the head and the skull was exposed. The coordinates from Bregma were identified and the site of injection was located relative to this. An electric drill was used to make a hole in the skull at the injection site, to expose the dura mater. The toxin (in solution) was slowly infused into the site of injection by monitoring the movement of an air bubble, deliberately introduced, through the plastic tubing which was connected to a 50 µl Hamilton syringe. Infusion of
the toxins was achieved by the steady depression of the plunger on the Hamilton syringe at a rate of 1 µl/min using an automated pump (Harvard Apparatus). The site for striatal injections were AP 0.0 mm, ML ± 3.7 mm, DV -5.0 mm and the site for nigral injections were AP -5.3 mm, ML ± 2.0 mm, DV -7.2 mm based on the brain rat atlas (Paxinos and Watson, 2006).

2.7 BEHAVIOURAL TESTS OF MOTOR IMPAIRMENT

2.7.1 CORRIDOR TEST

The Corridor Test measures contralateral sensorimotor neglect and was devised and characterised 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 during the testing period. The corridor apparatus consisted of two long, narrow chambers (length 50 cm, height 24.5 cm, width 7 cm). During the habituation period, animals were allowed to freely explore the first empty corridor with CocoPops® scattered on the floor. During the testing period, 10 pairs of pots were placed adjacently and at equal intervals along the floor edge of the second corridor and 2-3 CocoPops® were placed in each of these. Animals were allowed to freely retrieve CocoPops® from pots on either side (Fig 2.1A). The trial was deemed complete when an animal had made a total of 20 retrievals or after 5 mins had elapsed. The number of retrievals made by each rat from the ipsilateral and contralateral sides (relative to the lesioned side), was recorded and expressed as a percentage of the total number of retrievals made. This test is based on the premise that animals with a unilateral lesion will tend to ignore food on
their contralateral side and exclusively or predominantly retrieve from their ipsilateral side.

2.7.2 Stepping Test

The Stepping Test measures forelimb akinesia and was characterised as described previously (Olsson et al., 1995). Initially, animals were habituated to the test by restraining them in such a manner so that both forelimbs were resting on the edge of a table with their rear legs resting in the hands of the experimenter. This ensured the animal was comfortable with being handled in this manner and eliminates inaccurate results during testing (i.e. the animal could take additional steps in order to escape from the grip if it is not habituated). Once the rat 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 s). During this motion, the number of adjusting steps made by the ‘free’ paw was recorded in the forehand and backhand directions (Fig 2.1B). This was performed for both the ipsilateral and contralateral sides of the rat. Data was represented as the number of adjusting steps of the ipsilateral and contralateral limbs in the forehand and backhand direction, and again, unilaterally lesioned animals have a reduced number of adjusting steps on the contralateral side.

2.7.3 Whisker Test

The Whisker Test measures sensorimotor integration and has been characterised previously (Schallert et al., 2000). This test is based on the sensorimotor reflex underlying vibrissae-elicited movement whereby the animal’s whiskers are brushed against a surface (e.g. corner of a table) and this elicits a reflex which causes the rat’s
ipsilateral limb to move, placing its paw on the table surface. This response is impaired in animals with a dopaminergic lesion. Habituation to the task was performed initially and involved restraining the animal’s hind limbs and one forelimb in such a manner that the animal was comfortable, before gently brushing their whiskers against the corner of a table. Again, habituation is critical to ensure the animal is comfortable with being handled and restrained in this manner, in order to avoid false positive results. During testing, the number of vibrissa-elicited forelimb placings by the unrestrained forelimb was recorded when the animal’s whiskers were brushed against the corner of a table 10 times and this was performed on both ipsilateral and contralateral sides of the rat (Fig 2.1C). Data was represented as the number of forelimb placings.

2.7.4 CYLINDER TEST

The Cylinder test measures forelimb use and has been characterised previously (Schallert et al., 2000), and modified as described (Torres et al., 2008). In this test, there was no habituation period to maximise exploratory activity during testing. On testing days, animals were placed in a clear cylinder and were allowed to freely explore their environment, and each time they touched the side of the cylinder this was recorded as a forelimb placing. The test continued until a combined total of 20 forelimb placings of either the ipsilateral or contralateral limbs was made, or 5 mins had elapsed (Fig 2.1D). Data was represented as the number of contralateral placings expressed as a percentage of the total number of placings. Animals with a unilateral dopaminergic loss make less forelimb placings on the contralateral side.
Chapter 3: Characterisation of temporal CB$_2$ receptor expression in a genetic model of Parkinson’s disease

Fig 2.1 Behavioural tests of motor impairment. (A) The Corridor Test – Animals retrieved CocoPops® from 10 pairs of adjacent pots on either side of the corridor. (B) The Stepping Test - Animals were restrained on one forelimb and guided across a table surface at a steady pace and the number of steps taken by the unrestrained limb was recorded. (C) The Whisker Test – One forelimb of the animal was restrained and the whiskers on the same side as the unrestrained limb were brushed against the corner of a table, and the number of limb placings was recorded. (D) The Cylinder Test – The number of forelimb placings by the ipsilateral and contralateral limbs were counted until a total of 20 placings were recorded or 5 mins had elapsed.

2.8 IMMUNOHISTOCHEMISTRY

2.8.1 TISSUE PROCESSING

Rats were anaesthetised by injection of sodium pentobarbital (50mg/kg i.p. Dolethal, Vetoquinol, Dublin, Ireland) and then transcardial perfusion was performed with heparinised saline (5000 units/l) followed by 40 ml of ice cold paraformaldehyde. Brains were removed from the skull and post-fixed in 4% paraformaldehyde for 24 hours and cryoprotected in 25% sucrose with 0.1% sodium azide solution for long term storage (protocol in Appendix 1). Serial brain sections were cut on a freezing sledge microtome (Bright, Cambridgeshire, UK) and collected in a series of 1:12. For qualitative immunohistochemistry, a 1:12 series of sections was used, for quantitative analyses a 1:6 series was used.
Chapter 3: Characterisation of temporal CB$_2$ receptor expression in a genetic model of Parkinson’s disease

2.8.2 IMMUNOHISTOCHEMISTRY

Free-floating immunohistochemical staining was performed using a streptavidin-biotin-peroxidase method, on a continuous rocker (see Appendix 2 for protocol details) as has been described previously (Torres et al., 1993, Walsh et al., 2010, Hoban et al., 2013a). Briefly, sections were quenched in a solution composed of 3% hydrogen peroxide/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 species of the secondary antibody), in tris-buffered saline (TBS) with 0.2% Triton X-100 (TXTBS), at room temperature for 1 hour. The primary antibody was diluted in 1% serum (horse or goat) in TXTBS and allowed to incubate with the sections overnight at room temperature. The following day biotinylated secondary antibody was added to the sections and incubated for 3 hours.

Following this, a streptavidin-biotin-horseradish peroxidase solution (Vector, UK (PK6100)) was added to sections and allowed to incubate for 2 hours. Development of the staining was then performed using a 0.5% solution of diaminobenzidine tetrachloride (DAB) (Sigma, Ireland (D5637)) in TBS with 0.3 µl/ml hydrogen peroxide. Sections were then mounted onto gelatine-coated slides, and once dry were dehydrated in a series of ascending alcohols, cleared in xylene and coverslipped using DPX mountant (Sigma, Ireland). The specific antibodies used are detailed in Table 2.2.
Chapter 3: Characterisation of temporal CB$_2$ receptor expression in a genetic model of Parkinson’s disease

<table>
<thead>
<tr>
<th>Ab</th>
<th>Protein target</th>
<th>Description</th>
<th>Source</th>
<th>Host</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1$^\circ$</td>
<td>Tyrosine hydroxylase (TH) (rat)</td>
<td>Catecholaminergic neurons (including dopaminergic)</td>
<td>Millipore</td>
<td>Mouse</td>
<td>1:1000</td>
</tr>
<tr>
<td>1$^\circ$</td>
<td>OX42/CD11b (rat)</td>
<td>Microglia</td>
<td>Chemicon</td>
<td>Mouse</td>
<td>1:400</td>
</tr>
<tr>
<td>1$^\circ$</td>
<td>Glial fibrillary acidic protein (GFAP) (rat)</td>
<td>Astrocytes</td>
<td>DAKO</td>
<td>Rabbit</td>
<td>1:2000</td>
</tr>
<tr>
<td>1$^\circ$</td>
<td>α-synuclein (human)</td>
<td>Wild type α-synuclein protein</td>
<td>Millipore</td>
<td>Mouse</td>
<td>1:10000</td>
</tr>
<tr>
<td>2$^\circ$</td>
<td>Biotinylated horse anti-mouse</td>
<td>Targets mouse 1$^\circ$ IgG</td>
<td>Vector</td>
<td>Horse</td>
<td>1:200</td>
</tr>
<tr>
<td>2$^\circ$</td>
<td>Biotinylated goat anti-rabbit</td>
<td>Targets rabbit 1$^\circ$ IgG</td>
<td>Jackson</td>
<td>Goat</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Table 2.2 Antibodies used for immunohistochemistry. Ab, antibody; 1$^\circ$, primary; 2$^\circ$, secondary.

2.8.3 HISTOLOGICAL QUANTIFICATION

Histological quantification was performed as described previously (Dowd and Dunnett, 2005). All image analysis was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland USA). 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 and astrocytic staining was measured in both the substantia nigra and striatum using optical density. In all cases, three representative images were used for quantification purposes.

2.8.3.1 Quantification of tyrosine hydroxylase-positive cell bodies

The number of tyrosine hydroxylase-positive cell bodies in the substantia nigra was counted using the cell counter tool in ImageJ programme (Fig 2.2). Photomicrographs were taken of the site of interest on a Nikon SMZ800 microscope and DXM1200C.
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digital camera. The number of positively stained cell bodies were counted on both the ipsilateral and contralateral sides of the brain, relative to the lesion, and according to boundaries previously delineated (Kirik et al., 1998). In brief, immune-positive cells in the substantia nigra \textit{pars reticulata}, \textit{pars lateralis} and \textit{pars compacta} were counted and immune-positive cells in the ventral tegmental area (VTA) were excluded. Data was represented as a percentage of the intact (contralateral) side.

\textbf{Fig 2.2. 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.
2.8.3.2 Striatal fibre density measurement

Optical density measurement was used to analyse tyrosine hydroxylase-positive fibres in the striatum in ImageJ. Again, photomicrographs were obtained using the microscope mentioned in the previous section, and images were taken under the same conditions of exposure and pixel quality. For analysis, images were converted to 16-bit grayscale, the entire dorsal striatum was designated using the freehand drawing tool (Fig. 2.3), and the mean grey value was measured on both the ipsilateral and contralateral sides. To account for non-specific background intensity, an area of unstained tissue was measured also and this was subtracted from both intensity values for each striata. Mean grey values are converted to optical density values using a logarithmic function applied to the image pixel intensity (optical density = $\log_{10}(255/$mean grey value)). Data was then represented as a percentage of the intact side.
Fig 2.3. ImageJ analysis of nigrostriatal terminal density in the striatum. Screenshot of ImageJ software used to measure tyrosine hydroxylase-positive staining in the dorsal striatum. Area to be measured delineated by the yellow border in the grayscale image above. The area drawn excluded the nucleus accumbens at the border of the ventral tip of the ventricle. Mean gray value was measured in both striata and in non-stained background tissue.

2.8.3.3 Quantification of nigral and striatal microgliosis

Microglia were identified by staining for the microglial/macrophage marker CD11b (OX42). Again, photomicrographs were taken on a Nikon SMZ800 microscope with DXM1200C digital camera under the same conditions of exposure, magnification and pixel intensity. Optical density, as described in Section 2.8.3.2, was used to measure microglial staining. For nigral quantification, the area of microgliosis at the level of the substantia nigra was measured using a free-form shape which encompassed the same
anatomical boundaries used for tyrosine hydroxylase-positive staining, and a similar shape was used to measure the same anatomical area on the non-lesioned side (Fig 2.4). In the studies using immunohistochemistry as the primary outcome, the site of lesion was the substantia nigra. Thus a larger oval shape designated the area to be measured in the striatum, and the same shape was used to measure both striata. All data was represented as a percentage of the intact side.

**Fig 2.4. ImageJ analysis of microglial staining in the nigra and striatum.** Area to be measured delineated by the yellow border in the grayscale image above. In the substantia nigra (A), staining was measured using a free-form shape. In the striatum (B), measurement is taken surrounding the area of microgliosis, if any staining is present. If there is no staining then the area of the striatum is measured as indicated. Mean gray values were measured for both sides of the striatum and nigra.
2.8.3.4 Quantification of nigral and striatal astrocytosis

In the studies where quantitative immunohistochemical analysis was the primary outcome, astrocytosis was measured in the same manner as microgliosis, as detailed in the previous section. Astrocytosis was measured by immunostaining with glial fibrillary acidic protein (GFAP). GFAP-positive staining was measured in the substantia nigra and striatum (see Fig 2.4) on both lesioned and intact sides. All data was represented as a percentage of the intact side.

2.9 GENE EXPRESSION ANALYSES USING QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qRT-PCR)

2.9.1 TISSUE PROCESSING

Animals were sacrificed via decapitation under isoflurane anaesthesia (in 5% O₂) and brains were removed and snap frozen on dry ice. Brains were then stored at -80°C for processing. In order to obtain striatal and nigral tissue at the lesion site, a cryosectioning and micropunching procedure was used. Brains were mounted on a chuck in Tissue-Tek® O.C.T mounting medium (Sigma, Ireland) and coronal brain sections were cut at -20°C on a cryostat. The striatal and nigral sections were cut at thickness of 300 µm and collected on Superfrost™ microscope slides (Fisher Scientific, UK). To obtain tissue, punches were taken proximal to the site of injection of the toxin. This was determined based on (i) coordinates of the injection site and corresponding anatomical location using the rat brain atlas (Paxinos and Watson, 2006) and (ii) evidence of toxin infusion (needle track). Using a 2 mm internal diameter cylindrical puncher (Harvard Apparatus, UK) on a 1 ml syringe, tissue was excised and quickly displaced into RNase-free tubes.
and snap frozen on dry ice. 4-5 tissue punches from serial striatal sections were obtained for RNA isolation and qPCR analyses. An additional 4-5 tissue punches were obtained distal to the initial punches for mass spectrometry analyses of endogenous cannabinoids (Section 2.10). Nigral tissue was obtained in the same manner and 4 tissue punches were excised from serial sections, an area encompassing the substantia nigra \textit{pars reticulata, pars lateralis} and \textit{pars compacta} but not the VTA. In all cases, both the lesioned and non-lesioned (or intact where appropriate) sides of the striatum and nigra were punched.

2.9.2 RNA ISOLATION

Total RNA was extracted from striatal and nigral samples using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Fisher Scientific, Ireland) as described previously (Okine et al., 2014). 354 µl of RA1 lysis buffer containing 1% β-mercaptoethanol (M6250: Sigma-Aldrich, Ireland) was added to approximately 10 mg tissue prior to homogenisation with an Ultra-Turrax Polytron tissue disrupter (Fisher Scientific, Ireland). Homogenates were then transferred to a Nucleospin filter column (purple) and centrifuged at 11,000 x g for 1 min. 350 µl of 70% molecular grade ethanol (E7023: Sigma-Aldrich, Ireland) was added to the lysates and mixed by pipetting up and down 10 times. The samples were then transferred to another set of Nucleospin RNA II columns (blue) and centrifuged at 11,000 x g for 30 seconds to allow RNA bind to the column. Following centrifugation, the columns were placed in a new collection tubes and 350 µl of membrane desalting buffer (MDB, supplied with kit) was added prior to centrifugation at 11,000 x g for 1 min. Genomic DNA was digested using a 10% v/v rDNase solution prepared in DNase reaction buffer (supplied). 95 µl of the
rDNase solution was pipetted directly onto the centre of each column and allowed to stand for 15 mins at room temperature, following which 200 µl RA2 buffer was added to each column prior to 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 followed by centrifugation at 11,000 x g for 30 seconds. The eluent was discarded and 250 µl of RA3 wash buffer was added followed by centrifugation at 111,000 x g for 2.5 mins. The columns were then placed in RNase-free collection tubes and the RNA was eluted by the addition of 40 µl of RNase-free water (W4503: Sigma-Aldrich, Dublin) followed by centrifugation at 11,000 x g for 1 min. The eluted RNA was then stored at -80°C until quantification and reverse transcription.

2.9.3 ASSESSMENT OF RNA QUALITY AND CONCENTRATION

The quantity, purity and quality of RNA were assessed using a MaestroNano Micro-Volume Spectrophotometer (Maestrogen, Ireland). 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 approximately 1.6-2.2 was deemed indicative of pure RNA. Samples within this range were then used for cDNA synthesis. Where possible, samples were equalised to the same concentration of RNA (25 ng/µl) by addition of RNase free water, although in some cases low RNA yield did not allow for this. Equalised samples were then stored at -80°C until reverse transcription.

2.9.4 cDNA SYNTHESIS

For all studies, the amount of RNA transcribed to cDNA was 50 ng and a negative control (containing RNAase-free water) was used as a negative control for reverse
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transcription. cDNA synthesis was performed using Invitrogen Superscript™ reverse transcriptase custom kit (Biosciences, Dun Laoghaire, Ireland) as described previously (Okine et al., 2014). Initially, 1 µl random primers (250 ng/µl, Applied Biosystems) and 1 µl dNTP mix (10mM) was added to the sample containing 50 ng RNA (at a given volume), and made up to a reaction volume of 12 µl. Samples were then incubated for 5 mins to 65°C in a MJ Research Thermocycler (Bio-Rad, Fannin, Dublin, Ireland). 4 µl First Strand buffer (kit provided), 2 µl DTT (kit provided) and 1 µl RNaseOUT (Biosciences, Dun Laoghaire, Ireland) was then added to the sample and further incubation at 37°C for 2 mins. Finally, 1 µl Superscript™ reverse transcriptase was then added to the sample and a final incubation at 50°C for 50 mins, 70°C for 15 mins before cooling to 4°C. Samples were then diluted 1:4 with RNase-free water and stored at -20°C.

