Development and Characterisation of Novel Gene-Environment Interaction Rat Models of Parkinson's Disease

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Declaration

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

Behavioural data acquisition for the study outlined in Chapter 3 was performed by 4th year project students Niamh Moriarty, Jennifer Feehan, Deirdre Rooney and Aoife McNulty under my supervision.

Signed:…… Date:……..

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In examining the disease, we gain wisdom about anatomy and physiology and biology. In examining the person with the disease, we gain wisdom about life – Oliver Sacks

Dedicated to everyone who has courageously fought a neurodegenerative disease.

Abstract

Despite its discovery almost 60 years ago, levodopa remains the most effective therapy for the treatment of the motor symptoms associated with Parkinson's disease. However, chronic use of levodopa is associated with serious side effects such as the emergence of dyskinesias, on-off effects and is also limited by the fact that it is purely symptomatic and does not slow progression of the disease. One of the reasons suggested for the paucity of disease-modifying therapies for Parkinson's disease is the lack of relevant animal models which reliably recapitulate the features of the human disease. To date, Parkinson's disease has been modelled extensively in preclinical animals using selective catecholaminergic neurotoxins such as 6-hydroxydopamine and MPTP, and is typically induced using a single insult. Unfortunately, these models bear little etiological resemblance to the human condition as Parkinson's disease is thought to arise as a result of complex interactions between underlying genetics and exposure to environmental factors. Therefore, the overarching aim of this project was to develop a novel model of Parkinson's disease incorporating both genetic and environmental exposures. Not only would this shed further light on relevant gene-environments interactions in the context of the etiology of Parkinson's disease, but would also provide a novel gene-environment model for testing potential neuroprotective and disease-modifying therapies for this condition.

In order to facilitate development of a novel gene-environment model, we first characterised the motor and neuropathological impairments induced by the Parkinson's disease-associated pesticide, rotenone, and the bacterial inflammagen,

Lipopolysaccharide (LPS) and compared them directly to those induced by the 'gold standard' catecholamine neurotoxin, 6-OHDA. We then went on to assess the impact of dual exposure of rats to overexpression of the Parkinson's disease-associated protein, α-synuclein, and bacterial-like neuroinflammation and neurodegeneration driven by LPS. Finally, we investigated the impact of α-synuclein overexpression in combination with the agritoxin-driven Parkinsonism induced by rotenone. With the single lesion models induced by 6-OHDA, rotenone and LPS, we found that despite similar levels of neurodegeneration, the neurotoxic, environmental and bacterial triggers induced distinctly different patterns of motor dysfunction. With the dual exposure gene-environment studies, we found that exposing rats to both AAV-α-synuclein and LPS did not exacerbate the Parkinsonism caused by either toxin alone. In contrast, dual exposure to AAV-α-synuclein and rotenone significantly exacerbated the level of motor dysfunction and neurodegeneration caused by the genetic or environmental factor alone.

Overall, this research has shown that different Parkinson's disease-related neurotoxins can induce different patterns of motor dysfunction indicating the importance of the choice of lesioning agent in preclinical Parkinson's disease studies. We have also shown that dual exposure of rats to overexpression of the Parkinson's disease-associated protein, α-synuclein, and the Parkinson's disease-associated pesticide, rotenone, induced a highly relevant rat model of the condition with improved etiological relevance over existing models. Thus, this model may provide a suitable platform for testing novel neuroprotective and disease-modifying therapies for this condition.

Publications

Peer Reviewed Published Manuscripts

1. **C Naughton,** D O'Toole, E Dowd. (2016) Interaction between subclinical doses of the Parkinson's disease associated gene, α-synuclein, and the pesticide, rotenone, precipitates motor dysfunction and nigrostriatal neurodegeneration in rats. Under review at Neurobiology of Aging.

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3. J Samal, DB Hoban, **C Naughton**, R Concannon, E Dowd, A Pandit. (2015) Fibrin-based microsphere reservoirs for delivery of neurotrophic factors to the brain. Nanomedicine. 2015 Mar, 10(5):765-83.

Peer Reviewed Published Abstracts

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2. J Samal, N Moriarty, DB Hoban, **C Naughton**, RM Concannon, E Dowd, A Pandit (2014). Fibrin-based Hollow Microsphere Reservoirs for Controlled Delivery of Neurotrophic Factors to the Brain. Tissue Engineering Part A 21, S62-S62.

Other Research Dissemination

International Conferences Attended

1. **C Naughton,** D O'Toole, E Dowd. Intra-striatal administration of the environmental pesticide rotenone profoundly exacerbates motor dysfunction in the AAV−α-synuclein model of Parkinson's disease. Oral Presentation at Network of European CNS Transplantation and Restoration (NECTAR), Sweden, December 2015.

National Conferences Attended

1. **C. Naughton,** DB Hoban**,** RM Concannon, J Feehan, A McNulty, N Moriarty, D Rooney, E Dowd. Comparative assessment of the motor dysfunction induced by neurotoxic, inflammatory and environmental Parkinson's disease-related neurotoxins in rats. **Poster Presentation** at Young Neuroscientists Symposium, TCD, Sept 2014

2. **C. Naughton,** D O'Toole, E Dowd. Exacerbation of motor dysfunction in the AAV-α-synuclein model of Parkinson's disease after intra-striatal infusion of the pesticide rotenone. **Poster Presentation** at Neuroscience Ireland, DCU, Sept 2015

List of commonly used abbreviations

ANOVA	analysis of variance
COMT	catechol-O-methyl transferase
CNS	central nervous system
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
Fig.	figure
g	gram
GFP	green fluorescent protein
Gpe	globus pallidus externa
Gpi	globus pallidus interna
h	hour
i.p.	intraperitoneal
IHC	immunohistochemistry
kg	kilogram
LPS	lipopolysaccharide
MAO	monoamine oxidase
MFB	medial forebrain bundle
µg	microgram
mg	milligram
mg kg^{-1}	milligram per kilogram
min	minute
ML	mediolateral
mm	millimetre
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
ng	nanogram

6-OHDA	6-hydroxydopamine
s	second
SEM	standard error ± mean
SN	substantia nigra
STR	striatum
TBS	Tris-buffered saline
TH	tyrosine hydroxylase
UPDRS	Unified Parkinson's Disease Rating Scale

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 the second most common neurodegenerative disease affecting 3% of the population over 65 years of age (de Lau and Breteler, 2006) and in terms of incidence rates, it is second only to Alzheimer's disease (Mayeux et al., 2003). It is characterised by the progressive loss of dopaminergic neurons from the substantia nigra *pars compacta* and by the formation of intraneuronal cytoplasmic inclusions known as Lewy bodies (Eriksen et al., 2005a). The subsequent loss of dopamine from the striatum clinically manifests as tremor at rest, stiffness and slowness of movement (Jankovic, 2008). While the exact cause of the disease remains elusive to this day, it is widely accepted that Parkinson's disease is a multifaceted disease with a variety of environmental factors implicated in its etiology (Priyadarshi et al., 2001).

Despite decades of research, there is still no cure for Parkinson's disease. Current pharmacological treatments are based on increasing or replacing the endogenous neurotransmitter dopamine so as to minimise the motor fluctuations which manifest clinically when the loss of dopamine from the nigrostriatal pathway exceeds more than 80% (Cheng et al., 2010). One of the tragedies of Parkinson's disease is that by the time a patient presents with the clinical features of the disease, it has already progressed to an advanced stage where there is already significant nigrostriatal neurodegeneration and there is no therapy available which can either halt disease progression or replace the neurons already lost in the disease course.

One of the reasons put forth for the paucity of neuroprotective therapies for Parkinson's disease is the lack of animal models which faithfully reproduce the

etiological, neuropathological and motor features of the human condition (Beal, 2001). Despite decades of research, the etiology of idiopathic forms of the disease is still poorly understood but it is thought to arise as a result of complex interactions between a person's genetics and their environmental exposures, including exposure to pesticides and infection (reviewed in Liu and Bing, 2003). Although, this gene-environment hypothesis of idiopathic Parkinson's disease is widely accepted, the disease is still most commonly modelled in experimental animals using unitary, or single, neurotoxic insults which are not etiologically relevant to the clinical condition (reviewed in Blesa et al., 2012).

With this in mind, the work described in this thesis sought to develop and characterise more relevant gene-environment interaction models of Parkinson's disease. We focussed specifically on the interaction between pathological overexpression of the Parkinson's-related protein, α-synuclein and subsequent exposure to inflammation or pesticides. Ultimately, it is hoped that this work will shed further light on gene-environment interactions in the context of the etiology of Parkinson's disease and also provide a novel gene-environment model for testing potential neuroprotective and disease-modifying therapies for this condition.

1.1 Parkinson's disease

Parkinson's disease is one of the most common neurological diseases which affect approximately 3% of the population over 65 years of age and approximately 4-5% of people over the age of 85 (Satake et al., 2009, Whitton, 2007). Incidence rates of the disease increase sharply with age with more than 90% of idiopathic cases of the

disease occurring in persons over the age of 50 (Van Den Eeden et al., 2003). The remaining 10% of diagnosed patients have hereditary factors or genetic links which typically manifest before the age of 50 (Tarazi et al., 2014). Despite the fact that the clinical features only emerge in the advanced stages of the disease, the only conclusive confirmation of Parkinson's disease is *post-mortem* pathological examination of brains and subsequent identification of proteinaceous Lewy body inclusions which are still the defining feature of the disease (Michotte, 2003, Forno, 1996). Sadly, the onset of clinical symptoms only emerges once 40-50% of dopaminergic cell bodies have degenerated from the substantia nigra with almost an 80% loss of striatal dopamine (Lang and Lozano, 1998). The presence of two or more cardinal motor symptoms of tremor at rest, rigidity, akinesia or postural instability is the primary diagnostic process for the disease despite the identification of these symptoms by James Parkinson almost 200 years ago. Once referred to as a motor disease, a wealth of evidence has emerged in recent years confirming the presence of non-motor symptoms which have now been confirmed to precede the onset of the motor symptoms (Foltynie et al., 2004). The non-motor symptoms, whilst initially under-recognised, are now being reported extensively and they include neuropsychiatric issues such as depression, dementia and cognitive deficits. Patients have also reported significant disruption to their sleep patterns as well as a host of other autonomic, gastrointestinal and sensory disturbances (Chaudhuri et al., 2006). The consequences of these non-motor symptoms of Parkinson's disease are almost as disabling as the motor symptoms leading to increased healthcare costs in the years preceding onset of motor symptoms (Chaudhuri and Schapira, 2009).

1.1.1 An historical perspective of Parkinson's disease

The symptoms of Parkinson's disease were first described by an Indian civilisation as far back as 5,000 B.C where they referred to the motor features of the disease as Kampavata (kampa-tremors) (Lang and Lozano, 1998) derived from the words kampa (tremors) and vata (motor actions) (Singhal et al., 2003). This Indian civilization practiced a medical doctrine called Ayurveda which involved the concept of understanding a disease based on physical, physiological and psychological processes in order to devise suitable treatment strategies (Gourie-Devi et al., 1991). In order to treat Kampavata, a tropical legume was used called atmagupta (*Mucuna pruriens* or velvet bean/cowhage) which has since been shown to contain therapeutic quantities of L-DOPA (Vaidya et al., 1978). However, the first detailed description of the disease came from the ancient Greek physician, Galen (129-200 AD), who described a resting tremor in his book entitled 'De Tremore, Palpitatione, Convulsione et Rigore' (Raudino and Leva, 2012).

Then in 1817, a London based physician by the name of James Parkinson published a comprehensive and detailed description of six patients aptly named 'An Essay on the Shaking Palsy'. In his monologue, he refers to paralysis agitans and describes the condition as an 'involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the sense and intellect being uninjured'. He provides a comprehensive review of different shaking palsies with a passive trembling which can cease if the trembling limb is supported, however in the case of paralysis agitans, the reverse of this takes place with the agitation continuing

even when the limb is at rest. Although, the backgrounds of these six patients were significantly different from each other, the clinical symptoms he noted were strikingly similar to each other. He describes a 'slight sense of weakness with difficulty in preserving upright posture most observable whilst walking'. Interestingly, Parkinson also alluded to some of the non-motor symptoms of the condition: 'the saliva fails of being directed to the back part of the fauces, and hence, is continually draining from the mouth'. Ultimately, the motor complications would be the focus of this disease as 'debility increases and the influence of the will over muscles fade away and the tremulous agitation becomes more vehement' (Parkinson, 1817; republished in 2002).

Shortly thereafter, and unaware of James Parkinson's physical observations of paralysis agitans, Wilhelm von Humboldt (1767-1835) described his own medical history in a series of letters until his death in 1835. In them he provides a complex and detailed description of the manifestations of his disease which included a resting tremor with particular problems associated with writing which he referred to as a 'special clumsiness'. However, he equated this 'internal tremor' to an accelerated aging process related to the death of his wife (Horowski et al., 1995). Then, in 1861 the prolific French neurologist, Jean-Martin Charcot, suggested that paralysis agitans be renamed Parkinson's disease as a tribute to James Parkinson having provided the first clinical description of the disease. Moreover, Charcot also further characterised the behavioural aspects of the disease as he noted that patients presenting with Parkinson's disease did not necessarily present with a tremor. He also observed that long before rigidity actually develops, patients had significant difficulty in performing ordinary activities, although there was the possibility that this was the

consequence of another affliction. Charcot detailed how easy it was to identify the difficulty with which they performed normal tasks even though rigidity or tremor were not the limiting features of the physical observations made (Charcot, 1872). Interestingly, Charcot also classified the disease as ‘nervose’ meaning that it was a neurological disorder with no known pathological lesion (Goetz, 1986).

The intricate relationship between the substantia nigra and Parkinson’s disease was first alluded to in 1892 when a 38 year old man presented to Charcot’s clinic with tuberculosis but was also displaying Parkinsonian symptoms. Upon autopsy, it was discovered that this patient had a tumour in the substantia nigra (Parent and Parent, 2010). Then in 1912, the German-American neurologist Friedrich Lewy, described abnormal protein deposits in the brains of patients with Parkinson’s disease (Lewy, 1912). These inclusion bodies were termed Lewy bodies and have since become the distinguishing neuropathological feature of the disease and the only absolute diagnosis of the disease. Further support for the role of the substantia nigra in Parkinson’s disease came about when it was discovered that these Lewy bodies are most concentrated in the substantia nigra. Despite this observation, it took several decades for the biochemical changes underlying the motor and non-motor symptoms of this condition to be elucidated. The neurochemical nature of the disease was first demonstrated in 1960 when Ehringer and Hornykiewicz reported that there was a significant reduction in the concentration of dopamine in the caudate and putamen of Parkinsonian patients (Ehringer and Hornykiewicz, 1960).

Charcot’s medical intern was the first to describe the use of anticholinergic alkaloids, which were extracted from the root of the belladonna plant and the first well-

established treatment of Parkinson's disease (Ordenstein, 1972). Indeed, the use of anticholinergics was the primary focus of therapeutic intervention in Parkinson's disease until the 1950s when Arvid Carlson made the seminal discovery that dopamine was a neurotransmitter in its own right and not just a precursor to noradrenaline which had been previously thought and in addition to that he also discovered that the concentration of this neurotransmitter was highest in the striatum. Then in 1957, he demonstrated that the effects of reserpine-induced dopamine depletion (i.e. severe akinesia) could be reversed by administration of the dopamine precursor, L-DOPA, and this recovery correlated with a restoration of dopamine levels in the brain which provided the first conclusive report that dopamine was involved in modulating motor control (Carlsson et al., 1957). Following this discovery, George Cotzias conducted the first open label clinical trial in which he demonstrated that oral administration of L-DOPA could ameliorate the motor symptoms of Parkinson's disease (Cotzias et al., 1969a, Cotzias et al., 1969b). Since then, L-DOPA has remained the mainstay of treatment for the motor symptoms associated with Parkinson's disease.