2.9.5 Quantitative Real-Time Polymerase Chain Reaction (Q-PCR)

Gene expression of the genes of interest was determined using commercially available TaqMan gene expression assays (Applied Biosystems, UK) containing specific forward and reverse target primers and FAM-labelled MGB probes. GAPDH was used as an endogenous control to normalise gene expression and in all samples (and duplicates) a multiplex reaction was performed simultaneously measuring GAPDH expression using a VIC-labelled MGB probe (Cat #4308313, Applied Biosystems, UK) in addition to the target gene (Table 2.3), as described previously (Okine et al., 2013, Rea et al., 2014). A reaction master mixture was first prepared consisting of 5 µl TaqMan mastermix (Cat #4324018, Applied Biosystems, UK), 1.5 µl RNase-free water, 0.5 µl GAPDH control reagent, 0.5 µl target gene primers, per well. 2.5 µl of sample was pipette in duplicate
onto a MicroAmp® optical 96 well plate (Applied Biosystems, UK). 7.5 µl of the appropriate mastermix then was added to each well, giving a total reaction volume of 10 µl. Non-template controls (NTC) containing mastermix without cDNA was used for each target gene and a non-RNA control sample was also ran, controlling for the possibility of genomic DNA contamination. Plates were then sealed with optical adhesive covers and spun briefly at 1000 x g to ensure all liquid was at the bottom of the well and to eliminate any bubbles from the wells. The PCR reaction took place in an Applied Biosystems StepOne Plus and Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, UK) to a preset run with the following relative quantification cycling programme, Step 1: 50°C for 2 min, 95°C for 10 min, and Step 2: 40 cycles of 95°C for 15 s/ 60°C for 1 min. The level of gene expression was measured by the fluorescence amplification and measured during the annealing and extension phase at 60°C during the programme.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cannabinoid receptors and enzymes</strong></td>
<td></td>
</tr>
<tr>
<td>CB₁</td>
<td>Rn00562880_m1</td>
</tr>
<tr>
<td>CB₂</td>
<td>Rn03993699_s1</td>
</tr>
<tr>
<td>FAAH</td>
<td>Rn00577086_m1</td>
</tr>
<tr>
<td>MAGL</td>
<td>Rn00593297_m1</td>
</tr>
<tr>
<td><strong>Immune cells</strong></td>
<td></td>
</tr>
<tr>
<td>CD11b</td>
<td>Rn00709342_m1</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rn00566603_m1</td>
</tr>
</tbody>
</table>

Table 2.3. TaqMan® gene expression assays
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2.9.6 Analysis of qRT-PCR Data

Amplification plots and copy threshold (Ct) values were examined using Applied Biosystems StepOne Software v2.2 as described previously (Okine et al., 2014). The threshold was detected automatically by the software programme, at the linear exponential phase of the amplification curve, and this was manually adjusted if required (Fig 2.5). Ct values were then exported to Microsoft Excel for analysis. Relative gene expression was calculated using the formula $2^{-\Delta Ct}$. $\Delta Ct$ was determined using the formula $Ct_{\text{target gene}} - Ct_{\text{GAPDH}}$, where the average of Ct values were 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 control mean value. In the timecourse studies (Chapters 3-5) each treated and control sample was represented as a percentage of the mean control value for that timepoint, for example treated and control samples from Day 1 timepoint were expressed as a percentage of the mean control from Day 1. This method allowed us to determine the difference in expression of the target gene in treated animals compared to untreated animals.
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Fig 2.5 Sample amplification plots for (A) GAPDH and (B) CB₂
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2.10 QUANTIFICATION OF ENDOCANNABINOID AND N-ACYLETHANOLAMINE LEVELS USING LIQUID CHROMATOGRAPHY-TANDEMS MASS SPECTROMETRY (LC-MS/MS)

2.10.1 TISSUE PROCESSING

Striatal tissue was obtained using the protocol described in Section 2.8.1. Tissue punches distal to the site of lesion were used to measure levels of endocannabinoids and N-acylethanolamines in toxin-infused and vehicle-infused (control) striata. The protocol for preparation, quantitation and analysis is described previously (Olango et al., 2012, Kerr et al., 2012).

2.10.2 PREPARATION OF STANDARDS

Non-deuterated stock solutions were prepared in 100% acetonitrile for N-arachidonyl ethanolamide (AEA), 2-arachidonylglycerol (2AG), and N-palmitoyl ethanolamide (PEA) and N-oleoyl ethanolamide (OEA) (Cayman chemicals, Cambridge Biosciences, UK) at concentrations of 2.5 mg/ml for AEA, PEA, OEA and 0.5 mg/ml for 2AG. A single stock solution of all the above standards was then prepared containing 2AG 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, 2AG-d8, PEA-d4 and OEA-d2 and (Cayman Chemicals, Cambridge Biosciences, UK) were prepared in acetonitrile, each at a concentration of 100 µg/ml. From these stocks, a deuterated homogenising buffer was prepared containing 2AG-d8 at 100 ng/400 µl and AEA-d8, PEA-d4 and OEA-d2 at 5 ng/400 µl. The deuterated homogenizing buffer was then dispensed into 20 ml aliquots and stored with non-deuterated standards at -80°C.
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2.10.3 Preparation of standard curve and samples

A 10 point standard curve was prepared in acetonitrile by carrying out a 4 fold serial dilution of the undeuterated standard giving a concentration range of 71.5 fg to 18.75 ng for AEA, PEA and OEA and 187.5 fg to 715 fg for 2AG. 400 µl of the deuterated homogenising buffer was then added to each point of the standard curve.

2.10.4 Quantitation of endocannabinoid and N-acylethanolamine levels

Quantitation of the concentration of endocannabinoids and N-acylethanolamines was carried out essentially as previously described (Ford et al., 2011). Pre-weighed frozen tissue (~10 mg) was carefully homogenised in 400 µl of deuterated homogenising buffer, using a sonicator (Branson, UK). Following this, homogenates were centrifuged at 14,000 x g for 15 mins at 4°C and the supernatant (360 µl) was removed using a positive displacement pipette and evaporated to dryness along with the standard curve in a centrifugal evaporator (Thermo SPD131DDA-230, Fischer Scientific, Ireland). Lyophilised samples and standards were resuspended in 40 µl 65% acetonitrile and 2 µl were injected onto a Zorbax® C18 column (150 × 0.5 mm internal diameter) from a cooled autosampler maintained at 4°C. Mobile phases consisted of A (HPLC grade water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), maintained at a flow rate of 12 µl/min. Analytes were eluted under gradient elution (Table 2.4) and the total run time (per sample) was 30 min.
Table 2.4. Gradient used to elute analytes

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%B (CH$_3$CN, 0.1% formic acid)</th>
<th>%A (H$_2$O, 0.1% formic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>20.1</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>30</td>
<td>STOP</td>
<td></td>
</tr>
</tbody>
</table>

AEA, 2AG, PEA and OEA eluted at the following retention times: 11.4 min, 12.9 min, 14.4 min and 15.0 min, respectively. Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies, UK). Instrument conditions and source parameters including fragmentor voltage and collision energy were optimised for each analyte of interest prior to assay of samples.

2.10.5 QUANTIFICATION OF ANALYTES

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. In MRM mode the first quadrupole mass filter was set to allow only ions of the target mass (parent ions) to pass through into the second quadrupole collision cell where they collide with gas molecules producing product ions and neutral fragments. The third quadrupole was set to mass filter product ions produced in the collision cell, namely daughter ions. Thus retention times in combination with parent---daughter transition allowed the unique
identification of each analyte and its corresponding deuterated internal standard (Table 2.5).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Parent—Daughter transitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEA</td>
<td>348.3-------62.1</td>
</tr>
<tr>
<td>AEA(D8)</td>
<td>356.3-------63.1</td>
</tr>
<tr>
<td>2AG</td>
<td>379.3-------287.2</td>
</tr>
<tr>
<td>2AG(D8)</td>
<td>387.3-------294.2</td>
</tr>
<tr>
<td>PEA</td>
<td>300.3-------62.1</td>
</tr>
<tr>
<td>PEA(D4)</td>
<td>304.3-------62.1</td>
</tr>
<tr>
<td>OEA</td>
<td>326.0-------62.1</td>
</tr>
<tr>
<td>OEA(D2)</td>
<td>328.3-------62.1</td>
</tr>
</tbody>
</table>

Table 2.5. Parent-daughter transitions. Mass charge ratio (m/z) for deuterated and non-deuterated analytes.

2.10.6 Analysis of Analyte Concentrations

Quantification of each analyte was performed by determining the peak area response of each analyte against its corresponding deuterated internal standard (Fig 2.6A & B). This ratiometric analysis was performed using Masshunter Quantitative Analysis Software (Agilent Technologies, UK). The amount of analyte in unknown samples was calculated from a standard curve of Relative response vs. Relative concentration for each analyte i.e. (Peak Area analyte_{(undeuterated)}/Peak area analyte_{(deuterated)}) vs (Conc analyte_{(undeuterated)}/Conc analyte_{(deuterated)}). The limit of quantification was 1.32 pmol/g, 12.1 pmol/g, 1.5 pmol/g, 1.4 pmol/g for AEA, 2AG, PEA and OEA respectively.
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**Fig 2.6A.** Plot of Relative Response vs. Relative Concentration for 2-AG.

**Fig 2.6B.** Chromatograms of each analyte - anandamide, 2-AG, PEA and OEA
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2.11 STATISTICAL ANALYSIS

All data was expressed as mean ± standard error of the mean. One-way ANOVA was used to compare the mean of more than two groups on one factor, with post hoc Bonferroni analysis used to determine where the difference between groups lay in neuroprotection studies (Chapter 6). All other post mortem data was statistically analysed using two-way ANOVA to compare the means of two or more groups on two factors simultaneously, with post hoc Bonferroni analysis (Chapter 3-5). Behavioural data was measured using a two-way ANOVA repeated measures (with within subject-subject factor of time and between subject factor of group) in chronic neuroprotection studies (Chapter 6) and using a two-way ANOVA with post hoc Bonferroni test in studies where intermittent testing was performed (Chapters 3-5). Results were deemed significant if \( P < 0.05 \). Statistical analyses were performed using GraphPad Prism v5.0.
Chapter 3: Characterisation of temporal CB$_2$ receptor expression in a genetic model of Parkinson’s disease
3.1 INTRODUCTION

Parkinson’s disease is a severe and debilitating neurodegenerative disorder that is characterised by the pathological, progressive loss of nigrostriatal dopamine neurons and loss of striatal dopamine. This manifests clinically as a motor syndrome with symptoms including bradykinesia, resting tremor, rigidity and postural instability which reflects impairment in basal ganglia circuitry due to dopamine depletion. Another key characteristic of Parkinson’s disease is neuroinflammation. There is considerable evidence to suggest that inflammation is a key component in the pathophysiology of Parkinson’s disease – post mortem data, risk factor analyses and epidemiological studies all support the involvement of the immune system in disease pathogenesis (Hirsch and Hunot, 2009). Indeed, it has been suggested that inflammation may contribute to neurodegeneration and form a ‘self-sustaining’ cycle of neuroinflammatory and neurodegenerative events which accelerates the disease course (Tansey and Goldberg, 2010). Addressing neuroinflammation may therefore have some clinical benefit in slowing the disease course and in addition it may improve the efficacy of current or potential neurorestorative therapies.

In recent years, the cannabinoid type-2 receptor (CB$_2$) (Munro et al., 1993) has emerged as a potential anti-inflammatory target as it is highly upregulated in neurodegenerative diseases including Parkinson’s disease (Palazuelos et al., 2009, Benito et al., 2003, Yiangou et al., 2006, Garcia et al., 2015, Gómez-Gálvez et al., 2015) where it is
primarily expressed on microglial cells. The brain’s resident immune cells, microglia, are at a high density in midbrain (Lawson et al., 1990), and activated microglia have been identified in the substantia nigra of Parkinson’s disease patients post mortem (McGeer et al., 1988).

Thus targeting the CB$_2$ receptor to reduce inflammation in Parkinson’s disease may represent a viable therapeutic option, however much is still unknown about the role of this receptor in the pathogenesis of Parkinson’s disease and associated inflammatory events. In order to facilitate the development and testing of drugs targeting this receptor, preclinical evaluation of CB$_2$ receptor expression in relevant animal models of Parkinson’s disease is crucial. There are many well-characterised animal models of Parkinson’s disease, each of which recapitulates certain aspects of the clinical condition. In particular, viral-mediated over-expression of the α-synuclein protein in the midbrain of animals produces a disease phenotype that closely resembles that of the clinical condition including α-synucleinopathy, progressive dopaminergic degeneration and motor dysfunction (Decressac et al., 2011, Decressac et al., 2012a, Decressac et al., 2012b, Kirik et al., 2002a) as well as microglial activation (Chung et al., 2009, Theodore et al., 2008, Harms et al., 2013, Sanchez-Guajardo et al., 2010).

Given this robust preclinical phenotype, the aim of this chapter was to assess the temporal expression of the CB$_2$ receptor as well as other components of the endocannabinoid system in a viral-mediated α-synuclein model of Parkinson’s disease, and to compare these to the temporal pattern of microglial upregulation.
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3.2 METHODS

All methods are described in detail in Chapter 2.

3.2.1 EXPERIMENTAL DESIGN

The work presented in this chapter assessed the temporal expression of the CB$_2$ receptor in a genetic model of Parkinson’s disease. Animals were injected with an adeno-associated viral vector (AAV) expressing either human wild-type α-synuclein (α-syn) or green fluorescent protein (GFP), the latter of which served as a control, into the substantia nigra (AP -5.3, ML -2.0, DV -7.2). In order to assess any motor dysfunction as a result of injection of either virus, animals underwent the Stepping, Whisker and Corridor tests, and were subsequently sacrificed for qRT-PCR analyses of microglial and astrocytic markers, CB$_2$ receptor expression and expression of other endocannabinoid system genes at 8, 12 and 16 weeks post-surgery (AAV-α-synuclein: n=10; AAV-GFP: n=8 per time-point). In addition, one rat per group per timepoint was sacrificed for qualitative visualisation of α-synuclein expression and neuroinflammation in the substantia nigra and striatum.
3.3 RESULTS

3.3.1 UNILATERAL, INTRA-NIGRAL AAV-ALPHA-SYNUCLEIN INDUCES CONTRALATERAL MOTOR IMPAIRMENT

Before embarking on temporal qRT-PCR analyses (which was the main focus of this research), we first sought to determine if AAV-α-synuclein led to any impairment in motor function in the rats. One of the key elements of a Parkinson’s disease animal model is the induction of motor impairment following intra-cerebral delivery of a particular toxin, and in the case of unilateral administration the motor impairment is restricted to one side of the body which is contralateral to the side of the brain injected with the toxin. In line with previous studies, unilateral, intra-nigral, viral-mediated delivery of human wild-type α-synuclein induced motor impairment on the side of the body contralateral to the side of infusion (Fig 3.1). Animals that received AAV-α-synuclein into the substantia nigra displayed lateralised motor impairments in the Stepping test of forelimb akinesia (Group, $F_{(1,123)}=28.10$, $P<0.001$), the Whisker test of sensorimotor integration (Group, $F_{(1,122)}=25.12$, $P<0.0001$) and the Corridor test of sensorimotor neglect (Group, $F_{(1,124)}=16.82$, $P<0.0001$). Animals that received AAV-GFP into the substantia nigra did not display any lateralised motor impairment.
Fig 3.1. AAV-α-synuclein-induced contralateral motor dysfunction. Unilateral administration of AAV-α-synuclein into the substantia nigra induced contralateral motor deficits in the Stepping, Whisker and Corridor tests. Data are shown as mean ± SEM, n= 24-29 per group at 8 wk, n= 16-19 at 12 wk, n=8-10 rats at 16 wk. **P<0.01, ***P <0.001 vs. ipsilateral by two-way ANOVA with post hoc Bonferroni.
3.3.2 QUALITATIVE VISUALISATION OF ALPHA-SYNUCLEIN IN THE NIGROSTRIATAL PATHWAY

Once behavioural analyses were complete, we then went on to confirm α-synuclein protein expression in the nigrostriatal pathway after AAV-mediated delivery. Qualitative immunostaining for human α-synuclein at each timepoint revealed that intra-nigral delivery of AAV-α-synuclein, but not AAV-GFP, induced widespread expression of the human wild-type α-synuclein protein unilaterally in the midbrain (Fig 3.2; 16 weeks post-surgery is shown in the image).

Fig 3.2. Immunohistochemical staining for α-synuclein in the nigrostriatal pathway 16 weeks after AAV-α-synuclein infusion. Animals that received intra-nigral AAV-α-synuclein had extensive α-synuclein protein expression in the midbrain (A). Animals that received AAV-GFP did not display any α-synuclein-positive staining (B). All injections were on the right side. Scale bar = 2 mm.
3.3.3 **QUALITATIVE VISUALISATION OF NEUROINFLAMMATION IN THE NIGROSTRIATAL PATHWAY**

In addition to visualisation of α-synuclein expression, we also completed immunostaining for glial cells to determine if α-synuclein led to any visible neuroinflammation in the substantia nigra or striatum (Fig. 3.3). Despite the pronounced α-synuclein expression in the nigrostriatal pathway, this did not lead to any overt change in the density of microglia or astrocytes in either brain region at any timepoint.
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Fig 3.3. Immunohistochemical staining for microglia and astrocytes in the nigrostriatal pathway 16 weeks after AAV-α-synuclein infusion. Unilateral infusion of AAV-α-synuclein did not lead to any overt changes in the density of (A) OX42 (microglia) or (B) GFAP (astrocytes) immunostaining in either the substantia nigra or striatum. Scale bar = 2 mm. All injections were on the right side.
3.3.4 **AAV-ALPHA-SYNUCLEIN INDuces A REDUCTION IN MICROGLIAL MARKER GENE EXPRESSION**

Having completed the behavioural and qualitative immunohistochemical analyses, we then went on to assess and compare temporal changes in the expression of neuroinflammatory markers, and to compare these with changes in expression of endocannabinoid system genes.

We first assessed temporal changes in expression of the microglial marker, CD11b, and the astrocyte marker, GFAP, in nigral and striatal tissue by qRT-PCR (Fig. 3.4). Surprisingly, this revealed an overall reduction in mRNA levels of CD11b and GFAP in the substantia nigra of AAV-α-synuclein animals compared to control (AAV-GFP) (CD11b: Group, $F_{(1,42)}=8.87$, $P<0.01$; GFAP: Group, $F_{(1,41)}=5.39$, $P<0.05$), although post hoc analyses revealed that this was not significant at any particular timepoint. In contrast, CD11b expression remained unchanged in the striatum but GFAP was significantly elevated in the striatum (Group, $F_{(1,44)}=6.24$, $P<0.05$), although post hoc analyses revealed that this was not significant at any particular timepoint.
Fig 3.4. qRT-PCR analyses of microglial and astrocyte markers after AAV-α-synuclein infusion. In the substantia nigra (A), AAV-α-synuclein infusion led to an overall reduction in expression of the microglial marker CD11b as well as the astrocyte marker, GFAP. In contrast, in the striatum (B), CD11b expression remained unchanged while GFAP was elevated. ‘a’ indicates an overall group effect. Data are expressed as mean ± SEM.

3.3.5 CANNABINOID RECEPTOR 2 (CB2) GENE EXPRESSION IS REDUCED IN THE SUBSTANTIA NIGRA OF AAV-ALPHA-SYNUCLEIN-TREATED ANIMALS

Assessment of CB1 and CB2 receptor gene expression revealed changes in the expression patterns between the two receptors in this model (Fig. 3.5). Specifically, CB1 receptor expression was not altered in the substantia nigra or the striatum, whereas CB2 receptor expression was significantly reduced in the substantia nigra (Group,
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$F_{(1,40)}=5.571$, $P<0.05$), although post hoc analyses did not indicate a significant difference between treatment and control groups at any particular timepoint.

Fig 3.5. qRT-PCR analyses of cannabinoid receptors after AAV-α-synuclein infusion. Although CB$_1$ receptor expression in the substantia nigra (A) or striatum (B) was unaffected by AAV-α-synuclein infusion CB$_2$ gene expression was significantly reduced in the substantia nigra (A). ‘a’ indicates an overall group effect. Data are expressed as mean ± SEM.

3.3.6 AAV-ALPHA-SYNUCLEIN DOES NOT ALTER CANNABINOID ENZYME GENE EXPRESSION IN THE NIGROSTRIATAL PATHWAY

In addition to endocannabinoid receptors, we also assessed expression of endocannabinoid degrading enzymes as additional markers of the endocannabinoid system in this model (Fig. 3.6). This revealed that AAV-α-synuclein did not alter
mRNA levels of either the anandamide degrading enzyme, FAAH, or the 2-AG degrading enzyme, MAGL, in the substantia nigra or the striatum.

Fig 3.6. qRT-PCR analyses of endocannabinoid degrading enzymes after AAV-α-synuclein infusion. AAV-α-synuclein infusion did not lead to any changes in mRNA levels of either FAAH or MAGL. Data are represented as mean ± SEM.
3.4 DISCUSSION

This first chapter sought to investigate the temporal expression of the CB$_2$ receptor in an $\alpha$-synuclein-driven genetic model of Parkinson’s disease. To do so, animals received a unilateral, intra-nigral injection of an AAV virus carrying human wild-type $\alpha$-synuclein or GFP as a control. We assessed lateralised motor impairment, $\alpha$-synuclein expression, markers of immune cells and cannabinoid gene expression over a timecourse of up to 16 weeks post-surgery. We found that, in line with expectations, delivery of AAV-$\alpha$-synuclein induced overexpression of the $\alpha$-synuclein protein expression in the nigrostriatal pathway which led to impairment in contralateral motor function. However, $\alpha$-synuclein overexpression did not cause any overt (i.e. visible by immunostaining) cellular neuroinflammation in either the substantia nigra or striatum, but was associated with a reduction in both microglia and astrocyte transcripts in the substantia nigra, and conversely an elevation of astrocytic gene expression in the striatum. Interestingly, in parallel with the decrease in CD11b and GFAP expression, CB$_2$ receptor expression was also downregulated in the substantia nigra of AAV-$\alpha$-synuclein rats. Thus, this study has shown that CB$_2$ receptor expression is dysregulated in a genetic model of Parkinson’s disease.

The AAV-$\alpha$-synuclein model of Parkinson’s disease induces a relatively mild and variable phenotype characterised by development of $\alpha$-synuclein pathology and variable nigrostriatal cell loss, which precipitates a highly variable degree of motor decline (Kirik et al., 2002a, Decressac et al., 2012a). Thus, although widely accepted as the most relevant model of Parkinson’s disease available to preclinical researchers
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(Lindgren et al., 2012), it is limited by its unpredictability and variability. In terms of the neuroinflammatory response to AAV-driven α-synuclein expression, relative to other acute neurotoxic models, this model also induces a mild neuroinflammatory profile with subtle changes in glial morphology rather than overt gliosis per se (Sanchez-Guajardo et al., 2010, Watson et al., 2012). Moreover, there is also evidence to suggest that the microglial response to viral-driven overexpression of α-synuclein can vary depending on the degree of neuronal pathology and the level of cell death (Sanchez-Guajardo et al., 2010). Indeed, the relationship between microglia and α-synuclein is complex and not fully understood (reviewed in Sanchez-Guajardo et al., 2015). Given these findings, it was not surprising that qualitative immunohistochemistry for microglia and astrocytes did not reveal any overt neuroinflammatory lesion in the substantia nigra or striatum in this model. However, what was somewhat surprising was the finding of an overall decrease in the expression of CD11b and GFAP transcripts in the substantia nigra with a contradictory increase in GFAP expression in the striatum. Whether or not this reflects a decrease/increase in microglia and astrocyte numbers in the relevant anatomical region cannot be addressed without quantitative histological analyses, which was beyond the scope of the present study. Interestingly, the $CD11b$ gene encodes the CD11b receptor which is part of the complement receptor 3 complex (CR3) and this is critically involved in phagocytic microglial activity (Ross and Vetvicka, 1993). Since α-synuclein has previously been shown to alter the phagocytic function of microglia (Park et al., 2008, Roodveldt et al., 2010), the reduction in $CD11b$ gene expression observed in this study may also reflect such α-synuclein-mediated alterations in microglial phagocytic functionality. The reasons for the downregulation of nigral, and upregulation of striatal, GFAP mRNA
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observed over this timecourse study are not known, however, it is worth considering, that clinical evidence suggests that astrogliosis is highly variable in Parkinson’s disease patients and much more reserved than what has been observed in some experimental models (reviewed in Bruck et al., 2016).

The main purpose of this study however, was to determine if there were any changes in CB$_2$ receptor expression in the brain in response to AAV-driven, pathological $\alpha$-synuclein expression. The main quantitative method used in this study was qRT-PCR to assess expression of inflammatory and cannabinoid genes. This method was chosen instead of immunolabelling-based protein detection as, although CB$_2$ receptor expression has been detected in the diseased brain, the validity of commercially-available antibodies remains contentious due to non-specificity (Atwood and Mackie, 2010, Baek et al., 2013, Marchalant et al., 2014). Therefore, in order to accurately study any possible changes in CB$_2$ receptor expression induced by overexpression of $\alpha$-synuclein, we chose to use qRT-PCR. This also allowed us to assess multiple other aspects of the endocannabinoid system. Overall, there was no change in expression of the CB$_1$ receptor or expression of the anandamide degrading enzyme, FAAH, or the 2-AG degrading enzyme, MAGL. However, there was a decrease in expression of the CB$_2$ receptor in the substantia nigra. Given that CB$_2$ receptors are primarily associated with microglia in the brain, this reduction in CB$_2$ expression could simply reflect the decrease in microglia indicated by the reduction in CD11b transcripts. Alternatively it could reflect a pathological or compensatory response to $\alpha$-synuclein expression. Interestingly, only one previous study has investigated changes in CB$_2$ receptor expression in a genetic model of Parkinson’s disease (Palomo-Garo et al., 2016). In this
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study, transgenic mice overexpressing human mutated LRRK2 did not display altered CB$_2$ gene expression at any age (6, 12, 18 months) and other endocannabinoid system elements also remained unaltered in this model. However, despite an absence of overt neurodegeneration or neuroinflammation, LRRK2 animals exhibited poorer motor performance than wild-type mice which was ameliorated by chronic treatment with selective CB$_2$ receptor agonist HU308. This illustrates that even in a mild Parkinsonian disease phenotype, pharmacological targeting of the CB$_2$ receptor can ameliorate motor symptoms, although the mechanisms and substrates involved are still unknown.

In summary, the results presented in this chapter indicate that in this highly relevant, but mild and variable, AAV-α-synuclein rat model of Parkinson’s disease, α-synuclein overexpression led to a complex neuroinflammatory phenotype in which markers for microglia and astrocytes were reduced in the substantia nigra, while markers for astrocytes were increased in the striatum. In parallel with the decrease in microglial marker expression in the nigra, the only element of the endocannabinoid system (of those that were investigated) that was found to be altered in this region, was CB$_2$ receptor expression (which was decreased). This suggests that CB$_2$ receptor is dysregulated in response to pathological overexpression of the Parkinson’s disease-related protein α-synuclein. However, given the variability of the AAV-α-synuclein-induced phenotype and the relative mildness of its neuroinflammatory profile, the remainder of this thesis will focus on the temporal relationship between neuroinflammation and CB$_2$ expression in models with a more robust neuroinflammatory profile.
Chapter 4: Characterisation of the endocannabinoid system in neurotoxic models of Parkinson’s disease
4.1 INTRODUCTION

Neuroinflammation is a common feature of all preclinical animal models of Parkinson’s disease, as well as being present clinically; however the inflammatory profile can vary considerably from mild and transient to severe and sustained depending on the nature of the Parkinsonian agent. Preclinical modelling of Parkinson’s disease is an area of ongoing research and refinement and the majority of our knowledge of preclinical Parkinsonism has been drawn from studies using neurotoxins. The first of these animal models to study Parkinson’s disease were developed using neurotoxins such as the catecholaminergic toxin, 6-hydroxydopamine (Akiyama and McGeer, 1989, Depino et al., 2003), MPTP (Liberatore et al., 1999, Hurley et al., 2003b, McGeer et al., 2003, Barcia et al., 2004), and the organic pesticide, rotenone (Sherer et al., 2003a, Gao et al., 2002a), which both induce an immune response including microglial activation. These neurotoxic models also replicate many cardinal features of the clinical Parkinsonian phenotype, including neurodegeneration and motor dysfunction. These models have been well-characterised and utilised in the majority of studies examining the etiology and pathogenesis of Parkinson’s disease, as well as to examine the potential therapeutic benefits of emerging therapies.

6-hydroxydopamine is a catecholaminergic toxin that can induce selective degeneration of dopaminergic neurons in the nigrostriatal pathway, via the production of reactive oxygen species and quinones (Cohen, 1984). Downstream of these effects, 6-
hydroxydopamine has been shown to act via multiple pathways including the PI3K/Akt pathway to inhibit Nrf2-mediated anti-oxidant activity (Hanrott et al., 2008) and phosphorylation of JNK (c-Jun N-terminal kinases), p38 MAPK (mitogen-activated protein kinase) and ERK 1/2 (extracellular signal-related kinase) (Kulich et al., 2007, Fan et al., 2014) in addition to the activation of caspases (Choi et al., 1999). This ultimately induces dopaminergic cell death and activation of the immune system including microglial activation. 6-hydroxydopamine has been used to model Parkinson’s disease for almost 50 years (Ungerstedt, 1968) and is the most studied model as it produces a highly reproducible lesion. It is also useful as the degree of dopaminergic degeneration can be adjusted depending on the site of the injection. For example injection of 6-hydroxydopamine into the striatum induces a progressive but moderate degeneration (Kirik et al., 1998), whereas administration into the medial forebrain bundle (MFB) induces rapid and complete loss of dopaminergic neurons in the nigrostriatal pathway (Sauer and Oertel, 1994) and induces stable motor deficits.

Rotenone is an organic pesticide that inhibits Complex I of the electron transport chain in mitochondria and induces degeneration via oxidative stress mechanisms in dopaminergic neurons, including NADPH oxidase mediated production of superoxide free radicals and subsequent microglial activation (Gao et al., 2002b). Rotenone neurotoxicity is associated with multiple pathways including AMPK (AMP-activated protein kinase) and AKt (protein kinase B) signalling, (Xu et al., 2014) activation of GSK-3β (Hongo et al., 2012), MAPK signalling (Kamalden et al., 2012), and enhanced caspase activation (Sonia Angeline et al., 2012). Exposure to this pesticide has been found to increase susceptibility of developing Parkinson’s disease, and as such it is
often used to produce an environmental model of Parkinson’s disease (Priyadarshi et al., 2000, Firestone et al., 2005, Petrovitch et al., 2002, Betarbet et al., 2000). Initially systemic rotenone was found to induce Parkinsonian symptoms (Betarbet et al., 2000), however many alternative models using rotenone have been developed that are devoid of the systemic toxicity of the earlier approaches. These include stereotaxic infusion of rotenone into the striatum and substantia nigra which induces dopaminergic degeneration and motor deficits (Xiong et al., 2009, Mulcahy et al., 2011, Naughton et al., 2016).

These models recapitulate several aspects of Parkinson’s disease, including neuroinflammation, which makes them valid models for examining the relationship between CB$_2$ receptor expression and neuroinflammation. To date there have been no published reports on changes in CB$_2$ receptor expression in neurotoxic models of Parkinson’s disease, although there has been a report of a reduction in CB$_1$ receptor expression in terminal (striatal) 6-hydroxydopamine-lesioned rats (Walsh et al., 2010). For this reason, in order to fully evaluate the potential of the CB$_2$ receptor as a target for disease modification in Parkinson’s disease, any changes in expression of the receptor should be characterised in these widely-used neurotoxic models.

As such, the aim of this chapter was to assess the temporal expression of the CB$_2$ receptor (and other elements of the endocannabinoid system) and to relate this to temporal changes in neuroinflammatory markers, following intra-striatal infusion of the classic Parkinsonian neurotoxin, 6-hydroxydopamine, and the Parkinson’s disease-associated environmental pesticide, rotenone.
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4.2 METHODS

All methods have been described in more detail in Chapter 2.

4.2.1 EXPERIMENTAL DESIGN

Sixty-four adult male Sprague Dawley rats were used in this experiment. These first underwent behavioural habituation and baseline testing on the Stepping, Whisker and Cylinder test prior to surgery. Animals then received an intra-striatal stereotaxic infusion of 6-hydroxdopamine (10 µg in 2 µl in 0.02% ascorbate saline), rotenone (1.25 µg in 2 µl in DMSO:cremaphor: saline (1:1:18)) or their corresponding vehicles (2 µl of 0.02% ascorbate saline or 2 µl of DMSO:cremaphor:saline respectively). Behavioural testing was performed on day 7, 14 and 28 post-surgery. Animals were sacrificed on Days 1, 4, 14 and 28 post-surgery by decapitation under isoflurane anaesthesia for qRT-PCR and LC–MS/MS analyses (n=7, per timepoint), or transcardial perfusion-fixation under terminal pentobarbital anaesthesia for qualitative immunohistochemical analyses (n=1, per timepoint). As endocannabinoids are released on demand, sacrifice of the animals was carried out ~ 3 hours post-behavioural testing, to ensure any changes in striatal gene expression or endocannabinoid levels was not attributable to force motor behaviours. Given the low basal expression of the CB2 receptor in the brain, we chose to specifically examine CB2 receptor expression and inflammation at the lesion site, as to do so using the entire striatum could potentially dilute CB2 expression and mask any changes in these models. To do so, tissue was micropunched proximal to the site of
infusion for qRT-PCR analyses and tissue distal to this was micropunched for mass spectrometry analyses of endocannabinoid levels.

4.3 RESULTS

4.3.1 UNILATERAL, INTRA-STRIATAL 6-HYDROXYDOPAMINE INDUCES CONTRALATERAL MOTOR DYSFUNCTION

In order to assess the impact of unilateral administration of either 6-hydroxydopamine or rotenone, animals underwent a battery of tests to measure lateralised motor impairment during the experimental period. As expected, intra-striatal administration of the catecholaminergic neurotoxin, 6-hydroxydopamine, induced a pronounced motor impairment in motor function on the side of the body contralateral to the side of toxin infusion in the Stepping Test of forelimb akinesia (Group, $F_{(1,74)}=311.40, P<0.0001$), the Whisker test of sensorimotor integration (Group, $F_{(1,74)}=317.50, P<0.0001$), and the Cylinder test of forelimb use (Group, $F_{(1,74)}=194.50, P<0.0001$) (Fig. 4.1). Somewhat surprisingly, intra-striatal delivery of rotenone did not induce any contralateral motor impairment in any of the behavioural tests performed.
Fig. 4.1. Assessment of lateralised motor impairment following intra-striatal delivery of 6-hydroxydopamine and rotenone. Unilateral administration of 6-hydroxydopamine into the striatum induced contralateral motor deficits in the Stepping, Whisker and Cylinder Tests (A). Unilateral administration of rotenone did not induce any significant contralateral motor impairment in any of the behavioural tests. Data are shown as mean ± SEM with n=7 rats per group. 

\( ***P<0.001 \) vs. Vehicle by 2 way ANOVA with post hoc Bonferroni. 6-OHDA, 6-hydroxydopamine.
4.3.2 QUALITATIVE VISUALISATION OF NEUROINFLAMMATION IN THE TOXIN-INFUSED STRIATUM

Before embarking on the main focus of these studies (qRT-PCR of inflammatory and endocannabinoid genes), qualitative assessment of the neuroinflammatory response was performed using immunohistochemistry (Fig. 4.2). Initially, striatal neuroinflammation was visualised using qualitative OX42 immunohistochemistry for microglia. This revealed a pronounced microgliosis in the infused striatum after injection of both 6-hydroxydopamine and rotenone. Similarly, GFAP immunohistochemistry for astrocytes revealed partial astrocytosis in the 6-hydroxydopamine-infused striatum, and similarly in the rotenone-infused striatum.
Fig. 4.2. **Qualitative immunohistochemistry of microglia and astrocytes in the striatum.** Unilateral administration of (A) 6-hydroxydopamine and (B) rotenone induced a pronounced striatal neuroinflammation illustrated by microgliosis and astrocytosis in the toxin-infused side. Images illustrate injection of toxin on the right side, OX42-positive staining at Day 14 and GFAP-positive staining at Day 28 for both 6-hydroxydopamine and rotenone are shown. Scale in lower magnification photomicrographs = 2mm.
4.3.3 6-HYDROXYDOPMAINE AND ROTENONE INDUCED AN UPREGULATION OF MICROGLIAL MARKER GENE EXPRESSION

Striatal neuroinflammation was quantitatively assessed using qRT-PCR to measure levels of the microglial marker CD11b, and the astrocyte marker, GFAP. Gene expression analyses revealed an upregulation in CD11b mRNA levels at the 6-hydroxydopamine and rotenone infusion site. Upregulation of CD11b was most pronounced at Day 14 post-surgery, following either 6-hydroxydopamine (Group x Time, $F_{(3,45)}=19.16, P<0.0001$; post hoc Bonferroni confirmed significant difference at Day 14 with $P<0.001$) or rotenone (Group x Time, $F_{(3,38)}=2.50, P<0.05$; post hoc Bonferroni confirmed significant difference at Day 14 with $P<0.01$) infusion. Interestingly, upregulation of GFAP was observed in the 6-hydroxydopamine infusion site, most pronounced at Day 28 post-surgery (Group x Time, $F_{(3,44)}=4.16, P<0.05$; post hoc Bonferroni confirmed significant difference at Day 14 with $P<0.001$), whereas there was no significant upregulation of GFAP observed at the site of rotenone infusion in the striatum.
Fig. 4.3. qRT-PCR analyses of microglial and astrocyte markers in the striatum. Unilateral administration of (A) 6-hydroxydopamine or (B) rotenone induced a pronounced upregulation of the microglial marker CD11b and the astrocyte marker, GFAP at specific timepoints. Data are represented as mean ± SEM with n = 6-7 rats per group, **P<0.01, ***P<0.001 vs. Vehicle by 2 way ANOVA with post hoc Bonferroni.