1.1.2 Pathophysiology

Although Parkinson's disease has been classically defined as a movement disorder encompassing a broad range of motor manifestations which have been very well characterised, to date our understanding of the etiopathogenesis of the disease remains very limited (Chaudhuri et al., 2006, Schapira and Jenner, 2011). As previously mentioned, the cardinal features of the disease include a tremor at rest, rigidity, akinesia and postural instability and these motor symptoms become

progressively worse as the disease course progresses. There are also a host of secondary motor symptoms which include loss of spontaneous movements (akinesia), festinating gait, freezing and dystonia (Tarazi et al., 2014). It is the progressive degeneration of the nigrostriatal pathway of the basal ganglia circuitry which results in the clinical presentation of these motor fluctuations.

The heterogeneity among patients makes the disease very difficult to diagnose. Moreover, there are two different clinical phenotypes which differ in both their disease course and treatment (Rajput et al., 2009). These subtypes include tremor-dominant Parkinson's disease and akinetic-rigid Parkinson's disease. In the tremor-dominant subtype of the disease, the progression of neuronal cell loss is more extensive in the ventrolateral region of the substantia nigra, whereas with the akinetic-rigid subtype there is more severe neuronal loss associated in the lateral region of the substantia nigra (Jellinger, 1991, Jellinger, 1999). The neurodegenerative processes underlying disease progression are known to begin several years prior to the onset of any clinical symptoms or in the 'subclinical phase' of the disease but tragically by the time these symptoms appear, the disease is in the advanced stages where 70% of dopaminergic neurons have degenerated and there is no effective pharmacological treatment available which can halt or slow disease progression (Fearnley and Lees, 1991).

1.1.2.1 Nigrostriatal degeneration

Dopaminergic loss from the substantia nigra *pars compacta* has been identified as one of the primary neuropathological features of Parkinson's disease. There is a

spatiotemporal progression of neuronal cell loss which extends from the substantia nigra *pars compacta* and continues along a rostro-caudal axis finally terminating in the striatum (Damier et al., 1999a, Damier et al., 1999b, Braak et al., 2003). The resulting degeneration of the nigrostriatal pathway ultimately disrupts the delicate basal ganglia motor circuitry which is critical in modulating control of motor function.

1.1.2.1.1 The basal ganglia

The basal ganglia include several functionally different structures, namely, the striatum, which incorporates the caudate putamen and the nucleus accumbens, the subthalmic nucleus, the globus pallidus and the substantia nigra (Parent and Hazrati, 1995a, Parent and Hazrati, 1995b, Alexander et al., 1986). The most abundant neuronal type in the striatum are medium spiny neurons and these neurons are the target for nigrostriatal dopaminergic innervation (Deutch et al., 2007). These neurons utilise GABA as a neurotransmitter and controlled movement is facilitated by both direct and indirect striatopallidal GABAergic signalling (Lalchandani et al., 2013). GABAergic medium spiny neurons of the direct pathway express the excitatory D_1 subtype of dopamine receptor and project directly to the globus pallidus *pars interna* (DeLong and Wichmann, 2007) and the substantia nigra *pars reticulata* (SNr), while medium spiny neurons of the indirect pathway express the inhibitory D_2 subtype of dopamine receptor and pass through the external segment of the globus pallidus *pars externa* and the subthalamic nucleus (Fig. 1.1) (Lewis et al., 2003a). Nigrostriatal input to these GABAergic neurons, through release of dopamine, activates the direct pathway and inhibits the indirect pathway. This results (directly and indirectly) in

inhibition of the nigrothalamic GABAergic neurons which ultimately releases the inhibition of the glutamatergic thalamocortical neurons and facilitates voluntary motor activity (Wichmann and Delong, 2007).

In the Parkinsonian brain, degeneration of the nigrostriatal dopaminergic neurons reduces the availability of dopamine in the striatum and results in decreased activation of the direct pathway and increased activation of the indirect pathway (Fig. 1.2) (Lewis et al., 2003b). This results (directly and indirectly) in sustained activation of the nigrothalamic GABAergic neurons and sustained inhibition of the glutamatergic thalamocortical neurons thereby reducing voluntary motor control (Fisone et al., 2007).

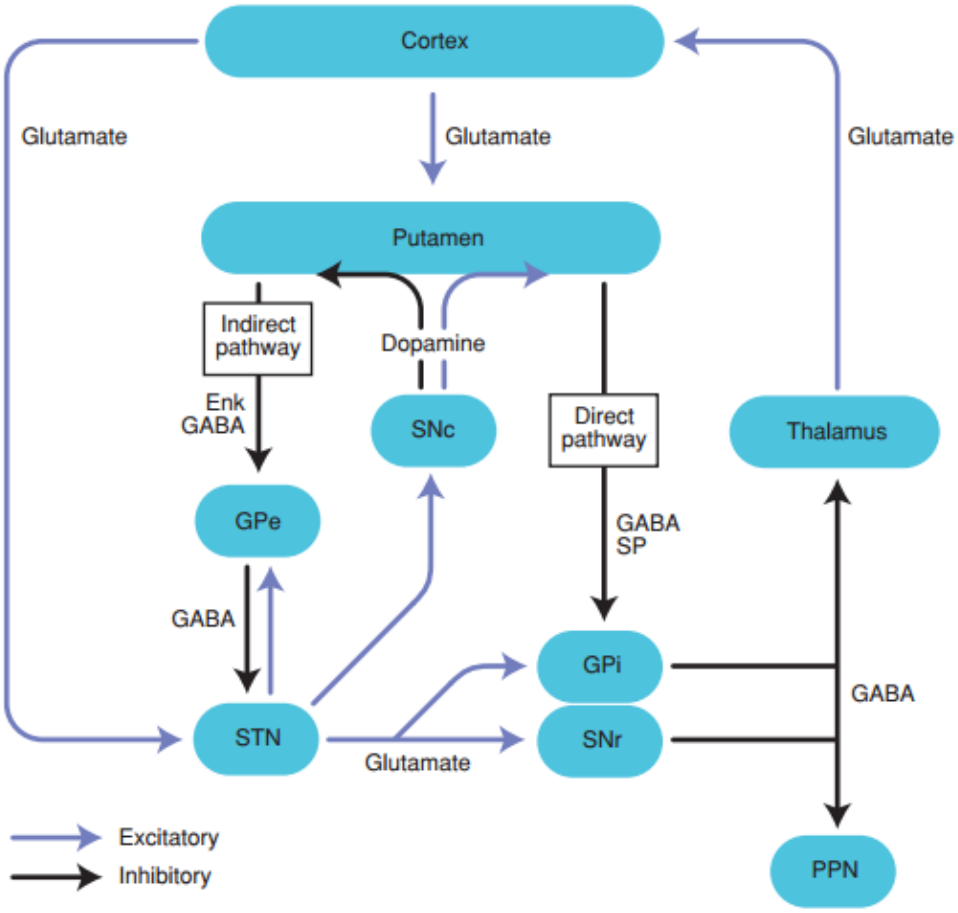


Fig. 1.1: A diagram of the basal ganglia circuitry in the normal brain. In the normal brain, the basal ganglia circuitry exerts opposing functions in order to facilitate movement through the direct and indirect pathways. Dopamine released from nigrostriatal neurons into the striatum stimulates D_1 receptors and activates the direct pathway thereby disinhibiting the thalamocortical neurons and thus facilitates motor function. Meanwhile, activation of D_2 receptors ultimately leads to increased inhibition of thalamocortical neurons of the indirect pathway reducing the excitation of motor cortical areas. Image taken from Lewis et al., (2003b).

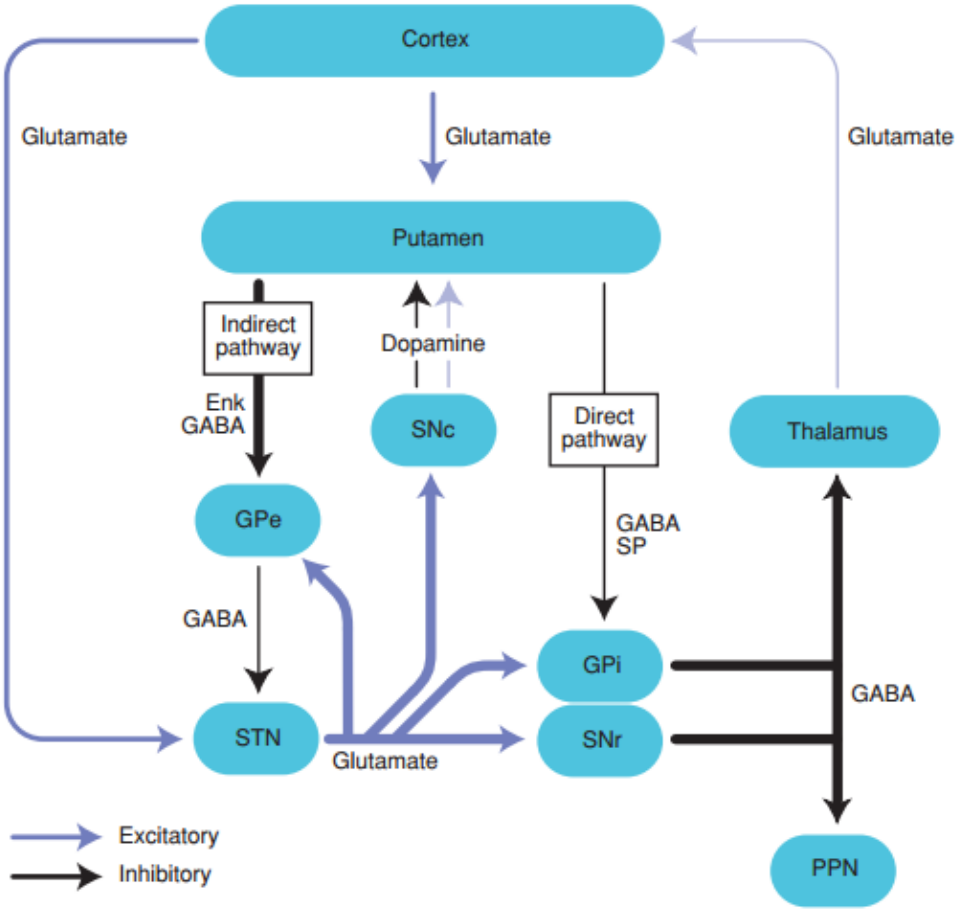


Fig. 1.2: A diagram of the basal ganglia circuitry in the Parkinsonian brain. In Parkinson's disease, the natural balance of the basal ganglia is disrupted and a decrease in the level of striatal dopamine results in decreased activation of the direct pathway and excessive stimulation of the indirect pathway which ultimately reduces movement. Image taken from Lewis et al., (2003b).

1.1.2.1.2 Loss of nigrostriatal dopaminergic neurons

At the onset of motor dysfunction, the disease has already progressed to a point whereby the concentration of striatal dopamine has decreased by almost 80% with a concomitant loss of almost 50% of nigral cell bodies (Hirsch, 1994, Fearnley and Lees, 1991). *Post mortem* analysis has revealed that neurodegeneration occurs over a

prolonged period of time and the delay in the emergence of the motor features of the disease are due to physiological mechanisms compensating for the reduction of striatal dopamine (i.e. the compensated stage of Parkinson's disease). When the loss of dopaminergic neurons becomes severe only then does the clinical syndrome become apparent (i.e. the decompensated stage). The loss of neurons from the Parkinsonian brain appears to be quite diverse with the most severe loss of dopaminergic neurons from the ventrolateral tier of the substantia nigra followed by partial damage to the medial ventral tier and dorsal tier (Damier et al., 1999a, Damier et al., 1999b). The reason proposed for the discrepancy between the progressive neurodegenerative effects of the disease and the onset of symptomatic features is the functional compensatory mechanisms of the surviving nigrostriatal terminals (Hornykiewicz, 1998, Zigmond et al., 1990a, Lee et al., 2000).

The compensatory mechanisms which allow for neurons to maintain control of striatal terminals and regulate homeostasis may be as a result of postsynaptic changes such as upregulation in dopamine receptor synthesis and membrane expression (Zigmond and Stricker, 1980, Creese and Snyder, 1979). Another compensatory mechanism postulated is that surviving nigrostriatal neurons synthesise and release more dopamine thereby maintaining dopamine striatal innervation (Hornykiewicz, 1975). As the disease progresses, the functional activity of remaining dopaminergic neurons becomes compromised with the result that structures outside the basal ganglia may then play a part in compensating for continued loss of striatal dopamine (Sokoloff et al., 1977, Bezard et al., 2001). Once a critical level of neurodegeneration has been reached, the functional organisation of the basal ganglia is compromised and surviving dopaminergic neurons become hyperactive in an attempt to maintain

dopamine homeostasis. This is believed to account for the akinesia and rigidity observed clinically in the disease (Zigmond et al., 1990b, Calne and Zigmond, 1991).

The degeneration of nigrostriatal dopaminergic neurons from the basal ganglia circuitry progresses relentlessly for years prior to the onset of motor fluctuations when patients are asymptomatic. This early onset pathology has also been characterised by the presence of morphologically aberrant, swollen axonal terminals in the putamen of early stage Parkinson's disease which are associated with α-synuclein deposition (Chu et al., 2009, Chu et al., 2012).

1.1.2.2 Lewy bodies and α-synuclein

Another defining neuropathological feature of Parkinson's disease is the presence of intraneuronal proteinaceous inclusions referred to as Lewy bodies, first described in 1912 by Friedrich Heinrich Lewy (Lewy, 1912). These eosinophilic inclusions are primarily located in the cytoplasm of dopaminergic cell bodies of the substantia nigra and their primary constituent is the protein α-synuclein (Spillantini et al., 1997). These inclusion bodies have been implicated in the degenerating Parkinsonian brain, be it genetic or idiopathic, with very little exception (Poulopoulos et al., 2012). Moreover, these cytoplasmic entities have also been identified in monoaminergic, cerebral cortical and other neurons, but are mostly concentrated in the substantia nigra (Ross and Poirier, 2004). A typical Lewy body is 8-30 μm in diameter and its conformation consists of a granular core surrounded by radiating filaments (McNaught, 2004).

α-synuclein is a 140 amino acid protein with a native unfolded structure and a hydrophobic core which increases its propensity to self-associate in neurons (Baba et al., 1998). This protein is heavily concentrated in presynaptic terminals and is found in both soluble and membrane-associated fractions in the brain (Iwai et al., 1995). Although the exact function of α-synuclein has yet to be elucidated, there have been several theories proposed as to its normal physiological role. α-synuclein has been associated with synaptic activity since the mid-1990s when it was shown to be upregulated during song acquisition in zebra finches, a period of enormous synaptic plasticity (Fig 1.3) (George et al., 1995). Subsequent studies have also revealed a role for this protein in synaptic processes, in the release of neurotransmitters, and perhaps interestingly, have highlighted a heightened sensitivity of nigrostriatal dopaminergic neurons to genetic loss of α-synuclein function (Abeliovich et al., 2000b, Scott et al., 2010, Gorbatyuk et al., 2010). Therefore, although the function of this protein has yet to be fully elucidated, it is clear that a basal level of α-synuclein expression is required in order to maintain neuronal homeostasis (Gorbatyuk et al., 2010).