4.3.4 6-HYDOXYDOPAMINE AND ROTENONE INDUCED AN ELEVATION IN CB2 GENE EXPRESSION

Following on from the initial neuroinflammatory qRT-PCR analyses, we next addressed expression of cannabinoid genes, namely the CB₁ and CB₂ receptor, and the endocannabinoid degrading enzymes, FAAH and MAGL (Fig. 4.4). We did not observe any changes in gene expression of the CB₁ receptor at the site of infusion of either 6-
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hydroxydopamine or rotenone, and there was also no changes in mRNA transcript levels of FAAH or MAGL following infusion of either toxin. However, we found that CB$_2$ receptor expression was significantly elevated in the striatum following intra-striatal infusion of either 6-hydroxydopamine or rotenone, and was most pronounced at Day 14 post-surgery (6-hydroxydopamine: Group x Time, $F_{(3,44)}=12.62$, $P<0.0001$; *post hoc* Bonferroni confirmed significant difference at Day 14 with $P<0.001$; rotenone: Group, $F_{(1,45)}=4.55$, $P<0.05$; *post hoc* Bonferroni confirmed significant difference at Day 14 with $P<0.05$).
Fig. 4.4. qRT-PCR analyses of endocannabinoid genes in the striatum. Unilateral administration of (A) 6-hydroxydopamine or (B) rotenone induced an increase in CB$_2$ receptor mRNA which peaked at Day 14. CB$_1$ receptor and degrading enzymes, FAAH and MAGL, were unaffected by either 6-hydroxydopamine or rotenone infusion. Data are represented as mean ± SEM with n = 6-7 rats per group, *P<0.05, ***P<0.001 vs. Vehicle by 2 way ANOVA with post hoc Bonferroni.
4.3.5 Correlation between 6-hydroxydopamine- and rotenone-induced neuroinflammation and CB$_2$ receptor expression

Given the similar temporal profile in the upregulation of microglial marker CD11b and CB$_2$ receptor expression and the evidence suggesting the CB$_2$ receptor is located on microglia, we performed correlation analysis to decipher any significant association between the increases in CD11b mRNA and increases in CB$_2$ receptor mRNA (Fig. 4.5). Indeed, we found that the extent of CB$_2$ receptor expression correlated significantly with CD11b expression at the site of 6-hydroxydopamine ($r = 0.88$, $P < 0.0001$) and rotenone infusion ($r = 0.93$, $P < 0.0001$).

Fig. 4.5. Correlation between 6-hydroxydopamine- and rotenone-induced microgliosis and CB$_2$ receptor gene expression. Increased striatal CB$_2$ receptor gene expression correlated significantly with increases in expression of microglial marker, CD11b, after striatal infusion of both 6-hydroxydopamine and rotenone. Data were analysed by linear regression analysis.
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4.3.6 6-HYDROXYDOPAMINE AND ROTENONE INDUCED ALTERATIONS IN ENDOCANNABINOID LEVELS

Given the evidence of endocannabinoid dysregulation in neurodegenerative disease and alterations in CB2 receptor expression evident in the current study, we sought to investigate if either 6-hydroxydopamine or rotenone infusion altered striatal endocannabinoid levels (Fig. 4.6). To do so, we measured striatal levels of the endocannabinoids, anandamide and 2-AG, and the related lipid mediators, PEA and OEA in the striatum, at sites distal to the site of toxin-infusion, by LC-MS/MS. Striatal levels of anandamide and 2-AG were not altered overall by infusion of either 6-hydroxydopamine or rotenone, although anandamide was elevated in the rotenone-infused striatum at Day 4 (Time, $F_{(3,43)}=3.127, P<0.05$, post hoc Bonferroni confirmed significance at Day 4, $P<0.05$). A similar trend for increased striatal anandamide was observed in the 6-hydroxydopamine-infused striatum, however this did not reach statistical significance. Interestingly, both neurotoxins induced an increase in striatal levels of the lipid immune mediators, PEA (6-hydroxydopamine: Time, $F_{(3,48)}=2.910, P<0.05$, post hoc Bonferroni confirmed significance at Day 4, $P<0.05$; rotenone: Time, $F_{(3,44)}=4.578, P<0.01$, post hoc Bonferroni confirmed significance at Day 1, $P<0.01$) and OEA (6-hydroxydopamine: Time, $F_{(3,48)}=3.021, P<0.05$, post hoc Bonferroni confirmed significance at Day 4, $P<0.05$; rotenone: Time, $F_{(3,44)}=3.635, P<0.05$, post hoc Bonferroni confirmed significance at Day 1, $P<0.01$) at specific timepoints.
Fig. 4.6. Mass spectrometry measurement of striatal endocannabinoid levels. Unilateral administration of (A) 6-hydroxydopamine and (B) rotenone did not alter levels of either anandamide or 2-AG in the infused striata overall. Both neurotoxins induced an increase in striatal levels of the N-acylethanolamines, PEA and OEA. Data are represented as mean ± SEM with n = 5-6 rats per group. *P<0.05, **P<0.01 vs. Vehicle by 2 way ANOVA with post hoc Bonferroni. AEA, anandamide.
4.4 DISCUSSION

The studies in this chapter sought to investigate changes in CB\(_2\) receptor expression after neurotoxin-induced neuroinflammation. To this end, we selected neurotoxins that are well established inducers of Parkinsonism in animal models, namely the catecholaminergic neurotoxin, 6-hydroxydopamine, and the organic pesticide, rotenone. We assessed the effects of unilateral intra-striatal administration of each toxin on lateralised motor function and neuroinflammation, as well as changes in endocannabinoid gene expression and striatal endocannabinoid levels. We found that unilateral administration of 6-hydroxydopamine, but not rotenone, induced persistent motor impairment on the side of the body contralateral to the side of toxin-infusion in all of the behavioural tests performed. We also observed a pronounced neuroinflammatory response that was associated with an upregulation in CB\(_2\) receptor expression in both the 6-hydroxydopamine- and rotenone-infused striata, which was most pronounced at Day 14 post-surgery. Dysregulation of the endocannabinoid system was also observed, with rotenone inducing an increase in striatal anandamide at Day 4 post-surgery. In addition elevated levels of lipid immune mediators, PEA and OEA emerged at distinct timepoints in both the 6-hydroxydopamine- and rotenone-infused striata.

Behavioural analyses were performed to confirm that motor impairment was induced by these toxins, as this is an important clinical feature of Parkinson’s disease. Although single site, intra-striatal 6-hydroxydopamine induced a stable motor deficit, rotenone did not. This suggests that rotenone did not induce sufficient neurodegeneration to
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precipitate a motor impairment and may be attributable to the infusion regime used. Previous work has employed a multi-site approach for intra-striatal rotenone administration (Mulcahy et al., 2011). However, in order to compare changes distal and proximal to the rotenone lesion site directly with those of the 6-hydroxydopamine study, it was necessary to use a single-site approach for both. Thus, this probably explains the lack of an overt motor impairment in rotenone-lesioned animals.

Despite this, the findings of both of these studies are very interesting in the context of neuroinflammation and the potential role of the CB$_2$ receptor. Although it has been well established that CB$_2$ receptor expression is upregulated in vitro (Ashton and Glass, 2007, Benito et al., 2008), these studies describe a novel finding of upregulated CB$_2$ receptor expression, strongly correlated with microglial upregulation, in response to striatal infusion of Parkinsonian toxins (Concannon et al., 2015, Concannon et al., submitted). Infusion of 6-hydroxydopamine induced a motor impairment that was associated with a neuroinflammatory response and a concomitant upregulation in CB$_2$ receptor expression. In rotenone-treated animals, evidence of pronounced microgliosis and associated CB$_2$ receptor upregulation emerged despite the absence of any motor deficits. Clearly, the CB$_2$ receptor is involved in the neuroinflammatory response to both an overtly degenerative stimulus (6-hydroxydopamine) and a minimally-damaging toxin (rotenone). Interestingly, changes in GFAP gene expression were only observed in the 6-hydroxydopamine-infused striatum. A delayed astrocytic response has been previously observed in mice and rats in response to 6-hydroxydopamine (Walsh et al., 2011, Stott and Barker, 2014), and the absence of an astrocyte response in rotenone-treated animals, based on gene expression analysis of GFAP mRNA, may be due to the
lack of neurodegeneration in this model. The upregulation of the CB$_2$ receptor and changes in both CD11b and GFAP gene expression may also reflect a regulation of inflammatory cells as overexpression of CB$_2$ has been shown to reduce astrocyte and microglial recruitment to the site of injury following intra-caudate 6-hydroxydopamine administration (Ternianov et al., 2012).

Having observed these interesting temporal changes in the neuroinflammatory and CB$_2$ receptor profile, our next focus was to investigate any temporal changes in other endocannabinoid genes. We did not observe any changes in striatal expression of the CB$_1$ receptor or degrading enzymes, FAAH and MAGL, in either the 6-hydroxydopamine- or rotenone-infused striata. Clinically, dysregulation of the CB$_1$ receptor remains a question with evidence to support and refute this theory (Lastres-Becker et al., 2001, Hurley et al., 2003a, Farkas et al., 2012, Van Laere et al., 2012). Considering the regulatory role of the CB$_1$ receptor in basal ganglia signalling (co-localising with D$_1$ and D$_2$ dopamine neurons on striatal medium spiny neurons, corticostriatal glutamatergic neurons and GABAergic projections from the globus pallidus to the subthalamic nucleus (Hohmann and Herkenham, 2000, Brotchie, 2003, Benarroch, 2007) and the clear, profound motor impairment (indicating dysregulated basal ganglia control of motor function), one would anticipate some alteration in CB$_1$ receptor expression following 6-hydroxydopamine-lesion, as has been identified in more pronounced 6-hydroxydopamine models (Romero et al., 2000), however this was not the case in this study and supports a previous finding of no alteration in striatal CB$_1$ receptor protein expression following intra-striatal 6-hydroxydopamine lesion (Chaves-Kirsten et al., 2013). A reduction in nigral CB$_1$ receptor expression has also been
reported in the terminal (intra-striatal) 6-hydroxydopamine model previously (Walsh et al., 2010), however striatal CB$_1$ receptor expression was not assessed and most likely the non-specific toxic effects of 6-hydroxydopamine on striatonigral projection neurons (non-dopaminergic) which express CB$_1$, may explain this finding. Also, as the work presented in this chapter assessed gene expression at the infusion site, it suggests CB$_1$ receptor is not involved in the initial response to toxin-induced neuroinflammation and neurodegeneration.

In addition to gene expression analyses, we also examined striatal endocannabinoid levels to determine if inflammation due to intra-striatal infusion of either toxin would alter these. Clinically, elevated levels of anandamide have been consistently observed in Parkinson’s disease (Pisani et al., 2005, Pisani et al., 2010) and 2-AG has been shown to be elevated following cerebral injury (Panikashvili et al., 2001). However, we did not observe any overall effect of either 6-hydroxydopamine or rotenone treatment, although anandamide levels were significantly elevated at Day 4 in the rotenone-infused striatum. Considering the absence of any change in gene expression of either FAAH or MAGL, it was not surprising to observe no significant changes in striatal endocannabinoid levels. An elevation in anandamide has been previously reported in the 6–hydroxydopamine-lesioned striatum however this was observed a much later timepoint (3 months), suggesting the endocannabinoid response to neurotoxic insult is delayed (Gubellini et al., 2002). However, the level of neurodegeneration may also affect the degree and mechanism of endocannabinoid dysregulation as severe dopaminergic denervation (~90%) does not alter FAAH activity but does reduce the activity of anandamide-synthesizing enzyme, N-acyltransferase (Fernandez-Espejo et al., 2004). A time-
dependent increase in the N-acylethanolamines, PEA and OEA, was observed in both toxin-infused striata, at Day 4 and Day 1 in 6-hydroxydopamine- and rotenone-treated animals, respectively. Given this early increase in levels in the striatum, this may reflect an anti-inflammatory response associated with increased activation of microglia. This is because both of these lipids are potent anti-inflammatory compounds which have shown anti-inflammatory efficacy in a Parkinson’s disease animal model, most likely acting at non-cannabinoid receptor targets (Esposito et al., 2012, Gonzalez-Aparicio et al., 2013). PEA and OEA can also enhance anandamide activity, via an ‘entourage effect’. Studies have shown that PEA and OEA, which predominantly binds to peroxisome proliferator alpha (PPARα), can enhance anandamide activity via competitive inhibition of FAAH-mediated hydrolysis of anandamide or allosteric binding of the TRPV1 receptor thereby potentiating anandamide binding at CB₁ receptors (reviewed in Hansen, 2010). This may partially explain the discrete temporal elevation in anandamide, PEA and OEA in the rotenone-infused striatum.

The main finding of these studies was that neuroinflammation is associated with an upregulation of CB₂ receptor mRNA following intra-striatal delivery of Parkinsonian neurotoxins, 6-hydroxydopamine and rotenone. This was observed despite the differences in motor dysfunction induced by either toxin, indicating CB₂ may play an important role in inflammatory events in both severe and mild Parkinsonian phenotypes. In comparison to the results detailed in the previous chapter, which described a genetic model that produced a behavioural impairment concomitant with downregulation of nigral microglial marker and CB₂ receptor mRNA, it would appear that the CB₂ receptor undergoes dynamic changes in response to the nature of the toxin or disease-
inducing protein. The most well-explored area of CB$_2$ receptor research has focused on inflammation studies, both in vivo and in vitro (reviewed in Ashton and Glass, 2007) however the role of the CB$_2$ receptor in inflammation with relevance to Parkinson’s disease has been less well explored.

In order to further unravel the role of the CB$_2$ receptor in inflammation-induced neurodegeneration in Parkinson’s disease, given the results presented in this and the previous chapter, we next sought to investigate CB$_2$ receptor expression following striatal infusion of potent inflammagens, both bacterial and viral. The results of these studies are presented in the next chapter.
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5.1 INTRODUCTION

In recent times, the importance of neuroinflammation in the pathogenesis of Parkinson’s disease, as well as the potential role of infection in the etiology of the condition, has garnered much interest. As a result, so too has the area of inflammatory modelling in Parkinson’s disease. Inflammatory stimuli can indirectly induce dopaminergic nigral cell loss and this has prompted the utilisation of inflamagens to induce dopaminergic neuron loss in the nigrostriatal pathway for the development of inflammation-driven models. Although the classic strategies employing intra-cerebral or systemic delivery of neurotoxins have pathological relevance to Parkinson’s disease, many do not fully recapitulate the extensive inflammatory profile that is often observed in the clinical condition. In order to examine the inflammatory hypothesis of Parkinson’s disease, inflammation-driven models have been developed and these include the LPS and Poly (I:C) models.

LPS is a bacterial endotoxin isolated from the outer cell membrane of Gram-negative bacteria which is. A ligand of TLR4, LPS can stimulate the activation and proliferation of microglia, and alter the production of cytokines and chemokines involved in the innate immune response, in addition to generating reactive oxygen species. Toll-like receptors (TLRs) are pattern recognition receptors highly expressed on immune cells that are responsible for recognising pattern-associated molecular pattern (PAMPs) which are present on microbes and there are a number of TLRs, which recognise
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distinct pathogens, for example TLR4 this senses LPS, along with its associated molecule, MD-2 (Poltorak et al., 1998). Activation of TLRs following pathogen binding causes oligomerisation, which induces cell signalling pathways that induce an inflammatory response. In the case of TLR4 stimulation, several pathways are initiated via adaptor molecules including the myeloid differentiation primary gene 88 (MyD88), TIR-domain-containing adaptor-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM) and TIR-domain containing adaptor protein (TIRAP) pathways which are extensively reviewed here (Akira and Takeda, 2004). In particular, activation of MyD88 induces translocation of NFκB and the production and release of proinflammatory cytokines including TNFα, IL-6 and IL-1, and activation of TRIF pathway induces production of type 1 interferons via activation of IRF3 (reviewed in Mogensen, 2009).

Increased susceptibility of dopaminergic neurons to LPS-induced degeneration was first observed by Bronstein and colleagues (1995), an effect which was mediated by microglia. Subsequently, numerous approaches have been employed to induce dopaminergic degeneration using LPS both in vitro (Le et al., 2001, Gao et al., 2002b, Gao et al., 2003, Li et al., 2009b) and in vivo (Castano et al., 1998, Gao et al., 2002b, Choi et al., 2009, Hoban et al., 2013a, Qin et al., 2007). In addition to inducing nigrostriatal dopaminergic loss, motor dysfunction is also observed in LPS-treated animals (Choi et al., 2009, Hoban et al., 2013a). It is also worth noting that both prenatal and neonatal exposure to LPS sensitises dopaminergic neurons to various degenerative stimuli in rat adulthood (Ling et al., 2004, Zhu et al., 2007, Tien et al., 2013, Fan et al., 2011). Thus, the susceptibility of dopaminergic neurons to
inflammation-induced degeneration, as illustrated experimentally in studies with LPS, suggests that inflammation is both pathologically and etiologically significant in Parkinson’s disease.