The central role that α-synuclein plays in the progression of Parkinson's disease is highlighted by the fact that Lewy bodies are a neuropathological hallmark of both idiopathic and genetic forms of the disease (Spillantini et al., 1997) and that missense mutations in the *SNCA* gene cause autosomal dominant Parkinson's disease (Polymeropoulos et al., 1997, Krüger et al., 1998, Zarranz et al., 2004). It has been suggested that α-synuclein exists in its native conformation in equilibrium and in an α-helical conformation (Dickson et al., 1989). However, under pathological conditions, the protein can undergo a profound conformational transition to form pathogenic species namely dimers, trimers and oligomers (Fig. 1.4). The pathogenic

intermediates of α-synuclein can aggregate into higher order structures such as protofibrils composed of β-pleated sheets and these higher order structures are the platform for the formation of the Lewy bodies (Irwin et al., 2013).

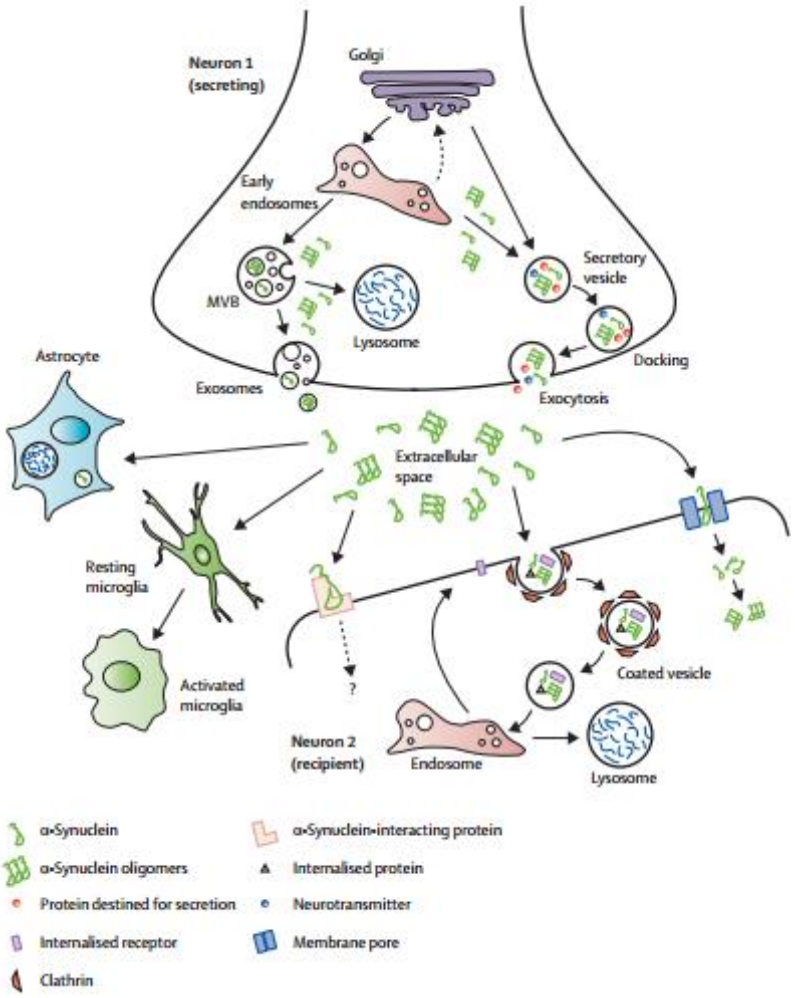


Fig 1.3: α-synuclein secretion and paracrine interactions. α-synuclein is secreted through non-classic endocytic or exocytic pathways. In early endosomes, protein material is either recycled back to the plasma membrane or sorted to MVBs. Cytoplasmic α-synuclein species can enter MVBs at this point, via inward budding of the limiting membrane of these vesicles. Subsequently, MVBs fuse with lysosomes for degradation or with the plasma membrane to release their content in the extracellular space as exosomes. Alternatively, α-synuclein species can be incorporated into secretory vesicles and be released via exocytosis. Once in the extracellular space, α-synuclein can affect the homoeostasis of recipient neurons by association with as yet unknown membrane proteins or receptors, endocytosis of clathrin-coated pits, or formation of pores on the recipient plasma membrane. In parallel, α-synuclein triggers neuroinflammatory responses via microglia activation. Finally, α-synuclein can be cleared through endocytosis by astrocytes. MVB=multivesicular body. Image taken from Vekrellis et al., (2011).

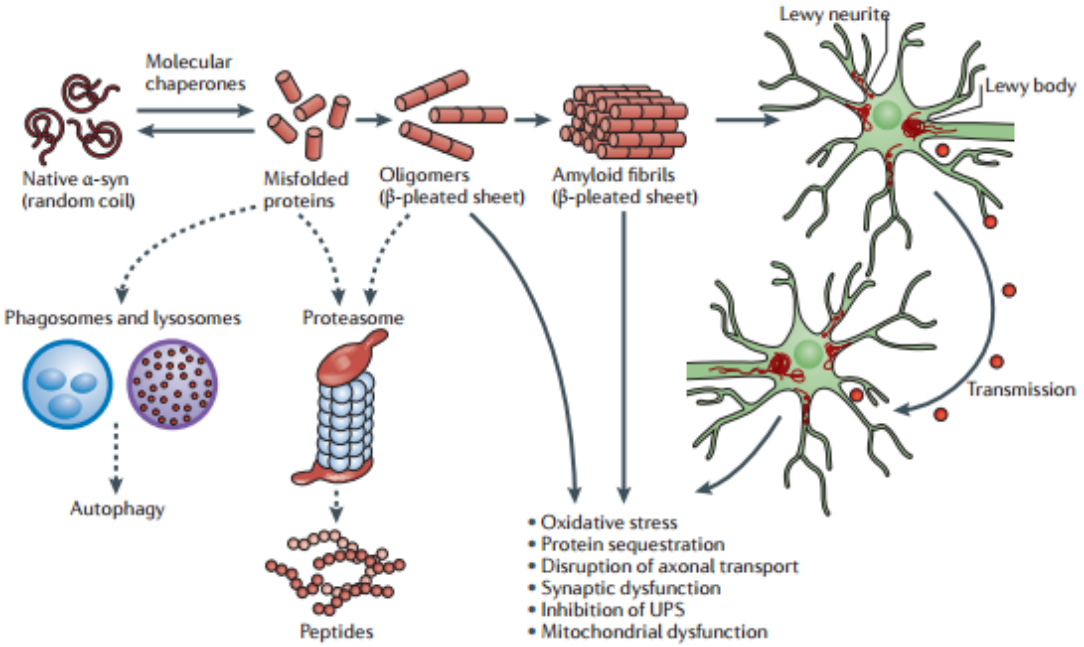


Fig. 1.4: Summary of the proposed mechanism by which the aggregation and spread of α-synuclein leads to dopaminergic cell death in Parkinson's disease. Under normal physiological conditions, α-synuclein exists as a monomer in a soluble unfolded state. However, under pathological conditions, the structure of the protein can change and it can fold into pathogenic species such as dimers, trimers and/or oligomers. These pathogenic species can aggregate into higher order structures such as protofibrils and these higher order structures are the platform for the formation of the pathological inclusion bodies termed Lewy bodies which ultimately compromise cell survival and results in dopaminergic cell death. Image taken from Irwin et al., (2013).

It has been proposed that Lewy body pathology first appears in the parasympathetic neurons of the peripheral nervous system, more specifically in the enteric neurons of the gut and from here progresses to lower brain regions via the vagal nerve (Braak et al., 2002). The resulting α-synuclein pathology in the dorsal motor nucleus and olfactory bulbs result in the onset of autonomic and olfactory deficits (i.e. the

subclinical phase). Once α-synuclein pathology reaches the lower brain regions it then spreads rostro-caudally to the mature neocortex and it is in this advanced stage of the disease that the motor aspects of the clinical syndrome manifest (i.e. the clinical phase) (Fig. 1.5).

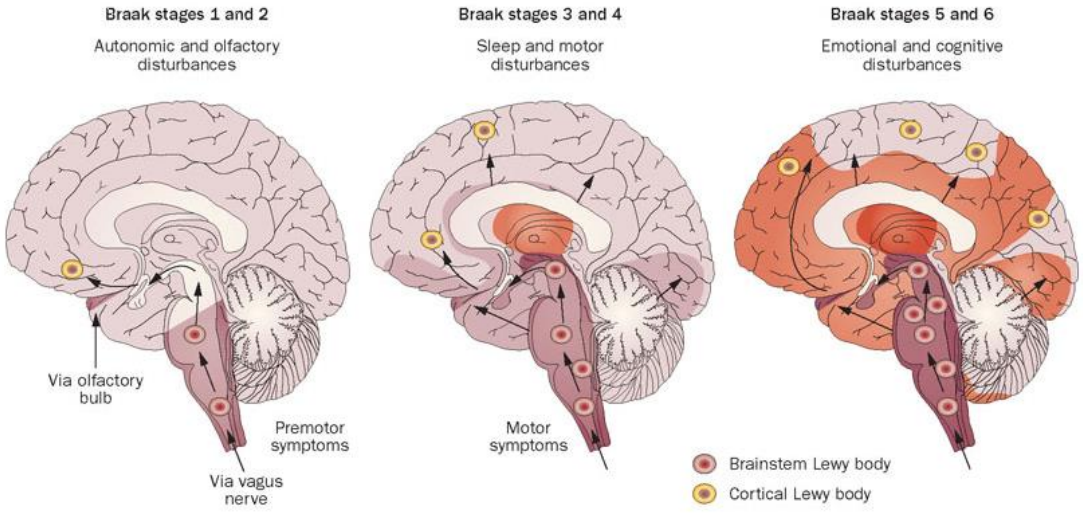


Fig. 1.5: Schematic representation of the Braak staging of Parkinson's disease. This diagram depicts the initiation sites of α-synuclein pathology in the olfactory bulb and the medulla oblongata which continues through to the later infiltration of Lewy pathology into the cortical regions. Image taken from Halliday et al., (2005).

It has been suggested that this stereotypical pattern of α-synuclein pathology advances via cell-to-cell transmission of the protein and this theory gained considerable momentum with results of clinical trials which suggest that α-synuclein is capable of spreading from one cell to another by the appearance of Lewy body pathology in grafted fetal neurons in recipients with Parkinson's disease (Li et al., 2008). Further compelling evidence regarding the prion like transmission of α-synuclein was supported by the observation made by Luk and colleagues who

demonstrated that injection of preformed α-synuclein fibrils resulted in widespread α-synuclein pathology in young asymptomatic α-synuclein transgenic mice (Luk et al., 2012a, Luk et al., 2012b). Moreover, *in vitro* analysis also confirmed the ability of mouse cortical stem cells, incubated with fluorescently-tagged α-synuclein, to endocytose extracellular α-synuclein resulting in the formation of inclusion bodies and subsequent cell death (Desplats et al., 2009, Hansen et al., 2011). Together, these data support the hypothesis that α-synuclein is a prion-like protein and the transcellular concept of α-synuclein seeding underpins abnormal α-synuclein deposits in the Parkinsonian brain.

In addition to nigrostriatal neurodegeneration and Lewy body pathology, Parkinson's disease is also associated with a host of other neuropathological features including neuroinflammation, mitochondrial dysfunction and oxidative stress, and these will be outlined in the coming sections.

1.1.2.3 Neuroinflammation

There is overwhelming evidence to support a role for neuroinflammation in the pathophysiology of Parkinson's disease. This was first postulated by McGeer and colleagues in 1988 who reported that activated microglia and T lymphocytes were detected in the substantia nigra of *post mortem* Parkinsonian brains (McGeer et al., 1988a). Subsequent follow-up studies indicated that there is also an upregulation in astrocytes in the substantia nigra of patients of *post mortem* Parkinsonian brains (Damier et al., 1993). In the intervening years, PET scans confirmed the persistent activation of microglia in patients with advanced idiopathic Parkinson's disease

(Gerhard et al., 2006), and since then, numerous studies have demonstrated a concomitant upregulation in the expression of proinflammatory cytokines such as tumour necrosis factor (TNF), interleukin-1 (IL-1) and interferon-γ (INFγ) in the CSF of patients with the disease (Banati et al., 1998, Gerhard et al., 2006, Lindqvist et al., 2013). Neuroinflammation has been implicated in a host of other neurodegenerative disease such as Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis and Guillain-Barre syndrome (Frank-Cannon et al., 2009, McGeer and McGeer, 2003, McGeer and McGeer, 2002a, Creange et al., 2002). The driving role that neuroinflammation plays in Parkinson's disease pathogenesis is highlighted by the epidemiological finding that there is a reduced risk of Parkinson's disease in people who chronically use non-steroidal anti-inflammatory drugs (Chen et al., 2003).

Microglia can be described as innate immune 'surveillance cells', comprising 5-20% of all glial cells, whose function is to patrol the CNS and safeguard it against infection and injury. They become activated in response to bacterial or viral infections and to other toxic insults such as aberrant protein aggregation (Jansen et al., 2014, Deleidi et al., 2010, Zhang et al., 2005). The microglial reaction in response to a neurotoxic insult comprises both an increase in the number of microglia and a morphological change in the cell from a resting to an activated or amoeboid state (Breidert et al., 2002, Brochard et al., 2009). This activation is required to maintain homeostasis of the CNS however overactivation, or sustained activation in response to cellular stress signals, is ultimately neurotoxic. Intriguingly, microglia are not uniformly distributed throughout the brain and there are regions which are more densely populated. One of the most densely populated regions of the brain is

the substantia nigra (Kim et al., 2000, Lawson et al., 1990) and it is in these densely populated regions that neurons are more vulnerable to persistent activated microglia-mediated cell death (Machado et al., 2011).

Whether neuroinflammation is a consequence of neurodegeneration or whether it initiates neurodegeneration is still debatable. However, it is generally accepted that ongoing active neurodegeneration harnesses a persistent neuroinflammatory response (Fig. 1.6) (Glass et al., 2010, Gao and Hong, 2008, Tansey and Goldberg, 2010). This proposal has been strengthened by the finding that 16 years following an initial insult with MPTP, activated microglia and reactive astrocytes were found in the human brain at *post mortem* (Barcia et al., 2004, Langston et al., 1999, Kanaan et al., 2008). Thus, whatever the initial insult is, be it genetic and/or environmental, there is a self-sustaining cycle of neuroinflammation and neurodegeneration where activated microglia release several neurotoxic factors that damage and kill neurons, and conversely, stressed and dying neurons activate microglia (Gao and Hong, 2008, Glass et al., 2010, Tansey and Goldberg, 2010).

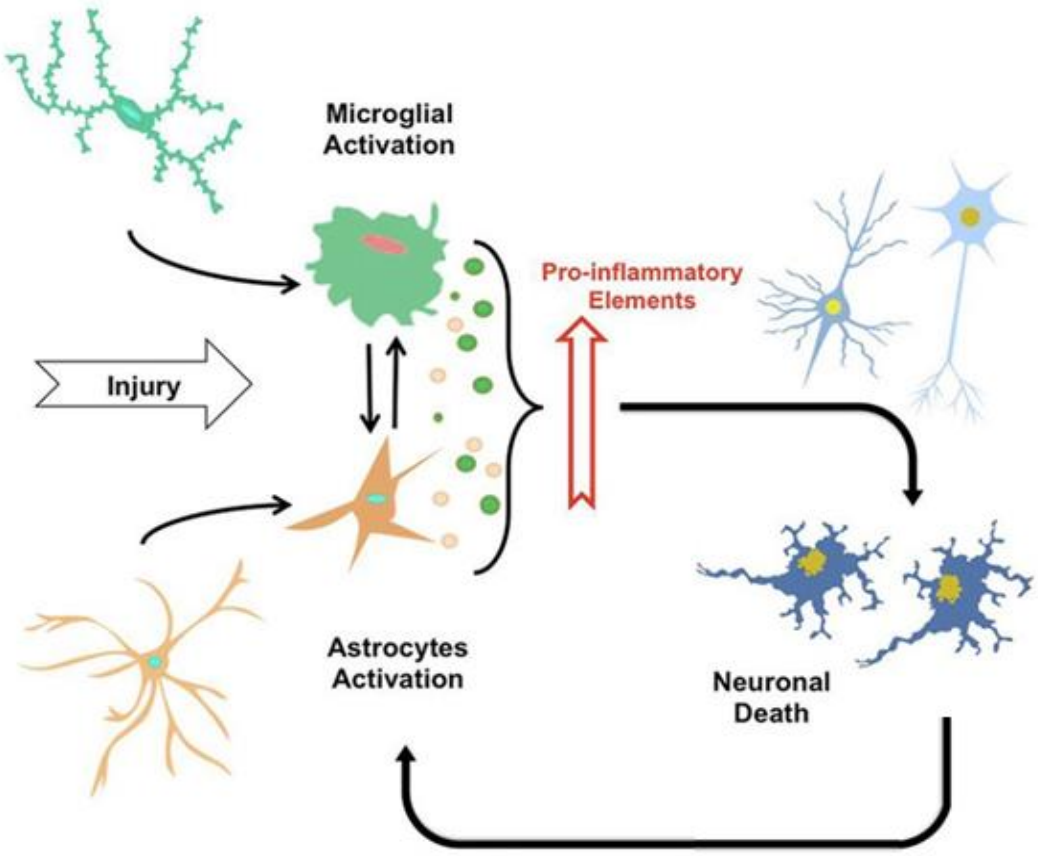


Fig. 1.6: The neuroinflammatory process. In response to stimuli that cause damage or injury to the CNS, astrocytes and microglia undergo significant alterations which result in a morphological change from a resting to an activated state. This results in the secretion of proinflammatory elements (cytokines, cytotoxic elements, ROS) which are ultimately toxic to neurons. Degenerating neurons then emit signals to further activate microglia (which in an uncontrolled activation are toxic to surrounding neurons) perpetuating a self-sustaining cycle of neuroinflammation and neurodegeneration. Image modified from Morales et al., (2014).

1.1.2.4 Mitochondrial dysfunction and oxidative stress

Mitochondria play a critical role in regulating cell survival and cell death, and there are many factors which contribute to mitochondrial dysfunction. Mitochondria are the powerhouse of the cell and are actively involved in the generation of cellular energy in the form of adenosine triphosphate (ATP). In order to facilitate production of ATP, the inner structure of mitochondria is folded which increases the surface area of the mitochondrion to produce cellular energy from the electron transport chain. ATP generated from mitochondria supplies over 95% of the total energy requirements for the eukaryotic cell (Erecinska and Wilson, 1982). The physiological functions of neurons require that they produce significant amounts of energy and in concert have considerable numbers of mitochondria. The dense concentration of mitochondria in neurons renders them highly vulnerable to neurotoxic insults which ultimately disrupt mitochondrial function (Winklhofer and Haass, 2010).

The first indication that mitochondrial dysfunction may be a feature in Parkinson's disease emerged in 1982 when a group of young drug addicts developed a form of Parkinsonism after self-administering merperidine contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahrdropyridine (MPTP). The clinical consequences of MPTP self-administration were reminiscent of patients who were in the advanced stages of Parkinson's disease (Langston et al., 1983, Ballard et al., 1985). It was subsequently revealed that the metabolite of MPTP (MPP^+), was selectively toxic for dopaminergic neurons, a feature conferred to it by virtue of its dependence on the dopamine transporter (DAT) for entry into neurons (Javitch et al., 1985). Once MPP^+ gains entry to dopaminergic neurons, it is concentrated in mitochondria where it

inhibits Complex I of the electron transport chain (Nicklas et al., 1985, Ramsay et al., 1986) causing nigrostriatal neurodegeneration. In addition to the evidence from MPTP studies, it is now well established that mutations in certain genes which encode mitochondrial proteins (i.e. *Parkin*, *PINK1* and *DJ-1*) result in familial Parkinson's disease (Dodson and Guo, 2007).

Mitochondrial dysfunction and inhibition of Complex I results in impaired ATP formation and production of reactive oxygen species and free radicals (Cleeter et al., 1992, Hasegawa et al., 1990) both of which are toxic to dopaminergic neurons (Fig 1.7). The mitochondrial electron transport chain has been recognised as one of the major cellular generators of reactive oxygen species which include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl free radical (OH) and quinones (Loschen et al., 1971, Boveris et al., 1972, Chance et al., 1979). Damage to the mitochondrial Complex I in the electron transport chain causes leakage of electrons which in turn reacts with molecular oxygen to form reactive oxygen species. In addition to this mitochondrial dysfunction, dopamine metabolism is also a source of oxidative stress as tyrosine hydroxylase and monoamine oxidase are reactive oxygen species generating enzymes (Hwang, 2013).

Dopaminergic toxicity as a result of Complex I inhibition is supported by the fact that there is an increase in basal lipid peroxidation in the substantia nigra of *post mortem* Parkinsonian brains (Dexter et al., 1989). There is also a decrease in the antioxidant, glutathione, in nigrostriatal dopamine neurons and the magnitude of this reduction parallels with disease severity (Riederer et al., 1989). In conditions which promote oxidative stress, radical-mediated oxidative damage occurs at various sites

within the cell which results in carbonylation of proteins. Protein carbonylation is the irreversible modification of amino acid side chains and is a well-used marker for oxidative stress. In the substantia nigra, basal ganglia and prefrontal cortex of *post mortem* Parkinsonian brains, an increase in these carbonyl groups was detected (Floor and Wetzel, 1998). Interestingly, it has also been suggested that there is a direct pathological link between mitochondrial dysfunction and pathogenic α-synuclein aggregation (Jenner, 1998) as mitochondrial dysfunction and enhanced oxidative stress can trigger α-synuclein accumulation within neurons. Moreover, it has also been demonstrated that α-synuclein is carbonylated in Parkinson's disease and that derangements in Complex I of the mitochondrial respiratory chain can cause α-synuclein aggregation sustaining aberrant protein aggregation (Dalle-Donne et al., 2006, Esteves et al., 2009).

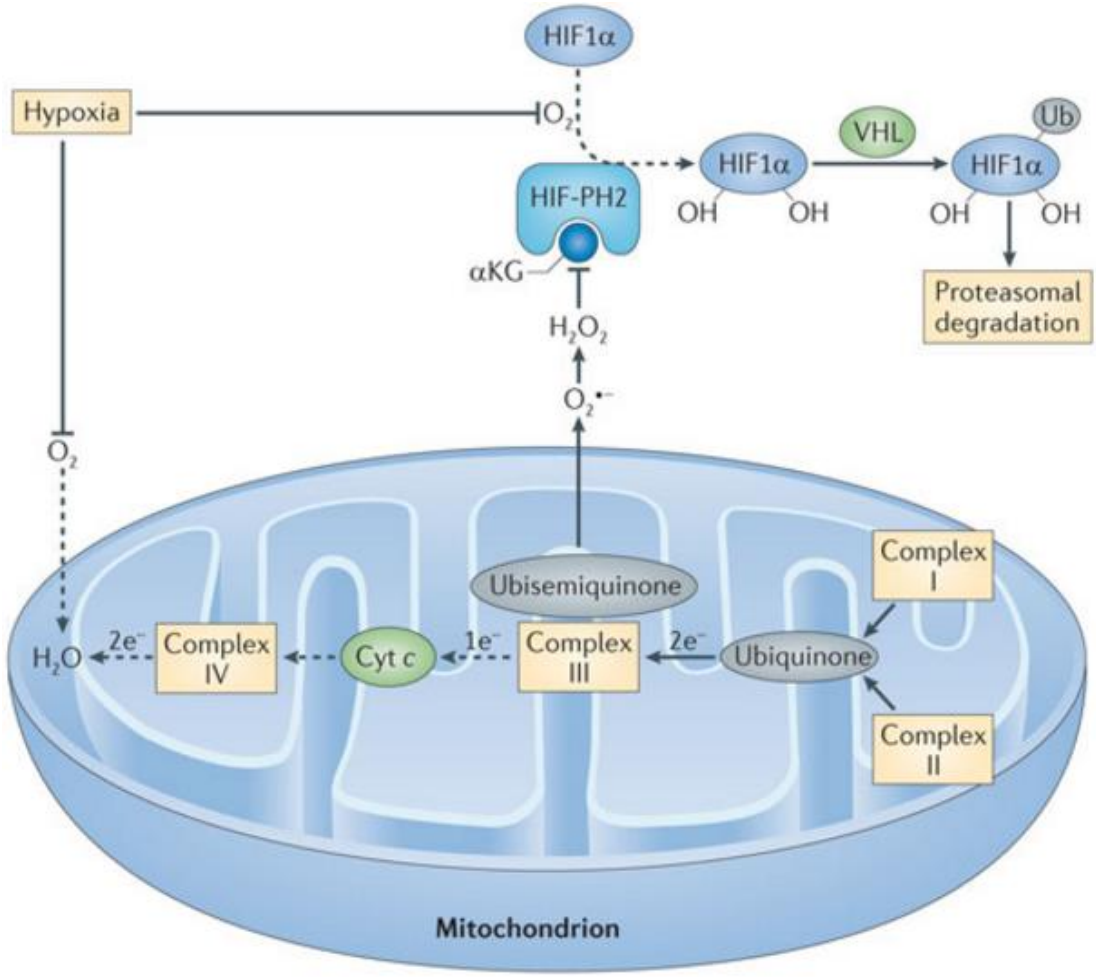


Fig 1.7: Overview of mitochondrial ROS production. ROS production by mitochondria can lead to oxidative damage to mitochondrial proteins, membranes and DNA, impairing the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions. Mitochondrial oxidative damage can also increase the tendency of mitochondria to release intermembrane space proteins such as cytochrome c and thereby activate the cell's apoptotic machinery. Consequently, it is unsurprising that mitochondrial oxidative damage contributes to a wide range of pathologies. In addition, mitochondrial ROS may act as a modulatable redox signal, reversibly affecting the activity of a range of functions in the mitochondria, cytosol and nucleus. Image taken from Nathan and Cunningham-Bussel., (2013).

1.1.3 Etiology

The etiology of idiopathic Parkinson's disease, which accounts for more than 90% of cases, remains elusive to this day. However, there is a growing body of literature which suggests that Parkinson's disease is influenced as a result of complex interactions between age, gender, genetics and exposure to largely unidentified environmental risk factors and is not limited to a single causative factor (Ascherio et al., 2006, Frigerio et al., 2006, Priyadarshi et al., 2000).

1.1.3.1 Age and gender

The concept that pathological neuronal loss observed in Parkinson's disease occurred with advancing age was first proposed by Hodge and colleagues in 1894 (Hodge, 1894). Aging remains the single largest risk factor for developing idiopathic Parkinson's disease (which affects 3% of the population over 60 years of age and 5% of the population over 85 years of age) indicating that the increasing longevity of our population will coincide with an increased risk of developing the disease (de Lau and Breteler, 2006). Recently, a meta-analysis of the worldwide data confirmed that there is a rising prevalence of Parkinson's disease with age where the number of people living with the disease increased from 428 per 100,000 in individuals aged 60 to 69 years to 1,087 per 100,000 in individuals aged 70 to 79 years (Van Den Eeden et al., 2003). Furthermore, it is anticipated that incidences of the disease will increase from 4.1 million to 9.3 million by 2030 (Dorsey et al., 2007) and with this ever increasing number, the global economic burden of Parkinson's disease will also increase. The mechanisms underlying age-related increases in Parkinson's disease have not been fully elucidated but it has been suggested that the substantia nigra (one of the

primary structures associated with degeneration in Parkinson's disease) may be more sensitive to the accumulation of age-related factors than other structures. One of the consequences of normal dopamine metabolism is the production of free radicals and this puts dopaminergic neurons under additional stress over time (reviewed in Sulzer and Schmitz, 2007). Normal aging has also been shown to be associated with Lewy body pathology (Jellinger, 2004) as well as the appearance of Parkinson's-like clinical features (Buchman et al., 2012) in non-Parkinsonian patients.

Another risk factor commonly associated with disease onset is gender. Epidemiological studies have indicated that the incidence rates of Parkinson's disease are 1.5 - 2 times higher in men than in women (Van Den Eeden et al., 2003, de Lau et al., 2004, Miller and Cronin-Golomb, 2010). The age of disease onset is also later in women than in men by approximately 2.2 years (Haaxma et al., 2007). Moreover, women also have better motor scores in the Unified Parkinson's Disease Rating Scale (UPDRS), but in concert, they also have higher incidences of dyskinesias (Chandran et al., 2014). One of the reasons postulated for this gender difference is based on the activity of oestrogen which modulates some aspects of dopaminergic function thus increasing the level of striatal dopamine and potentially exerting a neuroprotective effect on the nigrostriatal system (Joyce et al., 1982, Gordon and Perry, 1983, Disshon and Dluzen, 1997).

1.1.3.2 Environmental factors

Although the specific etiology of Parkinson's disease remains unknown, it has been widely accepted that disease onset is multifactorial with environment factors playing

a pivotal role in disease pathogenesis. Epidemiological and environmental studies have indicated that there are a number of factors which could increase the risk of developing Parkinson's disease (Tanner, 1992, Tanner and Langston, 1990). Factors associated with increased incidences of Parkinson's disease include exposure to bacterial and viral infections, exposure to industrial chemicals, well water, herbicides and pesticides, as well as farming and living in a rural environment (Olanow and Tatton, 1999). The evidence that exposure to environmental factors influences disease etiology dates back to just after World War 1 when patients developed a Parkinsonian-like syndrome secondary to viral encephalitis lethargica, or sleeping sickness. Moreover, these patients were very responsive to levodopa therapy (Foster and Hoffer, 2004, Sacks, 1983).

However, the most compelling evidence for exposure to an environmental factor as causative agent in Parkinson's disease relates to the incident outlined earlier where young drug addicts presented with symptoms typical of advanced idiopathic Parkinson's disease subsequent to having consumed MPTP-contaminated meperidine (Langston et al., 1983, Ballard et al., 1985). This unfortunate incident marked a seminal period in Parkinson's disease etiology research as it opened up the possibility of other, similar environmental factors as potentially triggering the disease.

1.1.3.2.1 Pesticides

Due to its structural similarity to MPTP/MPP^+, the herbicide, paraquat, was the first pesticide suggested as an environmental risk factor for Parkinson's disease. Since then, numerous epidemiological studies in humans have been conducted in order to determine the relationship between exposure to herbicides/pesticides and an increased susceptibility to Parkinson's disease. In the early 1990s, several studies reported that occupational use of pesticides greatly influenced the risk of Parkinson's disease (Semchuk et al., 1992, Seidler et al., 1996). This subsequently initiated a meta-analysis study which pointed to two pesticides in particular which were associated with a 3-fold increase in the risk of developing the disease, paraquat and rotenone (Tanner and Langston, 1990, Tanner et al., 2009, Tanner et al., 2011). Further research examining the effect of exposure to pesticides/herbicides, with particular reference to paraquat, identified a significantly greater risk among subjects who used paraquat rather than those who used pesticides/herbicides other than paraquat (Liou et al., 1997). It was also investigated as to whether or not there was a dose-effect relationship between occupational exposure to pesticides and the incidences of Parkinson's disease. A study performed by Elbaz and colleagues determined that there was a significant association related to the number of years of occupational exposure to pesticides and the onset of Parkinson's disease (Elbaz et al., 2009). Most recently, it was concluded that occupational exposure to pesticides was associated with a statistically significant odds ratio of developing Parkinson's disease ranging from 1.1 to 2.4 when compared to no pesticide exposure (Freire and Koifman, 2012).