Another emerging inflammatory model of Parkinson’s disease is that induced by the viral mimetic Poly (I:C). Poly (I:C) is considered a viral mimetic due to its structural similarity to double-stranded RNA viruses, and, like viruses, is a potent inflammatory stimulus acting at TLR3. TLR3 receptor downstream signalling is not mediated via MyD88, as with TLR4, but via TRIF pathway, and subsequently induces the production of type 1 interferons (Tatematsu et al., 2014). Poly (I:C) has been shown to activate microglia which is associated with increased sensitivity to Parkinsonian neurotoxins in animal models (Deleidi et al., 2010, Bobyn et al., 2012). Viral infection has been linked to the development of Parkinsonian pathology in preclinical animal models (Jang et al., 2009, Jang et al., 2012), and clinically in the emergence of postencephalitic Parkinsonism (Takahashi et al., 1995, Shoji et al., 1993). Post mortem data has also revealed immunolocalisation of influenza A virus with macrophages in the substantia nigra of Parkinson’s disease brains (Rohn and Catlin, 2011).

Investigation of the endocannabinoid system in genetic and neurotoxic models of Parkinson’s disease in previous chapters has presented clear evidence of dysregulation of the CB2 receptor, concomitant with changes in microglial marker expression in these varying Parkinsonian paradigms. If we are to consider the CB2 receptor as a potential target for anti-inflammatory disease modification, it is appropriate and essential to assess the endocannabinoid system in inflammation-driven models of Parkinson’s
disease and discern the impact of these models on endocannabinoid system signalling. As the LPS and Poly (I:C) models have improved etiological and pathological relevance over other models in this context, it was pertinent to determine if changes in CB$_2$ receptor expression also occurs in the brain in response to these inflammatory triggers. Thus, the aim of this chapter was to examine the temporal expression profile of the CB$_2$ receptor, in addition to inflammatory and other endocannabinoid changes, following intra-cerebral infusion of LPS or Poly (I:C). This work would serve to contribute to our knowledge of the CB$_2$ receptor in models of Parkinson’s disease, and upon completion of these characterisation studies, to identify the most suitable model for preclinical evaluation of pharmacological targeting of the CB$_2$ receptor.
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5.2 METHODS

5.2.1 EXPERIMENTAL DESIGN

The work presented in this chapter assessed the temporal expression of the CB$_2$ receptor in inflammation-driven models of Parkinson’s disease. Prior to surgery animals (male Sprague Dawley rats) underwent behavioural habituation and baseline testing on the Stepping, Whisker and Cylinder tests. During surgery a unilateral, intra-striatal stereotaxic injection of LPS (10 µg in 2 µl 0.9% saline), Poly (I:C) (20 µg in 4 µl 0.9% saline) or corresponding vehicle was performed. Behavioural testing was performed on day 7, 14 and 28 post-surgery. Animals were sacrificed on Days 1, 4, 14 and 28 post-surgery by decapitation under isoflurane anaesthesia for qRT-PCR and LC–MS/MS analyses (n=7 per group, per timepoint), or transcardial perfusion-fixation under terminal pentobarbital anaesthesia for qualitative immunohistochemical analyses (n=1 per group, per timepoint). Again, sacrifice of the animals was carried out ~3 hours post-behavioural testing, to ensure any changes in striatal gene expression or endocannabinoid levels was not attributable to force motor behaviours. In order to accurately compare the results with that of previous studies, we employed the same approach of specifically examining CB$_2$ receptor expression and inflammation at the lesion site. To do so, tissue was micropunched proximal to the site of infusion for qRT-PCR analyses and tissue distal to this was micropunched for mass spectrometry analyses of endocannabinoid levels.
5.3 RESULTS

5.3.1 Unilateral, intra-striatal LPS or Poly (I:C) induces contralateral motor dysfunction

As described in previous chapters, an important characteristic of a Parkinson’s disease animal model is the induction of motor impairment. In these unilateral models, animals underwent a battery of tests to assess lateralised motor function following stereotaxic surgery. As expected intra-striatal administration of the bacterial inflammagen, LPS, induced a pronounced and stable motor deficit on the side of the body contralateral to the side of infusion in the Stepping test of forelimb akinesia (Group, $F_{(1,64)}=1171.0$, $P<0.0001$), the Whisker test of sensorimotor integration (Group, $F_{(1,64)}=2490.0$, $P<0.0001$), and the Cylinder test of forelimb use (Group, $F_{(1,64)}=22.88$, $P<0.0001$). The emergence of a lateralised motor impairment is also important as it illustrates that motor dysfunction is a consequence of the site-specific delivery of LPS and not due to any LPS-induced sickness behaviour impeding motor performance. Similarly, Poly (I:C) infusion also induced profound motor impairment in the Stepping test (Group, $F_{(1,60)}=56.76$, $P<0.0001$), the Whisker test (Group, $F_{(1,64)}=22.48$, $P<0.0001$), and the Cylinder test (Group, $F_{(1,64)}=87.77$, $P<0.0001$), which provides one of the first reports of Poly (I:C) acting to induce motor impairment without any subsequent toxic challenge.
Fig. 5.1. Assessment of lateralised motor impairment following intra-striatal delivery of LPS and Poly (I:C). Unilateral administration of (A) LPS or (B) Poly (I:C) into the striatum induced contralateral motor deficits in the Stepping, Whisker and Cylinder Tests. Data are shown as mean ± SEM with n=7 rats per group. *P<0.05, **P<0.01, ***P<0.001 vs. Vehicle by 2 way ANOVA with post hoc Bonferroni.
5.3.2 Qualitative visualisation of neuroinflammation in the inflammagen-infused striatum

As both LPS and Poly (I:C) are potent inducers of inflammation, we sought to qualitatively assess neuroinflammation prior to the main quantitative outcome of these studies, namely qRT-PCR for endocannabinoid and inflammatory genes. OX42 and GFAP immunohistochemistry was performed for microglia and astrocytes, respectively (Fig. 5.2). This revealed a pronounced microgliosis in the infused striatum of LPS and Poly (I:C)-treated animals. Overt astrocytosis was not observed in the striata following infusion of either inflammagen.
Fig. 5.2. Qualitative immunohistochemistry of microglia and astrocytes in the striatum. Unilateral administration of (A) LPS and (B) Poly (I:C) induced a pronounced striatal neuroinflammation illustrated by microgliosis in the toxin-infused striata. Images illustrate injection of toxin on the right side, taken at Day 14 for OX42 and Day 28 for GFAP. Scale in lower magnification photomicrographs = 2 mm.
5.3.3 LPS AND POLY (I:C) INDUCED AN UPREGULATION OF MICROGLIAL AND ASTROCYTIC MARKER GENE EXPRESSION

Neuroinflammation in the infused striata was then quantitatively assessed using qRT-PCR to measure levels of CD11b (microglia marker) and GFAP (astrocyte marker) (Fig. 5.3). qRT-PCR analyses revealed that both LPS (Group x Time, $F_{(3,45)}=3.70$, $P<0.05$; post hoc Bonferroni confirmed a significant difference at Day 14 with $P<0.001$) and Poly I:C (Group x Time, $F_{(3,44)}=3.29$, $P<0.05$; post hoc Bonferroni confirmed a significant difference at Day 4 with $P<0.001$) induced a significant upregulation of CD11b receptor mRNA with differing temporal patterns. In addition, both LPS and Poly (I:C) induced an upregulation in GFAP mRNA expression which peaked at Day 14 in the LPS-infused striatum and Day 28 in the Poly (I:C)-infused striatum (LPS: Group x Time, $F_{(3,47)}=15.32$, $P<0.05$; post hoc Bonferroni confirmed a significant difference at Day 14 with $P<0.001$, and Day 28 with $P<0.01$; Poly (I:C): Group x Time, $F_{(3,43)}=21.74$, $P<0.0001$; post hoc Bonferroni confirmed a significant difference at and Day 28 with $P<0.001$. 

5.3.4 LPS AND POLY (I:C) INDUCED ALTERATIONS IN CB₂ GENE EXPRESSION

Having characterised the level of striatal neuroinflammation, we performed further gene expression analyses to determine the temporal profile of endocannabinoid system changes, in particular CB₂ receptor expression, in response to striatal infusion of these inflammmagen (Fig. 5.4). The most interesting observation of these analyses was a significant and pronounced upregulation of the CB₂ receptor in both the LPS- and Poly (I:C)-infused striata (LPS: Group x Time, F_{3,44}=5.60, P<0.01; post hoc Bonferroni
confirmed a significant difference at Day 4 with $P<0.05$, and Day 14 with $P<0.001$); Poly (I:C): Group x Time, $F_{(3,41)}=6.35$, $P<0.01$; *post hoc* Bonferroni confirmed a significant difference at Day 14 with $P<0.001$). In terms of the other endocannabinoid genes assessed, in the LPS infusion site, there was no overall change in CB$_1$ receptor expression although a trend for a reduction was observed at Day 4, however this did not reach statistical significance ($P=0.059$). There was no significant alteration in FAAH gene expression, although a trend for an increase was observed, and MAGL was also unchanged in the LPS-infused striatum. In the Poly (I:C) infusion site, there was an overall significant reduction in CB$_1$ receptor expression (Group x Time, $F_{(3,42)}=3.38$, $P<0.05$) although not at any specific timepoint. There was no significant alteration in FAAH gene expression, however a time-dependent increase in MAGL was observed in the Poly (I:C)-infused striatum (Time, $F_{(3,45)}=4.04$, $P<0.05$).
Fig. 5.4. qRT-PCR analyses of endocannabinoid genes in the striatum. Unilateral administration of (A) LPS or (B) Poly (I:C) induced an upregulation of CB₂ receptor gene expression, which was highest at Day 14 following infusion of both toxins. Poly (I:C) infusion also induced an overall reduction in CB₁ receptor mRNA, which was not significant at any timepoint. Data are represented as mean ± SEM with n = 6-7 rats per group. *P < 0.05, **P <0.001 vs. Vehicle by 2 way ANOVA with post hoc Bonferroni.
5.3.5 Correlation between LPS-induced neuroinflammation and CB$_2$ receptor gene expression

In order to elucidate the relationship between upregulation of microglia and CB$_2$ receptor expression, correlation analysis was performed to see if any significant correlation exists between these parameters (Fig. 5.5). We found that CD11b expression correlated significantly with CB$_2$ receptor expression in the LPS-infused striatum ($r=0.68$, $P<0.01$). Interestingly, we found no association between CD11b and CB$_2$ receptor expression in the Poly (I:C)-infused striatum ($r=0.14$, $P=n.s.$)

![Graphs showing correlation between CD11b and CB$_2$ expression](image)

**Fig. 5.5. Correlation between LPS-induced microgliosis and CB$_2$ receptor gene expression.** Increased striatal CB$_2$ receptor gene expression correlated significantly with increases in expression of microglial marker, CD11b, after striatal infusion LPS, however no significant association was observed in the Poly (I:C)-infused striatum. Data were analysed by linear regression analysis.
5.3.6 LPS AND POLY (I:C) INDUCED ALTERATIONS IN STRIATAL ENDOCANNABINOID LEVELS

Given the evidence of dynamic changes in expression of endocannabinoid genes, levels of endogenous cannabinoids were measured in the striata of LPS- and Poly (I:C)-infused animals (Fig. 5.6). Using LC-MS/MS, the endocannabinoids anandamide and 2-AG, as well as the N-acylethanolamines, PEA and OEA were measured in the striatum at sites distal to the site of infusion of either LPS or Poly (I:C). Interestingly, LPS induced a significant increase in both anandamide and 2-AG in the striatum (AEA: Group x Time, $F(3,47)=15.24, P<0.0001$; post hoc Bonferroni confirmed significance at Day 28 with $P<0.001$; 2-AG: Group, $F(1,47)=35.5, P<0.0001$; post hoc Bonferroni confirmed significance at Day 14 with $P<0.001$). Poly (I:C) did not induce any changes in either striatal anandamide, however there was an overall significant effect of treatment on the level of 2-AG, which saw a time-dependent increase in the Poly (I:C)-infused striatum (Group x Time, $F(3,46)=3.05, P<0.05$). Both inflammagens also induced alterations in PEA and OEA to varying degrees. LPS-infusion induced a sustained elevation of both PEA and OEA in the striatum which peaked at Day 28 (PEA: Group x Time, $F(3,47)=2.87, P<0.05$; post hoc Bonferroni confirmed significance at Day 28 with $P<0.001$; OEA: Group x Time, $F(3,47)=3.15, P<0.05$; post hoc Bonferroni confirmed significance at Day 1 with $P<0.05$ and at Day 28 with $P<0.001$. In the Poly (I:C)-infused striatum, PEA and OEA were not altered by treatment overall, however elevation of these lipid mediators was observed at Day 4 (PEA: Time, $F(3,48)=3.57, P<0.05$; post hoc Bonferroni confirmed significance at Day 4 with $P<0.001$; OEA: Time, $F(3,48)=2.92, P<0.05$; post hoc Bonferroni confirmed significance at Day 4 with $P<0.01$).
Fig. 5.6. Mass spectrometry measurement of striatal endocannabinoid levels. Unilateral administration of (A) LPS induced an increase in both anandamide or 2-AG in the infused striata, however Poly (I:C) (B) had no effect. Both inflammmagens induced an increase in striatal levels of the N-acylethanolamines, PEA and OEA. Data are represented as mean ± SEM with n = 5-6 rats per group, **P<0.01, ***P<0.001 1 vs. Vehicle by 2 way ANOVA with post hoc Bonferroni.
5.4 DISCUSSION

In this chapter, the aim was to investigate the temporal expression of the CB$_2$ receptor following a direct inflammatory challenge in the striatum. To do so, the bacterial inflammagen, LPS, and viral mimetic, Poly (I:C), were selected as the inflammatory stimuli and injected intra-striatally. We assessed the effects of unilateral administration of each inflammagen on lateralised motor function, neuroinflammation, changes in endocannabinoid gene expression and striatal endocannabinoid levels. We found that unilateral administration of LPS and Poly (I:C) induced stable motor dysfunction on the side of the body contralateral to the side of infusion across all behavioural tests employed. We observed a pronounced neuroinflammation in the striatum following infusion of both inflammagens, in addition to pronounced upregulation of striatal CB$_2$ receptor expression which correlated significantly in LPS-infused animals only. Alterations in other elements of the endocannabinoid system were also evident in both models, to varying degrees. Specifically, anandamide and 2-AG was elevated in the LPS-infused striatum, concomitant with an elevation in immune lipid mediators PEA and OEA. At the site of Poly (I:C) infusion, reduced CB$_1$ receptor expression and an increase in expression of the 2-AG-degrading enzyme, MAGL, was observed over time, along with a time-dependent increase in striatal 2-AG levels. PEA and OEA were not altered by Poly (I:C) treatment overall, however an elevation at Day 4 in the Poly (I:C)-infused striatum was noted.
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Behavioural analyses were performed to confirm motor impairment can be induced by these inflammmagens, thus supporting their relevance to Parkinson’s disease. As expected, we observed a stable motor deficit in the LPS-treated animals, as has been established previously (Choi et al., 2009, Hoban et al., 2013a). We also observed a contralateral motor impairment in Poly (I:C)-treated animals, which represents the first report of using Poly (I:C) as a single insult to elicit a Parkinsonian motor syndrome. This further highlights the role for neuroinflammation in the pathophysiology of Parkinson’s disease, and represents a novel model for investigation of neuroinflammatory events in vivo.

As expected, both LPS and Poly (I:C) induced a pronounced inflammatory response as indicated by OX42-positive immunostaining and upregulation of microglial marker CD11b gene expression in the striatum, however the temporal profiles differed slightly with peak elevation observed at Day 4 in the Poly (I:C)-infused striatum and Day 14 in the LPS-infused striatum. As a TLR4 agonist, which is highly expressed on microglia, LPS has been shown to induce a sustained upregulation of microglia in the nigrostriatal pathway (Choi et al., 2009). A previous report indicated maximal microglial response to TLR3 ligand, Poly (I:C), occurs at Day 12 post-infusion (Deleidi et al., 2010), which may explain the less pronounced upregulation of CD11b at the later timepoints. Overall, both LPS and Poly (I:C) induced a significant neuroinflammatory response in the infused striata.

The most important question of this chapter was the level of expression of the CB₂ receptor and how this might be altered by inflammatory challenge in the striatum.
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We observed elevation of CB₂ receptor expression in the infusion site of both LPS and Poly (I:C) in the striatum, which was far more pronounced than what had been observed in the neurotoxin studies detailed in the previous results chapter. Correlation analysis indicated that the increase in CB₂ receptor expression was associated with upregulation of microglial marker CD11b in the LPS-infused striatum which has been proposed in a number of studies (García et al., 2011, Gómez-Gálvez et al., 2015). Interestingly, the degree of CB₂ receptor upregulation in comparison to CD11b gene upregulation was far greater in these inflammation-driven models. Inflammatory stimulation of CB₂ receptor expression has been well characterised (reviewed in Ashton and Glass, 2007) and the data presented in this chapter suggests that increased CB₂ receptor expression is not simply reflective of an increase in number of microglia, but indicates that the CB₂ receptor is directly upregulated in response to inflammatory stimulus in the brain.

Interestingly, we also observed dynamic changes in other elements of the endocannabinoid system in both striata. Poly (I:C)-induced an upregulation in CB₁ mRNA at the lesion site, with a similar trend at the site of LPS infusion, indicating an inflammation-driven alteration in CB₁, which was not observed in neurotoxin-infused striata in the previous results chapter. CB₁ is located on both neurons and microglia and it also plays a role in neuroinflammation (reviewed in Kaplan, 2013). One of the most intriguing observations in this study has been the altered levels of endogenous cannabinoids in the striatum. 2-AG was elevated at all timepoints in the LPS-infused striatum, peaking at Day 14, and in the Poly (I:C)-infused striatum a delayed increase in 2-AG was observed. Clinically, 2-AG has been shown to be
Chapter 5: Characterisation of the endocannabinoid system in inflammation-driven models of Parkinson’s disease

elevated following cerebral injury (Panikashvili et al., 2001), suggesting a neuroprotective role in response to neuronal damage. Evidence from in vitro and in vivo studies has also observed elevated 2-AG in response to Parkinsonian toxins, and the administration of exogenous 2-AG ameliorates inflammation-induced degeneration (Lu et al., 2014, Mounsey et al., 2015). Interestingly, no change was observed in MAGL expression at the site of LPS infusion, however Poly (I:C) infusion induced an increase in MAGL over time.