1.1.3.2.1.1 Paraquat

Paraquat is a widely used agricultural herbicide with similar structural conformation to MPTP and belongs to a class of chemicals known as bipyridyl derivatives (Bus et al., 1976). The toxicity of this herbicide has been well established with two fatalities reported after accidental ingestion in the late 1960s (Bullivant, 1966). Patients who accidentally ingest this compound present with acute renal failure, hepatitis and subsequent death due to respiratory failure as a result of pulmonary inflammation and fibrosis (Dinis-Oliveira et al., 2008). The toxicity of paraquat is related to its redox cycling activity as paraquat undergoes an enzymatic monoelectron reduction to form a paraquat monocation free radical (Fukushima et al., 1993, Yamada and Fukushima, 1993). The class of enzymes which paraquat uses are termed cellular diaphorases and they transfer electrons from NAD(P)H to small molecules such as paraquat (Dicker and Cederbaum, 1991, Liochev and Fridovich, 1994). However, once NADH donates an electron to paraquat, it rapidly re-oxidises to its original state in the presence of molecular oxygen to form superoxide radicals (Dinis-Oliveira et al., 2008). Systemic administration of paraquat to animals reproduces the pathological features of Parkinson's disease including selective dopaminergic degeneration (McCormack et al., 2002), general motor dysfunction (Brooks et al., 1999) and α-synuclein pathology (Manning-Bog et al., 2002). Although paraquat and MPTP are structurally similar, the mechanism by which paraquat exerts its selective dopaminergic toxicity is still relatively unknown and even though paraquat interferes with the electron transport chain, it is not a specific Complex I inhibitor like rotenone (Richardson et al., 2005).

1.1.3.2.1.2 Rotenone

Rotenone is an organic pesticide which has been causally linked to the development of Parkinson's disease (Tanner et al., 2011). It is a non-specific broad spectrum insecticide and has also been utilised as a piscicide for fish eradication as part of water management (Goldman, 2014). Rotenone is extracted from the roots of two genera of the leguminosae family, the *Derris* in Asia and the *Lonchocarpus* in South America (Haag, 1931). It has been used extensively as a pesticide since 1848 when it was first applied to crops to control leaf-eating caterpillars. Due to its organic origin, rotenone was approved for commercial use in the UK against aphids on flowers, crops, fruit and vegetables until its use in the EU was banned in 2008 after it was causally linked to Parkinson's disease. Strikingly, although rotenone has been implicated in the etiology of Parkinson's disease, it has only been described as 'moderately hazardous' by the World Health Organization. To date, there have only been two reported fatalities to date which involved the accidental or intentional ingestion of rotenone (De Wilde et al., 1986, Wood et al., 2005). The mechanism by which rotenone elicits its toxicity is based on its ability to cause mitochondrial dysfunction. Rotenone is a highly lipophilic compound which can readily cross the blood brain barrier (Talpade et al., 2000) and once rotenone enters dopaminergic neurons, it accumulates in mitochondria where it acts as a potent Complex I inhibitor causing energy failure within the cell and eventual cell death (Betarbet et al., 2000).

1.1.3.2.2 Bacterial or viral infections

In addition to agritoxins, another environmental factor that is becoming more widely accepted as a risk factor for Parkinson's disease is exposure to viral or bacterial infection. Indeed, it is becoming increasingly evident that bacterial and/or viral

infection and the ensuing neuroinflammation may increase disease susceptibility and contribute to disease pathogenesis (Arai et al., 2006, Chao et al., 2014, De Chiara et al., 2012, Takahashi and Yamada, 1999). Several viral infections have been linked with Parkinsonism including influenza virus (Casals et al., 1998, Gamboa et al., 1973, Ravenholt and Foege, 1982, Takahashi and Wakabayashi, 2001, Takahashi and Yamada, 1999), herpes simplex virus (Hemling et al., 2003, Marttila and Rinne, 1981), polio virus (Nielsen et al., 2002), cocksackie virus (Kamei et al., 1990, Peatfield, 1987, Poser et al., 1969) and human immunodeficiency virus (de la Fuente-Aguado et al., 1996, Hersh et al., 2001, Koutsilieri et al., 2002, Maggi et al., 2000, Wang et al., 2004). Similarly, several bacterial infections have also been linked with the disease including *Helicobacter pylori* (Tan et al., 2015) and the soil bacterium, *Nocardia asteroids* (Kohbata and Beaman, 1991). Interestingly, despite the fact that numerous studies positively correlate the exposure to viral or bacterial infection to an increased susceptibility to Parkinson's disease, exposure to infections such as measles, mumps and chicken pox appear to be inversely related to the risk of developing the disease (Sasco and Paffenbarger, 1985).

1.1.3.3 Genetic Predisposition

As indicated above, in addition to environmental factors, genetic factors also play a role in the etiology of Parkinson's disease. To date, there have been 28 distinct chromosomal regions which have been causatively linked to autosomal dominant and autosomal recessive forms of the disease. These genes are termed PARK denoting their putative link to the etiology of familial forms of the disease (Klein and Westenberger, 2012) and are summarised in Table 1.1 below.

Gene Name	Gene product	Inheritance	Pathology	Clinical Phenotype	Progression	Onset	Reference
snca	α-synuclein	Dominant	Nigral degeneration with Lewy bodies	Aggressive progression Parkinsonism, cognitive and autonomic symptoms	Aggressive	Early	(Poulopoulos et al., 2012)
lrrk2	Leucine-rich repeat-containing kinase	Dominant	α-synuclein pathology (Variable)	Parkinsonism and variable additional symptoms	Typical	Late	(Paisan-Ruiz et al., 2004, Zimprich et al., 2004)
gba	Lysosomal glucocerebrosidase	Dominant	Nigral degeneration with Lewy bodies	Parkinsonism and variable additional symptoms	Typical	Late	(Aharon-Peretz et al., 2004, Neudorfer et al., 1996)
vps35	Vacuolar protein sorting 35	Dominant	Uncertain pathology	Parkinsonism with possible cognitive and behavioural symptoms	Typical	Late	(Vilarino-Guell et al., 2011)
parkin	E3 ubiquitin protein ligase	Recessive	Nigral degeneration without Lewy bodies	Young onset classical Parkinsonism	Slow	Early	(Kitada et al., 1998)
pink1	PTEN-induced mitochondrial serine/threonine kinase	Recessive	Nigral degeneration with Lewy bodies	Young onset classical Parkinsonism with various cognitive and psychiatric symptoms	Slow	Early	(Valente et al., 2004)
dj-1	Redox-dependent molecular chaperone in mitochondria	Recessive	No pathology reported	Classical Parkinsonism	Slow	Early	(Bonifati et al., 2003)
pla2g6	Phospholipase A2	Recessive	Lewy body pathology	Classical Parkinsonism or Parkinsonism-pyramidal syndrome	Unreported	Early	(Paisan-Ruiz et al., 2004)
fbxo7	E3 ubiquitin protein ligase	Recessive	Unknown	Classical Parkinsonism or pyramidal syndrome	Unreported	Early	(Di Fonzo et al., 2009, Shojaee et al., 2008)
dnajc6	Neuronal-specific clathrin-uncoating co-chaparone auxilin	Recessive	Unknown	Parkinsonism-pyramidal syndrome	Unreported	Juvenile	(Edvardson et al., 2012, Koroglu et al., 2013)

Table 1.1: Genes involved in the etiology of Parkinson's disease. Modified from Bonifati, (2014), Funke et al., (2013), Puschmann, (2013) and Spatola and Wider (2014).

1.1.3.3.1 *PARK1* – α-synuclein

Human α-synuclein (SNCA) encodes a highly conserved 140 amino acid protein which lies on chromosome 4q21.3 to 4q22. Maroteaux and colleagues first isolated α-synuclein from the neuromuscular junction of the electric eel and identified that the protein is primarily expressed in the brain, particularly in the neocortex, hippocampus, substantia nigra, thalmus and cerebellum (Maroteaux et al., 1988). They also confirmed its localisation to a restricted area of the nucleus and in presynaptic nerve terminals. Although the physiological function of the protein remains unknown, it is thought to play a role in synaptic plasticity and in learning processes. It is a natively unfolded protein however, once it interacts with negatively charged lipids such as phospholipids on cellular membrane, it adopts an α-helical structure due to its hydrophobic core and this results in the protein adopting a partially folded conformation (Li et al., 2001). In 1997, Polymeropoulos and colleagues described a mutation in the gene encoding α-synuclein (SNCA) in one large Italian family and three unrelated Greek families with autosomal dominant Parkinson's disease. This mutation involves an amino acid substitution at position 53 (A53T), changing an alanine for a threonine and 85% of patients who express this genetic mutation report clinical features of Parkinson's disease (Polymeropoulos et al., 1997). This genetic mutation has also been reported in over 60 familial cases since its description in 1997 and patients with this mutation experience an earlier age of onset, rapid disease progression and variable autonomic disorders (Petrucci et al., 2016). The second mutation was reported the following year in a small German family with an amino acid substitution at position 30 from alanine to proline (A30P). Disease onset is slightly later than that which occurs with the A53T mutation, typically in the fifth decade (Krüger et al., 1998). These findings also coincided with the discovery that the primary constituent of Lewy bodies in idiopathic cases of the disease

is the protein α-synuclein (Spillantini et al., 1997). More recently, a new mutation was described, the E46K mutation where there is an amino acid substitution at position 46 of glutamic acid for alanine (Zarranz et al., 2004). This genetic mutation was reported in a small Spanish family who presented with severe Parkinsonism and dementia with Lewy bodies. Age of disease onset ranged from 50 to 65 years with rapid disease progression. In addition to these observations, triplications and even duplications of the normal wild type α-synuclein gene have been implicated as a cause of familial Parkinson's disease indicating that overexpression of the normal protein is sufficient to cause the disease (Chartier-Harlin et al., 2004, Singleton et al., 2003).

The pathogenicity of both wildtype and mutant forms of α-synuclein is due to its structural conformation that confers to it a high propensity to aggregate. Studies have investigated this aggregation *in vitro* and determined that all mutant forms of α-synuclein generate insoluble fibrils more rapidly than wildtype α-synuclein (Conway et al., 1998, Conway et al., 2000, Li et al., 2001, Fredenburg et al., 2007). These pathological species induce toxicity by several different mechanisms such as; 1) disrupting the normal physiological function of α-synuclein in neurotransmission, 2) disrupting Complex 1 of the mitochondrial electron transport chain, 3) inducing endoplasmic reticulum stress by disrupting vesicular transport and, 4) interfering with protein degradation mechanisms (reviewed in Recasens and Dehay, 2014). As previously iterated, α-synuclein may self-associate into aggregates which are capable of traversing cellular membranes mediating 'prion-like' transmission of pathological α-synuclein to neighbouring neurons. Additional support for this cell-to-cell transmission comes from *in vitro* studies whereby two populations of distinct neuronal cells, one overexpressing α-synuclein, were cultured together and α-synuclein pathology was observed in the acceptor cells (Desplats et al., 2009).

There is unequivocal evidence which suggests that variability in the expression of α-synuclein is causally linked to familial Parkinson's disease and is also implicated in sporadic Parkinson's disease. However, studies have also demonstrated that α-synuclein is critical for normal brain homeostasis. Animal studies targeting α-synuclein knockout or knockdown display altered neurotransmitter release, rapid loss of dopaminergic neurons and impaired learning ability in tests of working and spatial memory (Kokhan et al., 2012, Abeliovich et al., 2000a, Gorbatyuk et al., 2010).

1.1.3.3.2 *PARK2* – Parkin

PARK2 was the first of three recessive genes identified to be causally linked to autosomal recessive juvenile Parkinson's disease. Parkin has been identified as an E3 ubiquitin protein ligase, is located in the cytosol and plays a role in the ubiquitin-proteasome system by maintaining mitochondrial integrity (Klein and Westenberger, 2012, Kitada et al., 1998). The distinguishing feature of autosomal recessive juvenile Parkinson's disease is the earlier age of onset. In contrast to idiopathic Parkinson's disease, the average age of onset for parkin-associated Parkinsonism ranges from 20 to 40 years of age with the mean age of onset at approximately 31 (Chien et al., 2006). There are some isolated instances where the age of onset is as early as 20 years old (Lucking et al., 2000). The parkin-associated phenotype is characterised by a predominant lower limb dystonia which is also the initial clinical symptom to emerge when patients present at the clinic (Khan et al., 2003). The disease progresses slowly and in some instances can span 50 years in patients who have experienced an earlier disease onset (Chung et al., 2006). Patients also experience an excellent and long-lasting response to low doses of levodopa although this is frequently associated with levodopa-induced dyskinesias (Bruggemann and Klein, 1993). Neuropathologically, this mutation

presents with a severe and protracted loss of dopaminergic neurons from the substantia nigra. Interestingly, Lewy bodies and Lewy body pathology are absent from parkin-associated Parkinson's disease but there are neurofibrillary tangles and deposits of tau protein which are important differences between Parkin-associated Parkinson's disease and typical idiopathic Parkinson's disease (Doherty and Hardy, 2013).

1.1.3.3.3 *PARK6* – PINK1

The *PARK6* gene encodes the PINK1 protein which is a serine-threonine kinase and when mutated is responsible for autosomal recessive Parkinson's disease (Valente et al., 2004). Unlike parkin which resides in the cytosol, PINK1 localises in the mitochondria and its function is to detect mitochondrial dysfunction. It regulates parkin by recruiting it to mitochondria in order to initiate mitophagy (Corti et al., 2011). Mutations in the PINK1 gene can result in mitochondrial dysfunction leading to toxicity of dopaminergic cells. Currently, a mutation in the *PARK6* gene encoding PINK1 relates to approximately 1-7% of early onset autosomal recessive Parkinson's disease (Pankratz and Foroud, 2007, Wang et al., 2007) and the distinguishing features of PINK1-linked Parkinson's disease include early onset, typically in the early thirties, good response to levodopa and slow disease progression (Shulman et al., 2011).

1.1.3.3.4 *PARK8* – LRRK2

Mutations of the leucine-rich repeat kinase 2 *(LRRK2)* gene result in autosomal dominant Parkinson's disease without dementia. It was first linked to a Japanese family in 2002 who all exhibited clinical features of idiopathic Parkinson's disease (Funayama et al., 2002). The *PARK8* mutation is linked with a late age of onset and mutations of this gene are the most common genetic form of the disease. To date, over 40 pathogenic

mutations of the LRRK2 gene have been identified (Dauer and Ho, 2010). However, despite the fact that LRRK2-linked Parkinson's disease exhibits the hallmark clinical features of the sporadic disease, there is diverse neuropathology in patients with LRRK2-mutations - studies have reported Lewy body pathology, tauopathy and neurofibrillary tangles (Zimprich et al., 2004). There is also significant nigral cell body loss with both α-synuclein and tau protein deposition evident within dopamine neurons. Interestingly, in some patients there is no evidence of Lewy body pathology. The physiological function of this protein is still relatively unknown as it is an unusually large protein with many interaction domains and therefore could be involved in a host of diverse functions (Kalinderi et al., 2016). Research investigating *LRRK2* mutations reveal that disease penetrance is age dependant and may also be ethnicity dependant (Kachergus et al., 2005, Healy et al., 2008). The prevalence of this mutation is highest in Italy, Portugal and Spain and very rare in Asia and Northern Europe. One of the most studied amino acid substitutions which accounts for almost 40% of LRRK related Parkinsonism is the G2019S substitution (Bonifati, 2006). The late onset and age dependent penetrance of LRRK2-G2091S presents a unique opportunity to track disease progression.

1.2 Current Pharmacological Treatment of Parkinson's disease

Although the identification of various environmental and genetic risk factors has led to considerable advances being made in our understanding of the etiology and pathogenesis of Parkinson's disease, unfortunately this has not as yet, led to any cure for the condition. The treatment approach for Parkinson's disease is complex and is based on the patient's age and degree to which the disease has progressed with the primary goal being to improve the physical and psychological symptoms as well as the quality of life of the individual patient. To date, most available treatments manage the symptomatic features of the disease and are based on dopamine replacement strategies which exert their therapeutic effects by mimicking the neurotransmitter and/or acting on dopamine receptors (Brooks, 2000). However, no treatment to date effectively targets the underlying neurodegeneration which drives disease progression.

1.2.1 Dopamine replacement strategies

1.2.1.1 Levodopa

Despite decades of research, levodopa is still the mainstay of treatment for patients of Parkinson's disease against which all new and emerging therapies are compared (Brooks, 2008). Levodopa therapy for the treatment of Parkinson's disease accounts for one of the most significant developments in the quest for a therapy for this incurable disease. It has greatly increased the quality of life of patients by significantly reducing their motor symptoms and improving some of the non-motor symptoms associated with the disease (Hoehn and Yahr, 1967).

Levodopa is biologically inert, however in 1938 it was discovered that the enzyme dopa decarboxylase converted it to dopamine (Holtz, 1939). At this point it was proposed that dopamine was only involved in the biosynthetic pathway of the catecholamines, noradrenaline and adrenaline. However, in 1957, Arvid Carlsson demonstrated the biological importance of dopamine in the brain and that administration of reserpine depleted striatal dopamine which led to a pronounced akinetic syndrome typical of that observed in patients with Parkinson's disease. He postulated that the 'tranquilizing' effect of reserpine was the result of the loss of dopamine and administered the dopamine precursor levodopa which reversed these symptoms (Carlsson et al., 1957). Then in 1960, *post mortem* analysis of patients with Parkinson's disease revealed a marked depletion of striatal dopamine, and from here, the era of levodopa therapy for the treatment of Parkinson's disease was born (Ehringer and Hornykiewicz, 1960). The first clinical trial involving the use of levodopa to treat Parkinson's disease was conducted in 1961 and it demonstrated positive anti-akinetic effects in patients who were rendered bedridden due to their disease (Birkmayer and Hornykiewicz, 1961). Subsequently, open label trials and double-blind placebo controlled trials followed confirming short term benefits of oral levodopa preparations. However, these initial reports failed to report complications associated with levodopa therapy such as levodopa induced dyskinesias (Cotzias et al., 1969a).

In the early stages of the disease there is a large therapeutic window where levodopa's efficacy responsiveness in ameliorating the motor complications is excellent. However, as the disease progresses it becomes increasingly difficult to

deliver a therapeutic dose of the drug which can provide symptomatic relief without patients experiencing dyskinesias and motor fluctuations (Cotzias et al., 1969b, Fahn et al., 2004). Dyskinesias are a result of pulsatile stimulation of dopamine receptors and typically occur in all patients within 10 years of levodopa treatment. Therefore, in an attempt to overcome this limitation the standard approach is to delay introduction of levodopa into the treatment regimen or to employ levodopa sparing strategies (Stocchi, 2006). One of these strategies involves the use of dopamine agonists which can delay treatment with levodopa or reduce the dose of when used as an adjunct to levodopa (Brooks, 2000).

1.2.1.2 Dopamine Agonists

The use of dopamine agonists for the treatment of Parkinson's disease dates back to the 1970s where first generation dopamine agonists were all ergot derivatives. It was almost 50 years later when the anti-Parkinsonian effects of the non-ergot derived agonist, apomorphine, was discovered. The main advantage of dopamine agonists as a monotherapy is their efficacy in treating the motor symptoms of the disease. They act by directly mimicking the action of dopamine, stimulating dopamine receptors, and their early use is associated with a reduced incidence of motor fluctuations and dyskinesias (Rascol et al., 2000, Bracco et al., 2004). Dopamine agonists such as bromocriptine, pergolide and cabergoline have a longer half-life than levodopa and the hypothesis is that drugs with longer half-lives provide a more continuous stimulation of dopaminergic receptors. When dopamine agonists are administered in adjunct to levodopa, there is a reduction in time spent in the 'off' state (Guttman et al., 1997). However, the use of ergot-derived dopamine agonists is no longer

recommended as the benefit-to-risk ratio is relatively poor. Peripheral side effects such as orthostatic hypotension, hypersexuality and excessive daytime somnolence are also more likely to be experienced whilst using ergot-derived dopamine agonists as a monotherapy (Biglan et al., 2007).

1.2.2 MAO/COMT Inhibitors

Monoamine oxidases A and B (MAO-A and MAO-B) are mitochondrial outer membrane bound enzymes which are involved in the metabolism of monoamine neurotransmitters including serotonin, histamine, dopamine, noradrenaline and adrenaline (Bortolato et al., 2008). Due to the fact that dopamine is preferentially targeted by MAO-B, inhibitors of this isoform increase striatal dopamine and attenuate dopamine deficits experienced in the Parkinsonian brain (Knoll, 2000). MAO inhibitors are the earliest drugs used to treat Parkinson's disease and have been used with or without levodopa to inhibit the metabolism of dopamine. Thus the increase in the availability of dopamine, as a result of the inhibition of its metabolism, should compensate for the deficits experienced in the nigrostriatal system as a result of neurodegeneration (Bortolato et al., 2008). As a treatment regimen for Parkinson's disease, MAO-B inhibitors are very effective in the early stages of the disease as they can delay the need to start levodopa therapy, prolong its efficacy and reduce the dosage of levodopa required for therapeutic effects (Riederer and Laux, 2011). Irreversible MAO inhibitors, or first generation MAO inhibitors, have been associated with serious side effects including hepatotoxicity, orthostatic hypotension and the potentially dangerous hypertensive crisis which occurs following ingestion of foods containing tyramine (Cooper, 1989). This is due to the fact that doses in excess of 20 mg also inhibit the activity of MAO-A in the gut

which requires a controlled low tyramine diet (Sunderland et al., 1994). However, at the dose required to provide symptomatic relief to patients with Parkinson's disease, a daily dose of 10 mg effectively leaves MAO-A intact and avoids the necessity of a tyramine restricted diet. MAO inhibitors have a well-established safety and tolerability profile as either a monotherapy or in adjunct to levodopa (Mann et al., 1989, Riederer and Laux, 2011).

1.2.3 Limitations of Current Treatments

Despite the symptomatic relief that is conferred by levodopa therapy, the developments of adverse reactions greatly limit its long term application in the treatment of Parkinson's disease. Chronic use of levodopa is associated with the development of motor side effects including disabling dyskinesias and on-off periods due to 'wearing off' of the medication (Lopez et al., 2010). These motor fluctuations occur in approximately 50-80% of patients receiving levodopa treatment within 5-10 years of starting treatment and in this vain, levodopa therapy is usually reserved for advanced stages of Parkinson's disease (Olanow et al., 2004). As previously iterated, the use of dopamine agonists as a monotherapy for Parkinson's disease dates back to the 1970s when bromocriptine was first used. As an initial therapy, dopamine agonists generally represent the first line of defence against the disease as they are associated with a reduced long-term incidence of motor complications seen in levodopa therapy (Antonini et al., 2009). However, there are concerns relating to the use of dopamine agonists, primarily non-motor issues such as peripheral oedema, nauseas, constipation, dizziness, impulse control disorders and heart valve fibrosis which is due to their interaction with receptors other than dopamine receptors

(Horvath et al., 2004). The most common early side effects of MAO-B inhibitors include orthostatic hypotension, dizziness, drowsiness and nausea. Some of these side effects can be attenuated with administering lower doses of the drug. However, there are also late side effects associated with these inhibitors including, but not limited to weight gain, muscle pains and hypersexuality (Davidson and White, 1983).

In addition to these side effects, current pharmacotherapies for Parkinson's disease are unable to slow, stop or reverse disease progression. They simply exert their anti-Parkinsonian effects by enhancing dopaminergic neurotransmission in the brain thereby compensating for the disruption of the basal ganglia circuit. However, there is no single therapeutic treatment, or combination of treatments, that can modify the disease pathogenesis in terms of aberrant α-synuclein expression/aggregation or nigrostriatal neurodegeneration. Thus, to date, a disease-modifying neuroprotective therapy for Parkinson's disease remains a major unmet clinical need in the treatment of this condition. One reason suggested for the lack of novel disease-modifying therapeutics is that animal models of the disease do not accurately depict the etiology or pathogenesis of the human condition (Beal, 2001, Blesa et al., 2012, Jackson-Lewis et al., 2012). The next section will review the historical and current approaches to modelling Parkinson's disease in preclinical animals.

1.3 Preclinical models of Parkinson's disease

1.3.1 Historical overview of PD animal models

One of the earliest animal models in Parkinson's disease research was the reserpine-induced model. It has been almost 60 years since it was discovered that systemic

administration of reserpine depleted the rodent brain of dopamine, and other catecholamines, by inhibiting vesicular monoamine uptake. The akinesias that emerged as a result of reserpine administration were successfully reversed by the endogenous dopamine precursor, levodopa (Carlsson et al., 1957, Carlsson, 1959). The link between depletion of monoamines and the reversal of Parkinsonian symptoms as a result of levodopa therapy paved the way for the first clinical trial investigating the efficacy of levodopa Parkinson's disease patients (Birkmayer and Hornykiewicz, 1961). Despite the fact that the reserpine model paved the way for the era of levodopa therapy, such pharmacological models are limited in their capacity to recapitulate key features of the disease. The effects induced by reserpine are transient which limit the usefulness of it as a model of a chronic neurodegenerative disease. As this model is not associated with any neuropathological features, it is essentially ineffective in assessing any strategies aimed at neurorepair or neuroprotection (Duty and Jenner, 2011). Nevertheless, this model has been successfully utilised to investigate the dopamine replacement agents including levodopa, dopamine agonists and monoamine oxidase inhibitors (Gossel et al., 1995).

Fortunately, in the late 1960s, identification of the selective toxicity of 6-hydroxydopmine introduced a revolutionary new era in toxin-based animal models of Parkinson's disease (Ungerstedt, 1968, Ungerstedt and Arbuthnott, 1970). This neurotoxin is selective for both dopaminergic and noradrenergic neurons due to the affinity of their plasma membrane transporter for the hydroxylated analogue of dopamine. The presence of the additional hydroxyl group is responsible for the toxicity of 6-hydroxydopmine and the reason as to why 6-hydroxydopmine cannot

freely cross the blood brain barrier (Bove et al., 2005). Indeed, in order to bypass the blood brain barrier, 6-hydroxydopmine is typically injected directly into the nigrostriatal pathway into the substantia nigra, the medial forebrain bundle or the terminal striatum. Depending on the region targeted, 6-hydroxydopmine can induce full or partial lesions and exert its neurotoxic effects via degeneration of dopaminergic neurons and thus mimicking Parkinsonian symptoms (Dauer and Przedborski, 2003).

In the early 1980s came the next breakthrough in Parkinson's disease modelling with the accidental discovery of MPTP as a nigrostriatal neurotoxin (Langston et al., 1983, Langston and Ballard, 1984). This discovery quickly led to the administration of MPTP to various animal species including non-human primates, mice and rabbits (Forno et al., 1993, Sonsalla and Heikkila, 1986). Interestingly, rats are relatively resistant to MPTP despite the fact that the toxic metabolite of MPTP, MPP+, accumulates in the brains of rats at higher levels than mice (Giovanni et al., 1991, Chiueh et al., 1984).

To study the role that neuroinflammation plays in the pathogenesis of Parkinson's disease, the lipopolysaccharide model of the disease was established. Whether neuroinflammation is a cause or a consequence of Parkinson's disease pathology has yet to be delineated, however the general consensus appears to be that neuroinflammation is secondary to the cause of cell death but further perpetuates the cycle of neurodegeneration (Tieu, 2011, Herrera et al., 2000).

More recently, as α-synuclein pathology became associated with both familial and idiopathic forms of the disease (Spillantini et al., 1997, Krüger et al., 1998, Zarranz et al., 2004, Singleton et al., 2003, Miller et al., 2004, Farrer et al., 2004), various genetic animal models have been established in order to investigate the central role that point mutations play in familial Parkinson's disease. These models are induced by viral gene transfer (Kirik et al., 2003) and transgenic overexpression of mutant genes which result in autosomal dominant Parkinson's disease (Martin et al., 2006). Both the advantages and the disadvantages of the animal models of Parkinson's disease are summarised in Table 1.2.

Model	Dose	Behavioral symptoms	Nigrostriatal degeneration	α-synuclein aggregation	Advantages	Limitations	References
6-OHDA (into nigra or MFB)	3µg in 1.5µl into the nigra or 4-8 µg in 1.5-2 µl into the MFB	Rotational behaviour after unilateral injection of methamphetamine and robust spontaneous behavioral phenotypes	Acute dose dependent loss of DA neurons and reduced striatal DA innervation	None	Full depletion of DA, mimics advanced PD, useful for screening therapies that may improve symptoms	Requires intracerebral injection, no synuclein pathology and rapid degeneration	(Deumens et al., 2002, Kirik et al., 1998)
6-OHDA (into the striatum)	20-30 µg in 2-2.5 µl	Rotational behaviour after unilateral injection of methamphetamine and robust spontaneous behavioral phenotypes	Progressive loss of striatal dopamine with retrograde degeneration of the nigral cell bodies	None	Progressive degeneration mimicking the progressive course of the disease	No Lewy body like inclusions, has no causal link with PD	(Deumens et al., 2002, Kirik et al., 1998)
MPTP	90mg/kg s.c. (single)	Motor dysfunction in non-human primates and less obvious motor impairments in rodent models with akinesia and bradykinesia of limb and trunk	Dose dependent loss of dopaminergic neurons reaching 95% in acute high dose conditions. Reduced striatal dopamine levels	None	Inhibits complex I activity, screen therapies that may improve symptoms and study the mechanisms of cell death	Non-progressive model of cell death. Inclusions are rare. Not reliable in rats	(Langston et al., 1999, Schober, 2004)

Rotenone	2.5mg/kg i.p for 1-5 weeks; 12 µg into the nigra; 3.6 µg at four sites of the striatum	Significant contralateral motor impairment	Reduction in dopaminergic cell bodies and reduced striatal dopamine innervation	Lewy body like inclusions from systemic administration but no evidence from intracerebral infusion	Test neuroprotective therapies	Significant toxicity associated with systemic rotenone, large variation in animal sensitivity, varied motor response	(Betarbet et al., 2000, Ferrante et al., 1997, Heikkila et al., 1985, Moreira et al., 2012, Mulcahy et al., 2011)
Paraquat	10mg/kg i.p for 3weeks	Reduced locomotor activity with stereotypical and rotational behaviour	Nigral cell body loss with a modest reduction of striatal DA innervation	None but there evidence of an increase of synuclein in DA neurons	More complete DA depletion when combined with Maneb. Test neuroprotective therapies	Not extensively used for testing or screening emerging therapies, inconsistent DA loss	(Manning-Bog et al., 2002, Nistico et al., 2011)
LPS	2 µg into the nigra, 10 µg into the striatum or nigra; 20 µg at four sites of the striatum	Modest contralateral motor dysfunction	Significant neurodegeneration	No appearance of synuclein immunoreactive inclusions	Test neuroprotective strategies	Associated with 'sickness' behaviour and variable motor dysfunction and neurodegeneration	(Castano et al., 2002, Herrera et al., 2000, Hoban et al., 2013, Naughton et al., 2016a)

Table 1.2: Parkinson's disease animal models and their associated features. Modified from Blesa et al., (2012); DA: Dopamine; MFB: medial forebrain bundle. Rodents used in experiments weighed 200-225g at time of surgery

1.3.2 Established models

1.3.2.1 6-hydroxydopamine

6-hydroxydopamine, a structural analogue of the catecholamines dopamine and noradrenaline, is selectively toxic for the catecholaminergic pathways via its affinity for plasma membrane transporters (Ungerstedt, 1968). Its selective toxicity has been a vital experimental tool in modelling Parkinson's disease. Due to the presence of the hydroxyl group, 6-hydroxydopamine cannot freely cross the blood brain barrier and so has to be injected directly into the nigrostriatal pathway (at the substantia nigra, the medial forebrain bundle or terminal striatum) on one or both sides of the brain. Unilateral lesions are almost exclusively employed due to the fact that bilateral 6-hydroxydopamine lesions result in marked aphagia and adipsia rendering tube-feeding necessary in order to maintain the welfare of animals (Ungerstedt, 1971, Sakai and Gash, 1994). As 6-hydroxydopamine displays a high affinity for both dopamine and noradrenaline transporters, it is often used in conjunction with systemic administration of desipramine to ensure selective toxicity for dopaminergic neurons as desipramine is a selective noradrenergic reuptake inhibitor (Martin et al., 1976).