Striatal anandamide was elevated following LPS but not Poly (I:C) infusion, with a trend for elevated FAAH expression at the LPS infusion site. Clinically, elevated anandamide has been repeatedly described in the cerebrospinal fluid of Parkinson’s disease patients (Pisani et al., 2005, Pisani et al., 2010) and has neuroprotective effects both in vitro and in vivo by mediating inflammation (Malek et al., 2015, Hernangomez et al., 2012). Indeed, anandamide has been shown to attenuate LPS-mediated increases in proinflammatory factors in astrocytes in vitro, which was inhibited by CB\textsubscript{2} receptor blockade indicating a CB\textsubscript{2} receptor-mediated neuroprotective effect of anandamide (Molina-Holgado et al., 1997, Molina-Holgado et al., 2002). This suggests a neuroprotective mechanism of 2-AG and anandamide in response to inflammatory challenge. It is important to remember, however, that endocannabinoid metabolism results in the generation of biologically active immune mediators which may mediate or potentiate inflammation, indeed deletion of MAGL in astrocytes in mice attenuates LPS-induced neuroinflammation which was attributed to reduced prostaglandin synthesis (via inhibition of 2-AG degradation) (Grabner et al., 2016). Enhanced endocannabinoid responses may represent a double-
Chapter 5: Characterisation of the endocannabinoid system in inflammation-driven models of Parkinson’s disease

edged sword and the complexities of endocannabinoid metabolic signalling and dynamic regulation in pathological conditions must be considered when interpreting preclinical data and developing strategies to modulate endocannabinoid levels.

PEA and OEA were elevated at Day 4 post-infusion of Poly (I:C) which may be attributable to their well-established role in immune modulation and as all acylethanolamines accumulate during neuronal injury (Hansen, 2010), it is probable that the discrete elevation observed in the Poly (I:C)-infused striatum is attributable to this (as the peak of neuroinflammation is also observed at this timepoint). LPS induced a pronounced and sustained increase of both PEA and OEA, which is unsurprising as their protective effects have been identified in vivo in response to LPS (Sayd et al., 2015), and in preclinical models of Parkinson’s disease (Esposito et al., 2012, Gonzalez-Aparicio et al., 2013). The pronounced elevation in PEA and OEA may also enhance anandamide activity by competitive inhibition of degradation (Jonsson et al., 2001) and/or potentiating anandamide action at CB1 or TRPV1 receptors (Costa et al., 2008).

In summary, this study revealed that both the bacterial inflammagen, LPS, and viral-mimetic, Poly (I:C) induced a neuroinflammatory response in the striatum which was associated with motor deficits, strong upregulation of the CB2 receptor and dynamic changes in striatal endocannabinoid levels. These results suggest that inflammation is a potent stimulus for endocannabinoid system dysregulation, far more so than what has been observed in genetic or neurotoxic models of Parkinson’s disease. Considering the clinical picture, endocannabinoid system dysregulation is a key
feature and some of the observations in this study parallel what has been observed in PD patients, specifically elevated endocannabinoid levels and increased CB_2_ receptor expression (Pisani et al., 2005, Pisani et al., 2010, Garcia et al., 2015, Gómez-Gálvez et al., 2015).

The overriding conclusion from these characterisation studies is that inflammatory stimuli elicit endocannabinoid system dysregulation to a greater extent than what was observed following neurotoxic challenge or by genetic induction of Parkinsonism. In particular, CB_2_ receptor elevation is more pronounced in inflammation-driven models and correlated with increased microglial activation. Taking this into consideration we believed it was appropriate to focus on these models for further investigation of the potential of targeting the CB_2_ receptor for anti-inflammatory disease-modification in Parkinson’s disease. In the next chapter, the findings of CB_2_ receptor pharmacological targeting in inflammation-driven models of Parkinson’s disease will be described and discussed.
Chapter 6: Pharmacological targeting of the CB$_2$ receptor in inflammation-driven Parkinson’s disease animal models
6.1 INTRODUCTION

The potential for targeting the CB$_2$ receptor to reduce neuroinflammation in Parkinson’s disease was initially postulated due to several converging lines of evidence including neuroinflammation and endocannabinoid dysregulation in the clinical scenario (McGeer et al., 1988, Pisani et al., 2005, Pisani et al., 2010), and the identification of CB$_2$ on microglia in a range of neurodegenerative diseases, including Parkinson’s disease (Benito et al., 2003, Palazuelos et al., 2009, Yiangou et al., 2006, Rodriguez-Cueto et al., 2014, Gómez-Gálvez et al., 2015). Some studies have indicated a potential for targeting CB$_2$ in animal models of Parkinson’s disease with varying results in terms of anti-inflammatory action and neuroprotection (Price et al., 2009, García et al., 2011, Gómez-Gálvez et al., 2015). Our studies have indicated that CB$_2$ receptor upregulation is pronounced in neurotoxic and inflammation-driven neurodegeneration, however a more pronounced upregulation, concomitant with microglial activation and dynamic endocannabinoid changes, is evident following infusion with inflammmagens, indicating a potentially important role for CB$_2$ in inflammation-driven degeneration in Parkinson’s disease (Concannon et al., 2015, Concannon et al., submitted). Taking this into consideration, we wanted to explore if activation of the CB$_2$ receptor could suppress neuroinflammation and provide neuroprotection to dopaminergic neurons in such inflammatory models of Parkinson’s disease.
Chapter 6: Pharmacological targeting of the CB2 receptor in inflammation-driven Parkinson’s disease animal models

There are many CB2 agonists that have illustrated anti-inflammatory activity, both natural and synthetic, with varying degrees of selectivity for CB2 over CB1. JWH133, a potent synthetic agonist with high selectivity for CB2 (CB2 Ki = 3.4 nM, 200 fold selectivity vs. CB1), has been shown to reduce inflammation and neuronal damage following cerebral injury (Amenta et al., 2014, Li et al., 2015a) and to reduce inflammation and ameliorate disease phenotype in animal models of Alzheimer’s disease (Martin-Moreno et al., 2012, Aso et al., 2013, Kofalvi et al., 2016). Although there are many synthetic CB2-selective drugs available, there are few natural compounds which act predominantly at CB2. However, one such compound is the dietary cannabinoid, β-caryophyllene (BCP). First isolated from Cannabis sativa by Gertsch and colleagues (2008), BCP is also found in many plants and spices with high binding affinity for the CB2 receptor (CB2 Ki = 155 nM, negligible affinity for CB1). It also has potent anti-inflammatory activity, although the mechanisms underlying this are still unknown. However, evidence from a small number of studies points to simultaneous modulation of CB2 and PPARγ receptors in the protective effects of BCP (Bento et al., 2011, Cheng et al., 2014).

We hypothesised that chronic administration of these CB2 agonists could reduce inflammation by targeting CB2 receptors on microglia thereby dampening inflammatory events leading to inflammation-driven neurodegeneration. To test the potential anti-inflammatory effects of CB2 activation using these compounds in inflammation-driven models of Parkinson’s disease, we performed two pharmacological studies. Firstly, we chronically administered the synthetic selective CB2 agonist, JWH133, to animals that received an intra-nigral LPS infusion, assessing motor function, nigrostriatal integrity
and neuroinflammation in these animals. Secondly, we chronically administered the natural CB$_2$-selective agonist, BCP, to animals that received an intra-nigral Poly (I:C) infusion, in the absence or presence of either a CB$_2$ receptor or PPAR$\gamma$ receptor antagonist. Again, assessment of motor function, neurodegeneration and neuroinflammation was performed.

In summary, the aim of this chapter was to assess if targeting the CB$_2$ receptor is a viable anti-inflammatory and/or neuroprotective strategy to address inflammation-driven neurodegeneration using animal models of Parkinson’s disease.
6.2 METHODS

All methods are described in more detail in Chapter 2. Methods specific to his chapter are also detailed below.

6.2.1 EXPERIMENTAL DESIGN

The studies presented in this chapter assessed the ability of pharmacologically targeting the CB₂ receptor to protect nigrostriatal dopamine neurons in inflammation-driven models of Parkinson’s disease. We chose two approaches to assess the neuroprotective potential of targeting the CB₂ receptor – using JWH133, a synthetic drug with high affinity and selectivity for CB₂, and BCP, a naturally-occurring selective CB₂ agonist with non-specific anti-inflammatory effects. Both CB₂ agonists were administered chronically to animals following intra-nigral administration of inflammmagens. Firstly, we assessed the effects of chronic treatment with JWH133 in an intra-nigral LPS model, and then we assessed the effects of chronic treatment with BCP in an intra-nigral Poly (I:C) model.

6.2.1.1 Effects of chronic treatment with a selective CB₂ agonist in an LPS model of Parkinson’s disease

The main aim of this study was to assess if administration of commonly used selective CB₂ agonist could ameliorate motor impairment, protect the nigrostriatal pathway and reduce neuroinflammation in an intra-nigral LPS model of Parkinson’s disease. Male Sprague Dawley rats were behaviourally habituated to the Stepping and Whisker tests and performance matched into their treatment groups. The dose of JWH133 was chosen
Chapter 6: Pharmacological targeting of the CB₂ receptor in inflammation-driven Parkinson’s disease animal models

based on evidence of preclinical efficacy in published reports (Murikinati et al., 2010, Zarruk et al., 2012). The treatment groups are described in Table 6.1 below:

<table>
<thead>
<tr>
<th>Group name</th>
<th>Drug</th>
<th>Surgery</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Vehicle</td>
<td>Naive</td>
<td>4</td>
</tr>
<tr>
<td>JWH133</td>
<td>JWH133</td>
<td>Naive</td>
<td>4</td>
</tr>
<tr>
<td>LPS &amp; Vehicle</td>
<td>Vehicle</td>
<td>LPS</td>
<td>10</td>
</tr>
<tr>
<td>LPS &amp; JWH133</td>
<td>JWH133</td>
<td>LPS</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 6.1 Groups used in this study. JWH133 (1 mg/kg i.p) was injected once daily for 14 days. The first injection was given 2 hours before LPS surgery (10 μg into the substantia nigra).

On the day of surgery, animals received an intra-peritoneal injection of either JWH133 (1 mg/kg) or vehicle (equivalent volume, ethanol:cremaphor:saline (1:1:18)). 2 hours post-injection, at the peak of drug serum concentration (Willecke et al., 2011), animals undergoing surgery received an intra-nigral infusion of LPS (10 μg in 2 μl 0.9% w/v sterile saline) on the left side at stereotaxic coordinates, AP: -5.3, ML: +2.0, DV: -7.2. For a further 13 days, animals received the same daily injection of either vehicle or JWH133, and were behaviourally assessed 2 hours-post injection (acute phase) and 18 hours-post injection (chronic phase). On Day 14 post-surgery, animals were sacrificed via transcardial perfusion fixation and post mortem immunohistochemistry was performed to assess neuroinflammation and neurodegeneration in the nigrostriatal pathway.
6.2.1.2 Effects of chronic treatment with an anti-inflammatory CB2 agonist in a Poly (I:C) model of Parkinson’s disease

The main aim of this study was to assess if administration of a natural selective CB2 agonist could ameliorate motor impairment, protect the nigrostriatal pathway and reduce neuroinflammation in an intra-nigral Poly (I:C) model of Parkinson’s disease. Male Sprague Dawley rats were behaviourally habituated to the Stepping and Whisker tests and performance matched into their treatment groups. In contrast to JWH133, BCP has been shown to act at both CB2 and PPARγ receptors (Cheng et al., 2014), and for this reason we included agonist-antagonist treatment groups to discern the receptor involved in any potential effect. The antagonists we chose were AM630, a CB2 antagonist and GW9662, a PPARγ antagonist. The vehicle used for all drugs was ethanol:cremaphor:saline (1:1:18). Drug doses were chosen based on evidence of preclinical efficacy in published reports (Choi et al., 2013, Toba et al., 2012, Navarrete et al., 2013). The treatment groups are described in Table 6.2 below:

<table>
<thead>
<tr>
<th>Group name</th>
<th>Drug(s)</th>
<th>Surgery</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>Vehicle &amp; Vehicle</td>
<td>Vehicle</td>
<td>4</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Vehicle &amp; Vehicle</td>
<td>Poly (I:C)</td>
<td>10</td>
</tr>
<tr>
<td>BCP</td>
<td>Vehicle &amp; BCP</td>
<td>Poly (I:C)</td>
<td>10</td>
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<tr>
<td>AM630 &amp; BCP</td>
<td>AM630 &amp; BCP</td>
<td>Poly (I:C)</td>
<td>10</td>
</tr>
<tr>
<td>GW9662 &amp; BCP</td>
<td>GW9662 &amp; BCP</td>
<td>Poly (I:C)</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 6.2 Groups used in this study. BCP (10 mg/kg i.p) was injected once daily for 14 days 30 min after antagonist administration (1 mg/kg i.p. for both). Poly (I:C) infusion (30 µg into the substantia nigra) was 1.5-2 hours after the first BCP injection.
On the day of surgery, animals first received an intra-peritoneal injection of either vehicle, AM630 (1 mg/kg i.p.) or GW9662 (1 mg/kg i.p.), followed by either vehicle or BCP (10 mg/kg i.p.) 30 mins later, according to treatment group. 1.5-2 hours after the BCP injection, animals received an intra-nigral Poly (I:C) infusion (30 µg in 3 µl 0.9% sterile saline) or vehicle (3 µl sterile saline) on the left side. Drug administration was performed in this manner for a further 13 days, with behavioural testing performed ~2 hours after the BCP injection. On Day 14 post-surgery, animals were sacrificed via transcardial perfusion with fixation, and brains were processed for immunohistochemical analysis of neurodegeneration and neuroinflammation in the nigrostriatal pathway.
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6.3 RESULTS

6.3.1 EFFECTS OF CHRONIC TREATMENT WITH A SELECTIVE CB\textsubscript{2} AGONIST IN AN LPS MODEL OF PARKINSON’S DISEASE

6.3.1.1 Assessment of chronic JWH133 treatment on LPS-induced motor impairment

As motor impairment is an important feature of Parkinson’s disease we wanted to assess if chronic treatment with JWH133 would improve any lesion-induced motor deficits. In order to assess any potential effects of JWH133 in this context, we chose to perform testing daily, at a time point that was considered acute to the time of injection (~2 hours post injection, at peak serum concentration) and at a time point that would represent any ‘chronic’ behavioural effects (~18 hours post injection). We did not observe any differences in the behavioural performance at either of these phases, so for the purposes of clarity the average scores across both testing periods is presented in Fig 6.1a.

As expected, we found that ipsilateral motor function was not altered by either LPS infusion or drug regime. We found that JWH133 did not ameliorate the contralateral motor impairment induced by intra-nigral infusion of LPS in the Stepping Test of forelimb akinesia. Similarly, ipsilateral motor function was not altered by either LPS infusion or drug regime and we did not observe any effect of JWH133 treatment on LPS-induced motor impairment in the Whisker test of sensorimotor integration, in either testing phase. Despite this, LPS induced a significant lateralised motor impairment in the Stepping as illustrated in Fig 6.1b, when taking into account the
average score for each parameter across the testing period (Group x Side, \(F(1,48)=7.27, P<0.001\)). ANOVA did not report a statistical difference between ipsilateral and contralateral sides overall however post-hoc Bonferroni did indicate a significant contralateral impairment in the ‘LPS & Vehicle’ group \((p<0.05)\), which was not observed in the ‘LPS & JWH133’ group.
Fig. 6.1a. Behavioural assessment of JWH133 on LPS-induced motor deficits in the Stepping and Whisker Test. Chronic administration of JWH133 did not affect LPS-induced contralateral motor dysfunction in the Stepping (A) and Whisker tests (B), when analysed over time. Analysis of the collapsed data, restricted to the first seven days post-operatively did not indicate effect of JWH133 (column graphs on right). Data are represented as mean ± SEM with n = 4-10 rats per group. Surgery was performed on Day 0.
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6.3.1.2 Assessment of JWH133-mediated effects on nigrostriatal integrity

In order to assess if chronic treatment with JWH133 could confer nigrostriatal neuroprotection following LPS infusion, quantitative immunohistochemical analysis was performed for dopaminergic neurons by staining tyrosine hydroxylase (TH), a dopamine synthesis enzyme (Fig. 6.2a,b). As expected, naive animals did not display any significant dopaminergic loss (Vehicle and JWH133 groups). Interestingly, intranigral LPS infusion resulted in a loss of TH-positive nigral cell bodies ($P<0.05$) and this was ameliorated by chronic treatment with JWH133 (Group, $F_{(3,21)}=3.70$, $P<0.05$, post hoc Bonferroni confirmed significant difference $P<0.05$), indicating a potential neuroprotective effect of JWH133. A similar trend was observed in the number of TH-positive, striatal terminals however this did not reach statistical significance.

Fig. 6.1b. LPS-induced lateralised motor deficits in the Stepping and Whisker test. Unilateral infusion of LPS induced a significant motor impairment contralateral to the side of infusion on average. Data are represented as mean ± SEM with n = 4-10 rats per group, *$P<0.05$, **$P<0.001$ vs. Vehicle by 2 way ANOVA with post hoc Bonferroni.
Fig. 6.2a. Immunohistological assessment of LPS-induced dopaminergic degeneration. LPS induced a reduction in dopaminergic neurons in the nigrostriatal pathway, as evidenced by a reduction in dopaminergic cell bodies in the substantia nigra. Data are represented as mean ± SEM, with n=4-10 animals per group, *P<0.05 vs. contralateral, by 2-way ANOVA with post hoc Bonferroni.
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Fig. 6.2b. **Immunohistological assessment of JWH133-induced effects on nigrostriatal integrity.** Chronic treatment with JWH133 prevented the LPS-induced reduction in dopaminergic cell bodies in the substantia nigra with a similar non-significant trend observed in dopaminergic nerve terminals in the striatum. Data are represented as mean ± SEM, with n=4-10 animals rats per group, *P<0.05 vs. LPS & Vehicle group, by one way ANOVA with post hoc Bonferroni. Scale bar = 2 mm.
6.3.1.3 Assessment of JWH133-mediated effects on neuroinflammation

Given the protection of dopaminergic neurons by chronic JWH133 treatment, we next sought to investigate if chronic JWH133 treatment altered the level of microgliosis in the nigrostriatal pathway following LPS-infusion. To do so, we performed immunohistochemical analysis using OX42 staining, which recognises the CD11b protein which is highly expressed on microglia and macrophages. We observed a subtle increase in OX42-positive staining in the ipsilateral substantia nigra (Side: $F_{(1,46)} = 5.684$, $p<0.05$) but not in the striatum in LPS-infused animals (Fig. 6.3a,b). Interestingly, we did not observe any difference between the level of OX42-positive staining in either the substantia nigra or the striatum of the ‘LPS & Vehicle’ and ‘LPS & JWH133’ groups. This may indicate that the effects of JWH133 on LPS-induced microglial activation may not involve a reduction in overall microgliosis, but via other anti-inflammatory mechanisms. We also performed GFAP immunohistochemical analysis to measure the astrocyte response in the nigrostriatal pathway (Fig. 6.4). We did not observe any change in levels of GFAP-positive staining in the nigrostriatal pathway following intra-nigral LPS infusion compared to naive animals as illustrated qualitatively and quantitatively.
Fig. 6.3a. Immunohistological assessment of LPS-induced microglial response. A mild increase in OX42-positive staining was observed in the substantia nigra of LPS-infused animals. Data are represented as mean ± SEM, with n=4-10 animals rats per group. *P<0.05 vs. contralateral, by 2-way ANOVA with post hoc Bonferroni
Fig. 6.3b. Immunohistological assessment of JWH133-induced effects on microglial activation. A mild increase in OX42-positive staining was observed in the substantia nigra of LPS-infused animals. Chronic treatment with JWH133 did not alter the level of microgliosis in the substantia nigra. Data are represented as mean ± SEM, with n=4-10 animals rats per group. Scale bar = 2mm
**Fig. 6.4. Immunohistological assessment of JWH133-induced effects on astroglial activation.** Overt astrocytosis was not observed in the nigrostriatal pathway of LPS-infused animals as assessed by GFAP immunostaining. Chronic treatment with JWH133 did not alter the level of astrocytosis in the substantia nigra or striatum. Data are represented as mean ± SEM, with n=4-10 animals rats per group. Scale bar = 2 mm.
6.3.2 Effects of chronic treatment with a CB₂ agonist in a Poly (I:C) model of Parkinson’s disease

6.3.2.1 Assessment of chronic BCP treatment on Poly (I:C)-induced motor impairment

In order to assess if chronic treatment with BCP could affect the motor function we performed behavioural testing across the course of the study (Fig. 6.5). As expected ipsilateral motor function was not altered following intra-nigral infusion of Poly (I:C) in either the Stepping (A) or Whisker test (B). Moderate contralateral motor dysfunction was observed over the timecourse following surgery however the duration of impairment varied between the groups in both behavioural tests. Poly (I:C) induced a mild impairment in contralateral motor function in the Stepping Test (Group, $F_{(4,39)}=4.052$, $P<0.01$). This was significantly ameliorated by chronic BCP administration ($P<0.05$). Animals receiving CB₂ antagonist AM630, in conjunction with BCP, did not differ significantly from the vehicle group which may suggest the protective effect of BCP was impeded by CB₂ receptor blockade, however there was no statistical difference between ‘BCP’ and ‘AM630 & BCP’ groups. However animals undergoing treatment with PPARγ antagonist GW9662, in conjunction with BCP, saw an improvement in contralateral motor performance ($P<0.05$). There was no statistically significant difference between any of the groups in the Whisker test.
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Fig. 6.5. Behavioural assessment of BCP on Poly (I:C)-induced motor deficits. Poly (I:C) induced a significant contralateral motor impairment in the Stepping (A) and Whisker Test (B). In the Stepping test, motor impairment was ameliorated by BCP treatment. Column graphs represent the collapsed data, restricted to the first seven days post-operatively. Data are represented as mean ± SEM with n = 4–10 rats per group, *P<0.05 vs. Sham group, *P<0.05, vs. Vehicle group by 1 way ANOVA with post hoc Bonferroni. Surgery was performed on Day 0.
6.3.2.2 Assessment of BCP-mediated effects on nigrostriatal integrity

We next addressed if chronic treatment with BCP could confer neuroprotection to dopaminergic neurons in the nigrostriatal pathway following intra-nigral Poly (I:C) infusion, and discern if any potential effects were prevented by blockade of CB$_2$ (with AM630) or PPAR$\gamma$ (with GW9662) receptors (Fig 6.6). To do so, we performed quantitative immunohistochemical staining for TH in the nigrostriatal pathway, as illustrated below. Animals that received intra-nigral Poly (I:C) had a reduction in TH-positive cell bodies in the substantia nigra (Group, $F_{(4,35)}=7.08$, $P<0.001$). However this was not prevented by chronic treatment with BCP. TH-positive striatal terminals were not significantly altered following intra-nigral Poly (I:C) infusion.
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Fig. 6.6. Immunohistological assessment of BCP-induced effects on nigrostriatal integrity. Poly (I:C) induced a partial reduction in dopaminergic cell bodies in the substantia nigra. Chronic treatment with BCP did not protect dopaminergic cell bodies from Poly (I:C)-induced degeneration. Data are represented as mean ± SEM, with n=4-10 animals rats per group, **$P<0.01$, ***$P<0.001$ vs. Sham, by one way ANOVA with post hoc Bonferroni. Scale bar = 2 mm.
6.3.2.3 Assessment of BCP-mediated effects on microglial activation

Although we did not observe any BCP-mediated neuroprotective effects, we wanted to assess if there were any changes in the microglial response and performed quantitative OX42 immunohistochemical staining in the nigrostriatal pathway (Fig. 6.7). Surprisingly we did not observe any significant microgliosis in either the substantia nigra or striatum, following Poly (I:C)-infusion, and no there was no effect of treatment with BCP or combination with AM630 or GW9662.
Fig. 6.7. **Immunohistological assessment of BCP-induced effects on microglial cells.** Poly (I:C) did not induce any overt microgliosis in the nigrostriatal pathway (A). Chronic treatment with BCP did not alter the level of OX42-positive staining (B). Data are represented as mean ± SEM, with n=4-10 animals rats per group. Scale bar = 2mm.
6.4 DISCUSSION

In this chapter we presented the results of two pharmacological studies that focused on pharmacological targeting of the CB$_2$ receptor for anti-inflammatory disease modification in inflammation-driven models of neurodegeneration. In the first study, we chronically administered the synthetic CB$_2$ agonist JWH133 following intra-nigral LPS lesion, and assessed lateralised motor performance, dopaminergic degeneration and neuroinflammation. We observed mild contralateral motor dysfunction in animals that received intra-nigral LPS, which was not prevented or ameliorated by JWH133 treatment in the Stepping Test of forelimb akinesia or the Whisker test of sensorimotor integration. However, at an anatomical level, LPS caused a loss of nigrostriatal dopaminergic cell bodies which was partially prevented by JWH133 treatment, indicating a neuroprotective effect. LPS also induced a significant increase in microglia in the substantia nigra but the level of microgliosis was not altered by JWH133 treatment, indicating the neuroprotective effects of JWH133 treatment did not involve a reduction in microgliosis. In the second study in this chapter, the effects of chronic administration of natural CB$_2$ agonist BCP was assessed following intra-nigral Poly (I:C) infusion. Assessment of lateralised motor function revealed that Poly (I:C) induced a moderate contralateral impairment in the Stepping test which did not manifest in Poly (I:C)-infused animals treated with BCP alone, but did emerge in those receiving co-treatment with CB$_2$ antagonist AM630. At a neuropathological level, we observed a partial reduction in dopaminergic cell bodies following intra-nigral Poly (I:C) infusion, which was not ameliorated by treatment with BCP alone. Surprisingly, we did not observe any overt microgliosis.
Chapter 6: Pharmacological targeting of the CB$_2$ receptor in inflammation-driven Parkinson’s disease animal models

in animals that received an intra-nigral infusion of Poly (I:C) and no difference between the drug regimes.

The evidence from these studies has provided some interesting and valuable insights into the neuroprotective potential of CB$_2$ activation. In the LPS study, JWH133 conferred neuroprotection to nigrostriatal dopaminergic neurons with little improvement in motor function and no alteration in overall microgliosis. JWH133 has been shown to provide functional neuroprotection in models of neurodegeneration, by reducing proinflammatory factors such as proinflammatory cytokines and oxidative stressors (Aso et al., 2013, Aso et al., 2016). Thus, although we did not observe a reduction in global nigral microgliosis in this study, the neuroprotective effects of JWH133 may be attributed to several other established effects of CB$_2$ agonism. Evidence shows that CB$_2$ agonism can stimulate activated microglia to reduce excessive inflammatory responses to injury (Merighi et al., 2012), again via a reduction in proinflammatory signalling or neurotoxic factors (Klegeris et al., 2003, Eljaschewitsch et al., 2006). Unfortunately, this could not be assessed in this study given the secretory nature of these various factors. Another potential anti-inflammatory and neuroprotective mechanism of JWH133 could be modulation of microglial phenotypes as evidence suggests a role for a JWH133-induced shift from the M1 to M2 macrophage state (Tomar et al., 2015) and has been observed in a model of stroke (Zarruk et al., 2012). As the immunohistochemical staining performed in this study could not differentiate between the populations of polarized microglia for technical reasons (i.e. the sections were too thick to discern discreet microglial morphology at the lesion site), it was outside the scope of this
Chapter 6: Pharmacological targeting of the CB₂ receptor in inflammation-driven Parkinson’s disease animal models

study to examine microglial morphology. Interestingly, a recent study reported that administration of another selective CB₂ agonist, HU308, is neuroprotective in a LPS mouse model, which was associated with reduced microglial activation, assessed by CD68 immunostaining (Gómez-Gálvez et al., 2015). As with all pharmacological studies, an important consideration is the mechanisms of action, and there is evidence of ‘biased agonism’ amongst CB₂ agonists, which suggests not all CB₂ agonists elicit the same response (Atwood et al., 2012) and indeed endocannabinoids, specifically 2-AG, may bind to CB₂ at sites distinct from those at which synthetic agonists or antagonists bind, so-called ‘agonist-directed trafficking’ which may mediate agonist activity (Shoemaker et al., 2005). This is particularly interesting in the context of this study, as we previously observed a pronounced elevation in 2-AG following intra-striatal infusion of LPS (Chapter 5). A recent study by Dhopeshwarkar & Mackie (2016) has provided a further insight into the functional selectivity of CB₂ ligands and downstream signalling, indicating a strong role for JWH133 in adenylyl cyclase inhibition. Overall, the mechanism of action of the CB₂ agonist in question may play an important role in these observations, particularly in the context of neuroprotection, however ultimately selective targeting of the CB₂ receptor has potential for disease modification in Parkinson’s disease.

In the Poly (I:C) study, chronic treatment with the natural, selective CB₂ agonist BCP produced some subtle improvement in behavioural impairment however it did not confer any significant neuroprotection in the nigrostriatal pathway or alter microgliosis. The behavioural impairment induced by Poly (I:C) in this study was acute and not very pronounced, however animals treated with BCP did not present
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with significant contralateral motor impairment on average. BCP has elicited functional protection previously at 10 mg/kg in a model of cerebral ischemic injury, reducing infarct size and edema (Choi et al., 2013), and in an Alzheimer’s disease model (Cheng et al., 2014) however the dose of BCP was much higher and the inflammatory profile in APP/PS1 mice is quite pronounced (Cheng et al., 2014). Interestingly, in the model of cerebral ischemia, the effects of BCP were mediated by CB$_2$, as blockade of CB$_2$ inhibited the effects of BCP, whereas in APP/PS1 mice, both CB$_2$ and PPAR$\gamma$ receptors were involved in BCP-mediated neuroprotection, which may have implications for the nature or origin of neurodegeneration (i.e. traumatic or acute injury vs. progressive, chronic inflammatory stimulation). It is important to note in this study that a reduction in dopaminergic cell bodies was observed in Poly (I:C)-infused animals and yet no overt microgliosis was evident following immunohistochemical analysis of OX42 protein expression. This was surprising given the wealth of evidence supporting Poly (I:C)-induced increase in microglia in animal models of Parkinson’s disease (Deleidi et al., 2010, Bobyn et al., 2012), but this could also be due to the time of assessment. Despite these caveats a key consideration would be the relative infancy of BCP in terms of our understanding of its actions. The effects of BCP still remain largely unexplored and although full agonism at CB$_2$ has been reported we cannot rule out alternative targets and mechanisms of action, although there is considerable promise for its therapeutic abilities (reviewed in Sharma et al., 2016).

In the first study, we observed neuroprotection mediated by CB$_2$ agonism, with no functional improvement in the behavioural tests and in the second study, CB$_2$
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agonism did not confer neuroprotection and but did elicit partial improvement in motor impairment in animals. This is an interesting observation, but undoubtedly the data could be more convincing. Overall, this chapter provides evidence that pharmacological targeting of the CB₂ receptor may be a viable option for anti-inflammatory disease modification in Parkinson’s disease. However, several key questions remain to be answered and the most critical of these will be firstly, what are the mechanisms underlying CB₂-mediated neuroprotection in models of Parkinson’s disease and secondly, to what degree is the CB₂ receptor response to neurodegeneration pathological or a compensatory protective response in both animal models and the clinical condition. Although it is impossible to fully ascertain this in clinical Parkinson’s disease, the results presented in this and previous chapters using animal models is encouraging for pharmacological targeting of the CB₂ receptor as an adjuvant neuroprotective therapy in Parkinson’s disease.
Chapter 7: General Discussion

The work presented in this thesis sought to determine the potential of targeting of the CB₂ receptor for anti-inflammatory disease modification in Parkinson’s disease. In order to fully evaluate the suitability of CB₂ as a pharmacological target in this regard, we focused on assessing the temporal response of CB₂ to various stimuli with pathological and etiological relevance to Parkinson’s disease, including genetic, neurotoxic and inflammatory triggers. These findings then served to inform our pharmacological studies which unmasked some of the functional effects of targeting the CB₂ receptor in inflammation-driven neurodegeneration.

The main findings presented in this body of work are: 1) CB₂ receptor expression is significantly upregulation following infusion of parkinsonian toxins with varying mechanisms of action, and correlates significantly with microglial marker upregulation 2) CB₂ receptor upregulation is more pronounced in response to inflammation-driven neurodegeneration (Concannon et al., 2015, Concannon et al., submitted) and 3) pharmacological targeting of the CB₂ receptor confers some neuroprotection in inflammation-driven models of Parkinson’s disease. Moreover, the neuroprotection mediated by CB₂ agonism in our study indicates the anti-inflammatory mechanisms of action does not involve a reduction in global microgliosis and may instead involve regulation of microglial activity, effects which have been previously well-established (reviewed in Ashton and Glass, 2007). These findings highlight the role of the CB₂ receptor, and the endocannabinoid system as a whole, in inflammation-driven neurodegeneration. It also provides encouraging evidence to support pharmacological targeting of the CB₂ receptor for modulation of
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the ‘self-sustaining’ cycle of neuroinflammation and neurodegeneration observed in Parkinson’s disease.

The need for novel targets and treatment approaches in Parkinson’s disease has never been so great, considering the aging population and socioeconomic burden associated with long-term care and treatment (Tarazi et al., 2014). All current treatments provide only short-term symptomatic relief and no disease-modifying therapies are available clinically (Worth, 2013). Although there are several promising neuroprotective therapies in the early stages of clinical trials (reviewed in Lotia and Jankovic, 2016), the majority of new treatment approaches are new pharmacotherapies for dopamine replacement or modulation - either reformulations of levodopa, new dopamine agonists or novel delivery approaches. In addition, restorative or regenerative approaches including gene and stem cell therapy are also garnering much interest (reviewed in Tarazi et al., 2014).

It is without doubt that Parkinson’s disease, in its primary clinical manifestation of motor dysfunction, is precipitated by dopamine deficiency; however our ever-increasing knowledge of the condition tells us that the picture is not that simplistic. At diagnosis, a symptomatic patient may present with 30-70% dopaminergic cell loss (Fearnley and Lees, 1991, Ma et al., 1997, Ross et al., 2004, Dauer and Przedborski, 2003, Greffard et al., 2006) which indicates that neurodegeneration has initiated and additional features such as neuroinflammation and oxidative stress are fully engaged and contributing to disease progression (Tansey and Goldberg, 2010). These phenomena may also hamper the efficacy of restorative therapies and as a result
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disease-modifying therapy is becoming increasingly more valued in the field of Parkinson’s disease research (Lotia and Jankovic, 2016). Neuroinflammation in particular is a key contributor to neurodegeneration, and anti-inflammatory disease modification has emerged as therapeutic interest in Parkinson’s disease, supported by a wealth of epidemiological evidence associating chronic anti-inflammatory drug administration with a reduced risk of developing the condition (Chen et al., 2003, Chen et al., 2005, Bower et al., 2006, Hernán et al., 2006). Activated microglia and microglial-derived factors including TNF-α, IL-1β and IL-6 are elevated in the substantia nigra of PD patients post mortem (McGeer et al., 1988, Mogi et al., 1994b), and this evidence has spurred the emergence of strategies to inhibit glial activity in preclinical models of Parkinson’s disease (reviewed in Tansey and Goldberg, 2010).