Once inside dopaminergic neurons, the neurotoxic effect of 6-hydroxydopamine is facilitated by a combination of mitochondrial dysfunction and oxidative stress (Glinka et al., 1997). 6-hydroxydopamine is readily oxidised by monoamine oxidase and forms reactive oxygen species such as hydrogen peroxide and free radicals (Mazzio et al., 2004). It also elevates levels of iron in the substantia nigra

(Oestreicher et al., 1994) and directly inhibits mitochondrial respiratory enzymes of Complex I and Complex IV leading to further oxidative stress (Glinka et al., 1997).

The magnitude and characteristics of neurodegeneration induced by 6-hydroxydopamine is dependent on the site and dose of injection. Unilateral injection of the neurotoxin into the substantia nigra or the medial forebrain bundle results in an immediate and almost complete loss (>95%) of dopaminergic neurons of the substantia nigra (Przedborski et al., 1995). While this model resembles the advanced stages of Parkinson's disease, the rapid nature of the toxicity induced is far removed from the slow progressive disease course (Duty and Jenner, 2011). Injection into the striatum results in a more protracted degeneration which results in striatal terminal loss within 48 hours of injection with maximum nigral cell body loss occurring approximately 2-3 weeks post injection (Sauer and Oertel, 1994). The main advantage of targeting the striatum is the improved validity of the model as a less extensive lesion is more clinically relevant. Also, the ease of which the striatum can be targeted over the much smaller structures also increases the chance of inducing the model (Tieu, 2011).

The major advantage of this 6-hydroxydopamine model is the fact that the motor deficit induced by the lesion is easily quantifiable and therefore provides a useful model for screening any pharmacological agents that could exert an effect on dopamine and its receptors (Yuan et al., 2005). Unilateral lesions involve the injection of the toxin into one hemisphere and the extent of dopaminergic asymmetry can be assessed via rotational circling behaviour after administration of

dopaminergic drugs (Ungerstedt, 1968). Through its ability to induce the release of dopamine, amphetamine creates an imbalance in dopamine transmission in favour of the intact striatum. This imbalance results in rotation towards the lesion, i.e. ipsiversive rotation. Partial lesions which produce a 50% loss of dopaminergic neurons will result in this spontaneous rotating behaviour (Hefti et al., 1980b, Hefti et al., 1980a). Apomorphine is a non-selective dopamine receptor agonist and at low doses favours stimulation of the lesioned striatum due to the hypersensitivity of postsynaptic neurons following nigral degeneration and this also results in rotation away from the lesion i.e. contraversive rotation. In contrast to ipsiversive rotations, contraversive rotational behaviour is only observed when there is at least 90% loss of dopaminergic neurons (Hudson et al., 1993).

In addition to this, the 6-hydroxydopamine model induces a profound and reproducible spontaneous behavioural profile. Unilateral 6-hydroxydopamine lesioned animals display forelimb akinesia in the adjusted stepping test (Olsson et al., 1995), impaired ability to make vibrissae-elicited forelimb placings in the whisker test (Schallert et al., 2000) and display significant asymmetry having a preference for their non-lesioned forelimb or non-lesioned side in the Corridor and Cylinder tests (Schallert et al., 2000, Dowd et al., 2005). In summary, the 6-hydroxydopamine lesion model of Parkinson's disease resembles the key features of Parkinson's disease in a number of ways. It has good pathogenic validity combining oxidative stress, mitochondrial dysfunction, nigrostriatal degeneration, dopamine depletion and motor dysfunction (Duty and Jenner, 2011). However, despite its clear advantages,

this model shares a common limitation with many other models as it does not have any associated α-synucleinopathy (Dauer and Przedborski, 2003).

1.3.2.2 MPTP

MPTP, as described previously, produced remarkably similar symptoms to advanced atypical Parkinson's disease in a group of young heroin addicts in California in the early 1980s (Langston and Ballard, 1984). It was discovered that these symptoms were as a result of degeneration of nigrostriatal dopaminergic neurons (Langston et al., 1999).

MPTP is highly lipophilic and readily crosses the blood brain barrier upon systemic administration and for this reason it has a competitive advantage over other animal models (Riachi et al., 1989). MPTP is frequently used in non-human primates where it is commonly administered in a multiple low dose regimen, via intraperitoneal or intramuscular injection. This treatment regime is typically employed for a prolonged period of time and results in a bilateral Parkinsonian syndrome (Emborg, 2007). However, in line with 6-hydroxydopamine bilateral lesions, systemic administration of MPTP requires treatment with levodopa in order to maintain the general health and well-being of animals (Bezard et al., 2013). In this respect, unilateral intracarotid infusion is recommended. Although the pattern of neuronal loss observed in MPTP treated non-human primates is remarkably similar to that observed in idiopathic Parkinson's disease (Dauer and Przedborski, 2003), due to the ethical and logistical issues associated with experimental research on non-human primates, rodent MPTP models of Parkinson's are widely used .

As rats are resistant to the toxic effects of MPTP, the mouse model of MPTP has become the most commonly used rodent model. However, mice strains vary greatly in their sensitivity to MPTP with C57BL/6 mice being more susceptible to the toxic effects of MPTP than BALB/c mice. In addition to MPTP strain sensitivity, the toxicity of MPTP is also age and gender dependant with older animals and females being more vulnerable (Filipov et al., 2009, Sedelis et al., 2000b, Sedelis et al., 2000a). Mice are also less sensitive to MPTP than non-human primates who require increasingly higher doses of the toxin to induce a sufficient magnitude of neurodegeneration. In comparison to CD-1 mice, C57BL/6 mice have a greater reduction in striatal dopamine levels and in the number of tyrosine hydroxylase positive nigral neurons (Muthane et al., 1994). The rate of neuronal death is rapid, begins 12 hours after administration and continues for 4-5 days which is in line with the 6-hydroxydopamine model and does not accurately reflect the progressive nature of the disease. Similarly to 6-hydroxydopmaine, MPTP induces its neurotoxicity via three main mechanisms: generation of reactive oxygen species as a consequence of intra or extracellular oxidation, formation of hydrogen peroxide as a result of dopamine metabolism and inhibition of mitochondrial enzymes causing energy failure within the cell (Blum et al., 2001).

Both the MPTP and 6-hydroxydopamine models of Parkinson's disease share several key features in that they result in significant degeneration of dopaminergic neurons and concomitant loss of striatal dopamine. There are also inflammatory events associated with both of these toxins with the proliferation of reactive microglia consistent with inflammatory features seen in idiopathic Parkinson's disease.

Although, there are obvious weaknesses associated with these models, these limitations do not negate the significance of the contribution that they have made to the field of experimental research. Nevertheless, the rapidity of the disease course, the lack of α-synucleinopathy and the lack of etiological relevance of these direct dopaminergic neurotoxins have led to efforts to develop other, more relevant, models of Parkinson's disease.

1.3.3 Newer models

1.3.3.1 Environmental toxin-induced: Rotenone

The observation that MPTP induced selective nigrostriatal degeneration, via inhibition of mitochondrial complex I of the respiratory chain, has underpinned the search for other mitochondrial I inhibitors. The agricultural pesticide, rotenone, was first used to model Parkinson's disease in 1985 when Heikkila and colleagues discovered that direct injection of rotenone into the medial forebrain bundle resulted in lesions to the nigrostriatal pathway (Heikkila et al., 1985). However, in the intervening years, this model was largely neglected until Schapira and colleagues reported a Complex I defect in *post mortem* Parkinsonian brains (Schapira et al., 1989). In 1997, Ferrante began the first experiment with systemic rotenone which resulted in brain lesions and peripheral toxicity when rodents were challenged with daily doses of rotenone in the range of 10-18 mg/kg (Ferrante et al., 1997). Interestingly, when the dose of rotenone was titrated downward to produce Complex I inhibition but to have minimal impact on Complex I-linked respiration, chronic infusion via cannula in the jugular caused selective degeneration of nigral

dopaminergic neurons. The degeneration observed began in the nerve terminals and progressed retrogradely to the cell bodies of the substantia nigra suggesting that dopaminergic nerve terminals are particularly sensitive to Complex I inhibition (Betarbet et al., 2000). Chronic systemic infusion of low doses (2 mg/kg) of rotenone was also achieved via osmotic mini pumps and produced a significant loss of striatal nerve terminals without overt loss of cell bodies from the substantia nigra (Zhu et al., 2004). In addition to this, the motor dysfunction observed in rats treated with systemic rotenone was suggested to be due to peripheral toxicity rather than degeneration of the nigrostriatal pathway (Lapointe et al., 2004, Hoglinger et al., 2003).

Due to the variability in the systemic rotenone model of Parkinson's disease, efforts were made to identify an alternative route of administration therefore rotenone was administered directly into the nigrostriatal pathway. Infusion of rotenone into the nigrostriatal pathway resulted in a significant loss of nigrostriatal neurons with a parallel reduction in striatal dopamine content and its metabolites (Heikkila et al., 1985, Antkiewicz-Michaluk et al., 2004, Sindhu et al., 2005, Ravenstijn et al., 2008, Mulcahy et al., 2011). This nigrostriatal loss occurred in a dose dependent fashion, with an increase in oxidative stress markers and significant motor dysfunction in motor tasks such as apomorphine/amphetamine induced circling behaviour and skilled reaching and walking (Sindhu et al., 2005, Klein et al., 2011, Mulcahy et al., 2011). Intranigral infusion of rotenone also produced depressive-like behaviour in the forced swim and saccharin preference tests (Santiago et al., 2010), as well as

memory (Dos Santos et al., 2013) and olfactory impairments (Rodrigues et al., 2014) thereby modelling non-motor symptoms of Parkinson's disease.

In comparison to the MPTP and 6-hydroxydopamine models, the rotenone model is also limited by a rapid disease course and a lack of α-synucleinopathy. However, because rotenone has been causally linked to idiopathic Parkinson's disease (Tanner et al., 2011), this model has the distinct advantage of being etiologically relevant to the human condition. Therefore, this model provides an excellent tool for researchers interested in environmental toxin-induced Parkinson's disease.

1.3.3.2 Inflammation driven: Lipopolysaccharide

In order to better understand inflammatory events of the nigrostriatal system in Parkinson's disease etiology, inflammation-driven lipopolysaccharide (LPS) models were established. LPS is a structural component of the outer membrane of Gram-negative bacteria and, as such, is detected by the bacterial recognition receptor, Toll-like receptor-4 (TLR4) on the cell membrane of microglia (Chow et al., 1999). Resident microglia in the healthy brain rest in a 'dormant' state and are activated in response to various stimuli including LPS (Nimmerjahn et al., 2005). Given that the substantia nigra is densely populated with microglia (Kim et al., 2000, Lawson et al., 1990), it renders neurons in this region highly sensitive to inflammation-driven neurodegeneration. The inflammatory response induced by LPS involves the release of pro-inflammatory cytokines and other cytotoxic factors, such as reactive oxygen species and free radicals, all of which are toxic to nigrostriatal dopaminergic neurons (Kim et al., 2000).

The pioneering reports of direct administration of LPS causing nigral microglial activation with nigrostriatal neurodegeneration came from two independent laboratories in quick succession (Castano et al., 1998, Herrera et al., 2000). They also demonstrated that nigrostriatal degeneration was permanent and exclusive to dopaminergic neurons as both serotonergic and GABAergic neurons remained intact. Subsequent studies also demonstrated an increase in the levels of pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6, in the substantia nigra after LPS administration (Lu et al., 2000). Unlike 6-hydroxydopamine, MPTP or rotenone which causes dopaminergic cell loss as a result of direct neurotoxicity, LPS causes an inflammation-driven neurodegeneration. It has been determined that there is a temporal relationship between microglial activation and the degeneration of nigral neurons where significant microglial activation occurred 1-2 weeks post injection which was earlier than the onset of neurodegeneration which followed 4-6 weeks post injection (Gao et al., 2002). When LPS was infused into the striatum there was a progressive retrograde degeneration of dopaminergic cell bodies, a reduction in striatal dopamine content and detectable motor deficits (Choi et al., 2009, Hunter et al., 2009).

In addition to the MPTP, 6-hydroxydopamine and rotenone models, the LPS model is also limited by a relatively rapid disease course and a lack of α-synucleinopathy. However, because bacterial infection as well as chronic neuroinflammation have been linked to the etiology and pathogenesis of Parkinson's disease (Gao and Hong, 2008, Glass et al., 2010, Tansey and Goldberg, 2010), this inflammation-driven

model provides an excellent tool for researchers interested in infection and inflammation as drivers of this condition.

1.3.3.3 Genetic models: α-Synuclein transgenic mice

Familial forms of Parkinson's disease have been linked to several genetic mutations, and the identification of these genes has formed the basis for the development of many transgenic models of Parkinson's disease. These models include overexpression models for mutant genes such as α-synuclein or LRRK as well as knockdown or knockout models for Parkin and DJ-1 (Lim and Ng, 2009). As α-synuclein is relevant to the work described in this thesis, attempts to develop α-synuclein transgenic models will be outlined in more detail.

Several transgenic α-synuclein mouse models have been developed which are driven by different promoters, all which have produced variable phenotypes. Using the platelet-derived growth factor-β (PDGFβ) promoter to drive expression of wild type α-synuclein, overexpression of α-synuclein resulted in striatal loss of dopamine and motor impairment in the rotarod test. However, the inclusion bodies present were not typical of Lewy bodies, resided in atypical locations and there was no loss of cell bodies from the substantia nigra (Masliah et al., 2000). Similarly, mice overexpressing wildtype α-synuclein under the thymus cell antigen 1 (Thy1) promotor, displayed α-synuclein accumulation and the emergence of early motor deficits, but not significant nigrostriatal neurodegeneration (Fleming et al., 2004a, Fleming et al., 2008). A53T and wild type α-synuclein transgenic mice, under the

Thy1 promotor, displayed a progressive deterioration in motor dysfunction as a result of neuronal degeneration. However, the synuclein pathology observed in these animals was in the anterior spinal cord and not in nigral neurons (Kahle et al., 2000). In order to achieve expression of α-synuclein in the nigrostriatal pathway, the dopamine specific tyrosine hydroxylase promoter was used to drive expression of mutant A30P α-synuclein in mice. While this model exhibited significant α-synuclein pathology throughout the nigrostriatal pathway there was no associated neurodegeneration, motor dysfunction or true Lewy bodies (Matsuoka et al., 2001).

It is interesting that although α-synuclein mutations cause familial Parkinson's disease with 100% penetrance, transgenic α-synuclein mouse models result in "Parkinsonism" but not Parkinson's disease (Dawson et al., 2010). One possible theory suggested for the consistent lack of nigrostriatal degeneration in these models is that the different lifespans between mice and humans and the shorter lifespan of murine neurons may underlie the resilience of murine substantia nigra dopaminergic neurons to transgenic α-synuclein overexpression (Crabtree and Zhang, 2012, Kreiner, 2015).