The endocannabinoid system has emerged as a promising target for anti-inflammatory disease modification in Parkinson’s disease due to its dual role in basal ganglia circuitry and immune modulation (Tanasescu and Constantinescu, 2010), and in particular the CB₂ receptor has received much attention due to its location on microglial cells (Núñez et al., 2004) and enhanced expression in response to injury/degeneration (reviewed in Ashton and Glass, 2007). The endocannabinoid system is a very relevant target in Parkinson’s disease given the dysregulation observed in the clinical scenario including elevated anandamide (Pisani et al., 2005, Pisani et al., 2010) and altered CB₁ (Lastres-Becker et al., 2001, Hurley et al., 2003a, Farkas et al., 2012, Van Laere et al., 2012) and CB₂ expression (Grünblatt et al., 2007, Garcia et al., 2015, Gómez-Gálvez et al., 2015). Despite this, not much is
known about dysregulation of the endocannabinoid system in preclinical Parkinsonism, including the response of the microglial CB₂ receptor, which is critical in order to fully evaluate its therapeutic validity. Increased CB₂ receptor expression has been observed clinically and preclinically in a host of neurodegenerative diseases including Alzheimer’s disease (Benito et al., 2003, Wu et al., 2013), multiple sclerosis and amyotrophic lateral sclerosis (Yiangou et al., 2006, Shoemaker et al., 2007, Lou et al., 2011), spinocerebellar ataxia (Rodriguez-Cueto et al., 2014), Huntington’s disease (Palazuelos et al., 2009, Dowie et al., 2014, Fernandez-Ruiz et al., 2007, Sagredo et al., 2009, Bouchard et al., 2012) and very recently, Parkinson’s disease (Garcia et al., 2015, Gómez-Gálvez et al., 2015, García et al., 2011). The large majority of these studies utilised immunostaining methods to visualise CB₂ receptor expression which is concerning, due to the controversy surrounding commercially available CB₂ antibodies. This was highlighted in a review by Atwood and Mackie (2010), where the issue of non-specific staining in CB₂ knockout mice was discussed, and further methodological studies have corroborated the non-specificity of commercially-available CB₂ antibodies (Baek et al., 2013, Marchalant et al., 2014). Given the questionable reliability of quantifying CB₂ receptor expression in this manner, and the lack of data of CB₂ receptor expression patterns in animal models of Parkinson’s disease, we felt it prudent to investigate this and performed a series of characterisation studies which addressed the temporal profile of CB₂ receptor expression following infusion of Parkinsonian agents; the results of which are detailed in Table 7.1.
In Chapter 3, we induced a genetic model of Parkinson’s disease mediated by viral over-expression of α-synuclein and assessed the temporal profile of CB₂ receptor expression. We observed reduced expression in addition to reduced microglial marker expression. Although the reduction in microglial marker expression was not expected, genetic models often present a subtle inflammatory profile dependent on the level of neurodegeneration (Sanchez-Guajardo et al., 2010) and the downregulation may reflect α-synuclein-mediated suppression of phagocytic activity (Roodveldt et al., 2010). The pathological significance of reduced CB₂ receptor expression in this model may be a response to α-synuclein or reflective of the reduction in microglia given CB₂ is primarily associated with microglia in the brain. The CB₂ receptor has not been extensively investigated in genetic models of Parkinson’s disease, in fact only one other report in LRRK2 transgenic mice investigated CB₂ receptor and no changes in expression were observed in that study (Palomo-Garo et al., 2016). Even though our findings are in conflict with our anticipation that increased CB₂ receptor expression in the presence of microglial activation would be evident in this model, the data is highly valuable as it provides an indication for CB₂ receptor dysregulation in response to α-synuclein overexpression. Although this model is very efficient at recapitulating the progressive neurodegeneration in Parkinson’s disease, the neuroinflammatory profile that it precipitated was mild. Taking this into consideration, full characterisation of the CB₂ receptor and other aspects of the endocannabinoid system was required in order to discern the role of CB₂ in other models of Parkinson’s disease with a stronger neuroinflammatory profile.
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<table>
<thead>
<tr>
<th>Model</th>
<th>CB₂</th>
<th>CD11b</th>
<th>eCB levels</th>
<th>Other eCB changes</th>
<th>Motor function</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-synuclein</td>
<td>↓</td>
<td>↓</td>
<td>n/m</td>
<td>←</td>
<td>↓</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>↑PEA &amp; OEA*</td>
<td>↓</td>
</tr>
<tr>
<td>Rotenone</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>↑PEA &amp; OEA*</td>
<td>↔</td>
</tr>
<tr>
<td>LPS</td>
<td>↑</td>
<td>↑</td>
<td>↑AEA, 2-AG</td>
<td>↑PEA &amp; OEA←</td>
<td>↓</td>
</tr>
<tr>
<td>Poly (I:C)</td>
<td>↑</td>
<td>↑</td>
<td>↑2-AG</td>
<td>↓CB₁, MAGL</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑PEA &amp; OEA*</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.1 Summary of main results observed in Chapter 3-5. Various elements of the endocannabinoid system were altered in each model, alterations in CB₂ receptor expression was observed in all models. *elevation was observed at discrete timepoint.

Neuroinflammatory responses can vary due to the nature of the toxin and site of infusion in models of Parkinson’s disease and so in Chapters 4 and 5, we chose to assess the temporal expression of the CB₂ receptor following striatal infusion of more established Parkinsonian toxins. Lesions of the striatum have been shown to produce motor impairment and neuropathological features typical of Parkinson’s disease (Walsh et al., 2011, Stott and Barker, 2014, Hoban et al., 2013a, Choi et al., 2009, Mulcahy et al., 2011) and this strategy also allowed us to perform multiple post mortem analyses because of the larger size of the region for tissue sampling. In Chapter 4 we demonstrated that the CB₂ receptor is significantly upregulated in response to infusion of catecholaminergic and environmental neurotoxins, which is correlated significantly with microglial marker upregulation (Concannon et al., 2015, Concannon et al., submitted). In Chapter 5, we made a similar observation of increased CB₂ receptor expression, concomitant with microglial marker upregulation, in response to infusion of the bacterial inflammasogen, LPS, and the viral-mimetic,
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Poly (I:C), however in the case of these inflammation-driven models, the response was far more pronounced. Correlation analyses revealed CB$_2$ receptor expression correlated significantly with CD11b expression in the 6-OHDA-, rotenone- and LPS-infused striata, but not the Poly (I:C)-infused striatum. Of course, correlation statistics describe an associative relationship but cannot assess if a causal relationship exists, so this may represent a direct upregulation of CB$_2$ expression or alternatively, upregulation of microglia may derivatively upregulate CB$_2$. However, interestingly, in the inflammation-driven models, the degree of CB$_2$ receptor expression upregulation was much greater than the degree of CD11b overexpression, providing a strong indication that CB$_2$ receptor upregulation is not consequential of microglial activation, but specifically upregulated in response to inflammation-driven events. This is encouraging given that CB$_2$ expression has been strongly associated with microglial phenotypes in vitro (Cabral and Marciano-Cabral, 2005). We must also consider that CB$_2$ receptor expression may not be exclusive to microglia in the brain, as CB$_2$ has been identified on astrocytes (Molina-Holgado et al., 2002) and CB$_2$ receptor expression on neurons is also debateable. Initially identified by Gong et al. (2006), the prospect of neuronal CB$_2$ receptors has been tainted by the issue of non-specific antibody staining, and conflicting reports continue to emerge. In particular, one comprehensive study identified CB$_2$ receptors on ventral tegmental dopamine neurons in mice (Zhang et al., 2014). Conversely, a study which generated CB$_2$-GFP BAC transgenic mice observed CB$_2$ receptor localisation on microglia but not neurons in the murine brain (Schmole et al., 2015a). We cannot comment definitively on the cellular localisation of CB$_2$ expression in this work however it remains a point of interest. Recently, CB$_2$ receptor fluorescent probes have been developed for in
**Chapter 7: General Discussion**

*in vitro* and *in vivo* imaging (Wu et al., 2014, Zhang et al., 2015) and this holds much promise as a strategy for preclinical assessment of CB$_2$ receptor expression, as an alternative to immunolabelling methods, however this approach is still in its infancy.

An important aspect in evaluating the potential role of CB$_2$ in neuroinflammation and neurodegeneration is the response of the endocannabinoid system as a whole to various stimuli. In Chapter 4 and Chapter 5 we have shown that striatal endocannabinoids are differentially altered depending on the toxin. In the neurotoxic studies, discrete changes in striatal endocannabinoid levels was observed, however in the inflammation-driven studies, elevation of endocannabinoids was more sustained, particularly in the LPS-infused animals. Administration of exogenous 2-AG and anandamide are neuroprotective following inflammatory stimuli *in vitro*, and this protection is mediated via CB$_2$ receptor signalling (Correa et al., 2010, Lu et al., 2014, Malek et al., 2015). Indeed, enhancement of endogenous cannabinoid signalling *in vivo* is also neuroprotective in a CB$_2$ receptor-dependent manner (Nader et al., 2014, Wen et al., 2015). PEA and OEA are lipids with a well-established role in immune signalling, and we have shown that, similar to endocannabinoids, they are enhanced in the striatum of LPS-infused animals and this may represent multiple neuroprotective functions of PEA and OEA including enhancement of anandamide signalling (Hansen, 2010). The burning question is whether dysregulation of the endocannabinoid system in these models represents a pathological or protective series of events, that is, are these changes in endocannabinoid signalling and CB$_2$ receptor expression driving neuroinflammation and neurodegeneration, or are these events a compensatory, neuroprotective mechanism to limit degeneration. It is
difficult to discern this, given the limitations of the various models, however the consensus is that endocannabinoid dysregulation is indicative of compensation to neurodegeneration and promotes neuroprotection by stimulating anti-inflammatory activity and acquisition of non-cytotoxic microglial phenotype (Mecha et al., 2015). However, in chronic neurodegenerative disease, prolonged immune stimulation can shift from protective to destructive, and as such, the same could be true for sustained endocannabinoid dysregulation. In Parkinson’s disease the endocannabinoid system also plays an important role in basal ganglia circuitry, thus we must also take into account that sustained neurodegeneration in the midbrain will inherently alter endocannabinoid levels, not inclusive of immune-mediated responses.

Ultimately the overriding question is does the endocannabinoid system, and the CB2 receptor in particular, represent a viable target for anti-inflammatory disease modification in Parkinson’s disease. The first indications for pharmacological targeting of CB2 in Parkinson’s disease was presented by Price et al., (2009), where the CB2 agonist, JWH015, ameliorated MPTP-induced motor impairment, neuroinflammation and dopaminergic degeneration in mice. A second report by Garcia and colleagues (2011) showed that HU308, another CB2 selective agonist, attenuated a partial reduction in dopaminergic cells induced by LPS, although no behavioural testing or assessment of the neuroinflammatory profile was performed.

Observations from our characterisation studies indicate that dysregulation of the endocannabinoid system, in particular pronounced CB2 receptor upregulation and elevated endocannabinoid levels, is inflammation-driven and indeed in Chapter 6,
we have shown that selective agonism of CB₂ using JWH133 protects the nigrostriatal pathway from inflammation-driven degeneration. We have shown for the first time that JWH133 is neuroprotective in this model of Parkinson’s disease, which has been supported by recent work in LPS-lesioned mice (Gómez-Gálvez et al., 2015). In addition, JWH133-mediated neuroprotection was not concomitant with a reduction in global microglial density suggesting that CB₂ agonism may induce multiple ‘anti-inflammatory’ effects which may involve altered microglial responsivity, adaptive cytokine/chemokine profiles, altered microglial phenotype, enhanced or suppressed endocannabinoid release or some combination of all of these events. Thus, pharmacological targeting of the CB₂ receptor has potential for neuroprotective therapy in Parkinson’s disease although the mechanisms underlying CB₂-mediated anti-inflammatory activity needs to be discerned.

**Future directions**

The studies presented in this thesis have provided an interesting insight into endocannabinoid system dysregulation and the pathophysiological aspect of neuroinflammation in preclinical Parkinson’s disease. In the future it will be pertinent to investigate the cellular localisation of the CB₂ receptor when suitable antibodies or other analytical techniques become available, to absolutely discern several key questions including: 1) are CB₂ receptors located on microglia, 2) are CB₂ receptors upregulated on microglia in response to stimulation or is CB₂ receptor upregulation a marker of microglia activation and 3) what other cells, if any, express CB₂ receptors?
Chapter 7: General Discussion

The focus of this work was on the pathophysiological role of neuroinflammation however it may also be a critical etiological factor in Parkinson’s disease. Considering the dynamic patterns of CB$_2$ receptor upregulation in the pathology of preclinical Parkinson’s disease models, it may also be worthy of investigation in the context of the etiological contribution of neuroinflammation to the development of Parkinson’s disease. Preclinical evidence has shown that exposure to environmental toxins or systemic infections increases dopaminergic sensitivity to subsequent degenerative stimuli (Tien et al., 2013; Fan et al., 2011; Cai et al., 2013). It would be very interesting to investigate if changes in CB$_2$ receptor expression, and the endocannabinoid system in general, is evident in the nigrostriatal pathway following the initial challenge and also following the initiation of dopaminergic degeneration, to assess if the CB$_2$ receptor is differentially regulated.

In terms of pharmacological targeting, the evidence presented in this work supports a neuroprotective effect of pharmacological targeting of the CB$_2$ receptor. The pharmacological action of selective CB$_2$ agonists is diverse and they have varying anti-inflammatory effects via multiple signalling pathways (Atwood et al., 2012; Dhopeshwarkar and MacKie, 2016). Further studies would be highly beneficial to assess the effects of diverse CB$_2$ agonists in Parkinson’s disease models.

Although the anti-inflammatory effects of CB$_2$ receptor targeting has been characterised in vitro and in vivo, the effects elicited are often restricted to measurement of immune cells, cytokines, chemokines and oxidative stressors, and thus far, measurement of endocannabinoid levels in response to CB$_2$ agonism in
neurodegenerative models has not been explored to the best of our knowledge. Given the evidence of neuroprotective effects of the endocannabinoids themselves, and to unlock their role in the anti-inflammatory effects of CB₂ receptor stimulation, we would suggest that this would be an important focus area for continuing investigation. In addition, it would be interesting to assess the effects of enhancement of endocannabinoid tone, which has been shown to be neuroprotective (Fernandez-Suarez et al., 2014), on CB₂ receptor expression, and to assess if CB₂ receptor blockade alters the effects of inhibiting the degrading enzymes FAAH and MAGL in preclinical models of Parkinson’s disease.

**Concluding remarks**

Anti-inflammatory therapy for disease-modification in Parkinson’s disease is an exciting area for preclinical research, and the prospects for pharmacological modulation of the CB₂ receptor remains attractive. Numerous lines of evidence both *in vitro* and *in vivo* have provided evidence that is very encouraging for CB₂-mediated neuroprotection, including the work presented in this thesis. Parkinson’s disease research is predominantly focused on developing approaches to restore dopaminergic input and integrity and for many years the sole interest was on dopamine replacement strategies. As our understanding of the condition has grown so has the importance of contributing factors, such as neuroinflammation, to disease progression and this has seen a drive for disease-modifying strategies. Although reduction of neuroinflammation will probably not be sufficient to solely halt disease progression, it may serve to slow progression and by ameliorating the inflammatory milieu it may enhance the effectiveness of current and emerging restorative
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treatments. Thus, the development of novel strategies for anti-inflammatory disease modification in Parkinson’s disease has tremendous potential to improve the treatment and quality of life for patients living with this condition.
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preliminary autoradiographic study with the selective dopamine D<sub>2</sub>/D<sub>3</sub> antagonist [<sup>3</sup>H] raclopride and the novel CB<sub>1</sub> inverse agonist [<sup>125</sup>I] SD7015. *Brain research bulletin*, 87, 504-510.


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References


References


References


References
Appendices

Appendix 1

Buffers for Perfusion-Fixation

0.2M Phosphate buffer

**Stock A**
Sodium dihydrogen phosphate monohydrate \((\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O} \text{ MW}=137.99)\)
1M=137.99g in 1L dH\(_2\)O
0.1M=13.799g in 1L dH\(_2\)O
0.2M=27.598g in 1L dH\(_2\)O

**Stock B**
Disodium hydrogen phosphate dehydrate \((\text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O} \text{ MW}=177.99)\)
1M=177.99g in 1L dH\(_2\)O
0.1M=17.799g in 1L dH\(_2\)O
0.2M=35.598g in 1L dH\(_2\)O

→For 100ml PB
9.5ml stock A + 40.5ml stock B + 50ml dH\(_2\)O =100ml PB

→For 1000ml PB
95ml stock A + 405ml stock B + 500ml dH\(_2\)O =1000ml PB

→For 2000ml PB
190ml stock A + 810ml stock B + 1000ml dH\(_2\)O =1000ml PB

Stock A: 27.598g/L x 0.19L = 5.24362g in 190ml dH\(_2\)O
Stock B: 35.598g/L x 0.81L = 28.83438g in 810ml dH\(_2\)O
Alternatively add both to 1L of dH\(_2\)O and dissolve
Make up to 2L with water

**NOTE:** Weights must be multiplied by 2 to take into account the 1:1 dilution with PFA

Fixative: 4% Paraformaldehyde (4L) (in fume hood)

1. Heat 1.5L of dH\(_2\)O to 60 °C
2. Add 160g of PFA powder
3. Add a few NaOH pellets in order to dissolve PFA
4. Stir until clear
5. Fill to 2L with dH\(_2\)O
6. Stir until clear
7. Add 2L of 0.2M PB to give a final volume of 4L
8. PH to 7.4 and cool to 4 °C
**Heparinised Saline**
1 ml heparin is added per 1L saline
Each small vial of heparin contains 25,000/5 ml i.e. 1 ml of heparin has 5000 units

**25% w/v Sucrose Solution (1L)**
1. Dissolve 5 PBS tablets in ~500ml dH₂O (1 tablet per 200ml water)
2. Add 250g of sucrose
3. Stir until dissolved, apply heat if necessary
4. Make up to 1L with dH₂O

**0.1% w/v TBS-Azide**
1. Weigh out 12g Trizma, 9g NaCl, 1g sodium azide
2. Add to 1L dH₂O and dissolve
3. PH to 7.4
## Appendix 2

### Solutions for Immunohistochemistry

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

Suitable for 30 µm free-floating sections. Cut from tissue which has been perfused with phosphate buffer, fixed in 4% buffered paraformaldehyde, and then equilibrated with 25% 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.

1. Wash 1*5 min in TBS.

2. Quench for 5min.

   - Methanol 5 ml
   - 30% H₂O₂ 5 ml
   - Dist H₂O 40 ml

3. Wash 3*5 min with TBS.

4. Incubate sections in 3% NHS* for 60 min.

   - 30 µl/ml NHS in 970 µl/ml TXTBS (freshly made-up)
   - *serum dependent on 2° host

5. Draw off excess and incubate in primary in 1% NHS* at room temperature overnight (e.g. 1:1000):

   - 1 µl/ml of 1° in 10 µl/ml NHS* in 989 µl/ml TXTBS

6. Wash 3*10 min with TBS.

7. Incubate in biotinylated secondary in 1% NHS* for 3 hours (e.g. 1:200):

   - 5 µl/ml of 2° in 10 µl/ml NHS* in 985 µl/ml TBS

8. Make ABC Complex. Wash 3*10 min with TBS.

   - 5 µl of solution A and 5 µl of solution B per ml in 10 µl/ml NHS in 980 µl/ml TBS.

9. Incubate in ABC Complex for 2 hours.

10. Wash 3*10 min with TBS.
11. Wash with TNS (freshly made-up) overnight @ 4°C.

12. Incubate in H₂O₂/DAB solution until colour develops.
   - TNS 40 ml
   - DAB 20 mg (frozen in 2 ml aliquots).
   - 30% H₂O₂ 12 µl
   - Dilute 1/5 and use.

13. Wash 3*5 min with TNS

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

15. Dehydrate in an ascending series of alcohols
   - 50% EtOH for 5 min
   - 70% EtOH for 5 min
   - 100% EtOH for 5 min
   - 100% EtOH for 5 min

   - 1st Xylene for 5 min
   - 2nd Xylene for 5 min