1.3.3.4 Genetic models: Viral α-synuclein gene transfer

Harnessing viral vectors for gene transfer to the CNS offers an alternative approach to transgenesis for overexpression of α-synuclein in the nigrostriatal pathway. A particular advantage of viral gene transfer is the possibility of targeting gene expression to specific brain areas. Currently, the most utilised viral vectors for gene

delivery in animal models of Parkinson's disease are recombinant adenovirus, recombinant adeno-associated viruses (AAV), herpes simplex viruses and lentiviruses (Mandel et al., 2008). Since AAV-mediated transfer of α-synuclein to the substantia nigra was used in this thesis, this will be described in more detail.

AAVs are small nonenveloped viruses of the parvovirus family and categorised in the *Dependovirus* genus as they depend on coinfection with a helper virus either adenovirus or herpesvirus for replication (Daya and Berns, 2008). They have a cloning capacity of 4.7 kb and their genome is composed of single-stranded DNA which encodes two genes essential for replication and packaging (*rep* and *cap*) flanked by two inverted terminal repeats (ITR) (Sonntag et al., 2010) (Fig. 1.8 and Fig. 1.9). The advantages of the AAV vector system include; 1) the lack of disease associated with wildtype AAV in either human or animal systems, 2) the ability to transduce both dividing and non-dividing cells, 3) the long term expression of the delivered transgene, and 4) an attractive safety profile as it is non replicating with no risk of insertional mutagenesis (Denyer and Douglas, 2012). To date, there have been 9 serotypes characterised in the literature, AAV1 to AAV9, and these are all associated with variable tropism (i.e. preference for tissue/cell types). AAV_1, AAV_2 and AAV_5 have been shown to be most efficient in transducing neurons, and to a lesser extent, glia (Mandel and Burger, 2004). For neurodegenerative diseases such as Parkinson's disease, newer serotypes or 'pseudotypes' are being explored for their potential to increase the neural tropism and transgene expression over standard vectors. The pseudotype vector which offers the greatest transduction efficiency and longevity of transgene expression is the $AAV_{2/5}$ vector system as AAV_2 has a greater

tropism for neurons and AAV_5 is the most efficient at transducing cells (Monahan and Samulski, 2000). This vector system was created by using *rep* from AAV_2 and *cap* from AAV_5 to produce the resulting $AAV_{2/5}$ vector (de Backer et al., 2011).

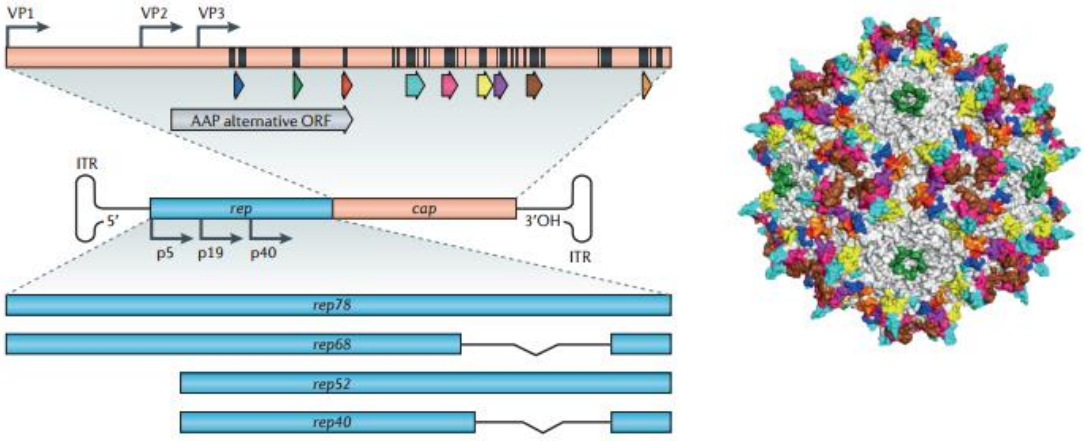


Fig 1.8: Overview of the AAV plasmid system. The AAV genome is packaged within a non-enveloped icosahedral capsid and contains three open reading frames (ORFs) flanked by inverted terminal repeats (ITRs) which form T-shaped hairpin ends. The rep ORF encodes four non-structural proteins (Rep40, Rep52, Rep68 and Rep78) that are essential for viral replication, transcriptional regulation, genome integration and virion assembly. The cap ORF encodes 3 structural proteins (VP1, VP2 and VP3) that form the viral capsid with the aid of the assembly-activating protein (AAP) which is encoded by an alternative ORF (grey arrow) located within cap. Crystal structure of the AAV capsid is shown and hypervariable regions of VP3 are coloured to match the corresponding genetic regions. Image taken from Kotterman and Schaffer, (2014).

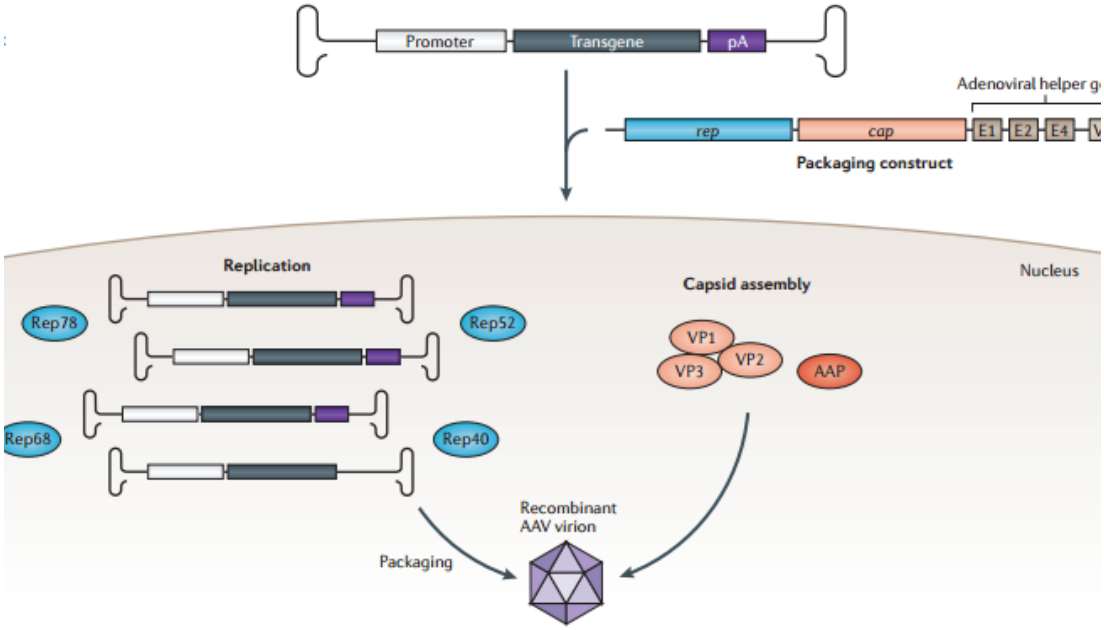


Fig 1.9: Overview of the AAV plasmid system. To generate recombinant versions of AAV, a gene of interest is inserted between the ITRs and replaces both rep and cap, which are provided in trans on 'packaging constructs' along with adenoviral helper genes that are needed for replication. The viral capsid determines the ability of the resulting AAV vector to transduce cells, from initial cell surface receptor binding to nuclear entry and genome release, which can lead to stable transgene expression in post mitotic tissue. There are 11 naturally occurring serotypes and more than 100 variants of AAV, which differ in their amino acid sequence of the capsid and thus in their gene delivery properties. pA, poly(A) tail. Image taken from Kotterman and Schaffer, (2014).

Using this viral gene transfer approach, overexpression of α-synuclein with AAV revealed the ability of wildtype and mutant α-synuclein to induce nigrostriatal pathology (Kirik et al., 2002). Infusion of AAV-α-synuclein into the rodent substantia nigra yielded significant neurodegeneration 8 weeks post injection and there was a quantifiable motor dysfunction observed in animals whose dopamine depletion exceeded a threshold of 50-60%. The variability in behavioural deficits was likely due to the high variability in the magnitude of neurodegeneration observed in the nigrostriatal pathway. The patterns of neuropathology and behavioural deficits observed in the AAV-α-synuclein rat model were subsequently reproduced in non-human primates (Kirik et al., 2003, Eslamboli et al., 2007).

In comparison with the α-synuclein transgenic models, the AAV gene transfer model has a major advantage in that it causes α-synuclein-induced nigrostriatal neurodegeneration. Moreover, relative to the neurotoxic, environmental and inflammatory models described above, it is also associated with a more clinically relevant and protracted disease course as well as consistent α-synucleinopathy (albeit without *bona fide* Lewy body formation). Thus, the AAV-α-synuclein model is now widely accepted as the most relevant animal model of Parkinson's disease currently available to preclinical researchers (Lindgren et al., 2012).

1.3.3.5 Gene-environment models

In addition to the recent developments in Parkinson's disease modelling using environmental pesticides, inflammagens and genetic alterations, there has also been a

drive to develop gene-environment interaction models of the disease which should have further improved etiological and neuropathological relevance. These gene-environment models have the potential to, 1) elucidate relevant gene-environment interactions in Parkinson's disease, 2) determine the pathological consequences of gene-environment interactions in Parkinson's disease, 3) determine the mechanisms underlying gene-environment interactions in Parkinson's disease, and 4) provide relevant models for testing of novel therapies for the condition (Horowitz and Greenamyre, 2010).

In this regard, the Dowd laboratory have recently embarked on a series of studies to develop animal models of Parkinson's disease which are triggered by dual exposure to genetic and environmental factors relevant to the human condition (Mulcahy et al., 2012, Mulcahy et al., 2013) . These studies have revealed that intra-nigral infusion of rotenone can exacerbate nigrostriatal neurodegeneration and motor dysfunction induced by AAV-α-synuclein thereby highlighting the potential importance of this interaction in the etiology of Parkinson's disease. However, the fact that Parkinson's is now being regarded as a retrograde 'die-back' disease in which impaired terminals retrogradely degenerate resulting in nigral cell body death and that previous studies in our lab focused on 1) sequential intranigral AAV-α-synuclein and rotenone and, 2) intranigral AAV-α-synuclein and systemic rotenone, we opted to target the terminals of the striatum in an attempt to model this feature of the disease.

1.4 Hypotheses

Thus, given that gene-environment approaches have the potential to provide improved Parkinson's disease animal models, the overarching aim of this thesis is to develop and characterise novel gene-environment rat models of Parkinson's disease which we hypothesise will recapitulate the main neuropathological and motor impairments of the human condition. Therefore, with these hypotheses in mind, the specific aims we undertook are outlined in the following section.

1.5 Aims of this study

Initial studies were performed in order to characterise new and emerging animal models of the disease, namely those driven by the bacterial inflammagen LPS and the agritoxin, rotenone and compare these models to the gold standard model of the disease, the 6-OHDA model (Chapter 3). Following on from the results of these experiments, we then focused on the interaction between pathological α-synuclein expression and inflammatory insults (Chapter 5) or environmental toxins (Chapter 6).

Chapter 2: Materials & Methods

2.1 List of materials used

Section	Product	Supplier	Catalogue code
Animals	Sprague Dawley rats	Charles River	Strain code: 400
Virus	DMEM	Sigma Ireland	D6429
	Penicillin/Streptomycin	Sigma Ireland	P0781
	Trypsin-EDTA	Sigma Ireland	T4174
	T-175 Flasks	Sarstedt	83.1812.002
	15cm Culture Dish	Sarstedt	83.1803
	Sterile filters	Sarstedt	83.1826.001
	Cell Scrapers	Sarstedt	83.183
	10 ml Pipettes	Sarstedt	86.1254.001
	JetPEI	Polyplus	
	25 ml Pipettes	Sarstedt	86.1685.001
	Dulbecco's PBS	Sigma Ireland	D8662
	Optiprep	Sigma Ireland	D1556
	DNase solution	Sigma Ireland	DN-25-100mg
	Benzonase	Sigma Ireland	E1014
	Giga Plasmid Kit	Qiagen	12191
	LB Broth	Sigma Ireland	L3022
	TE Buffer	Millipore	574793
	Isopropanol	Sigma Ireland	534021
Surgery	Isofluorane	VSS Co	IsoFlo 250ml

	Burs-steel round HP½	Claudius Ash	GCL0096
	Burs-steel round HP 3	Claudius Ash	GCL0069
	22G s/steel tubing	Coopers Needle Works	
	23G s/steel tubing	Coopers Needle Works	
	26G s/steel tubing	Coopers Needle Works	
	30G s/steel tubing	Coopers Needle Works	
	Polyethylene tubing	Harvard Apparatus	BS4 59-8326
	Curved Haemostat	Fine Science Tools	13003-10
	Straight Haemostat	Fine Science Tools	13018-14
	Rotenone	Sigma Ireland	R8875
	LPS	Sigma Ireland	L.012
	6-OHDA	Sigma Ireland	
	Cremophor-EL	Sigma Ireland	C5135
	Ethanol 200 proof	E7023	E7023
	Dimethyl Sulfoxide	Sigma Ireland	D260
Histology	Pentobarbital	Vetoquinol	Dolethal 250ml
	Heparin	Wockhardt	5,000 I.U./ml
	Paraformaldehyde	BDH Chemicals	28794.295
	Trizma Base	Sigma Ireland	T1530
	Sodium Azide	Sigma Ireland	S2002
	Sodium Chloride	Sigma Ireland	A3597
	Sodium Hydroxide	Sigma Ireland	30620
	Cutting Microtome	Bright Instruments	8000
Microtome	Grenier Plastic Pots	Cruinn Diagnostics	203-170

Hydrogen Peroxide	Sigma Ireland	H1009
Triton-X 100	Sigma Ireland	T9284
Diaminobenzidine	Sigma Ireland	D12384
ABC Kit	Vector Laboratories	PK-6100
Xylene	Lennox	A0663
DPX mountant	BDH Chemicals	360294H

2.2 Ethical statement

All procedures were 1) approved by the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland, Galway (Approval ID: 012/08), 2) completed under licence by the Irish Department of Health and Children (Dr. Eilis Dowd's Project Licence Number: B100/3827) and the Irish Health Products Regulatory Authority (Ms. Carol Naughton's Individual Authorisation Number: AE19l25\I050) and, 3) were carried out in accordance with European Union Directive 2010/63/EU and S.I. No. 543 of 2012. Any experiments involving the administration of viral vectors were completed under a license from the Irish Environmental Protection Agency (GMO Register Number: 290).

2.3 Animal husbandry

A total of 176 male Sprague Dawley rats sourced from Charles River, UK (6 weeks of age and weighing 225-250g on arrival) were used in this body of research. Rats were maintained on a 12:12 hour light:dark cycle (lights on at 08:00h) under regulated temperature (21 ± 2ºC) and humidity (40-70%) conditions. Rats were housed (4 per cage) in plastic bottom cages (50.5 x 13 x 24 cm) with a wire grid lid

until they reached a body weight which required them to be housed in pairs. Standard rat chow and water were available *ad libitum* except when food restriction was required for behavioural experiments. In this case, rats were given enough food to maintain their weight at 85-90% of their free feeding weight, assessed by comparison to animal growth curve supplied by Charles River. All behavioural testing and *ex vivo* quantification were completed blind to the treatment of the rats.

2.4 Global Experimental Design

The overall aim of this thesis was to develop and characterise novel gene-environment rat models of Parkinson's disease that recapitulate the nigrostriatal neurodegeneration, α-synuclein pathology and progressive motor dysfunction reminiscent of the human condition. After first characterising emerging inflammatory and environmental single-insult models (Chapter 3), we then went on to investigate dual-exposure models by assessing the interaction between pathological α-synuclein expression and inflammatory insults (Chapter 4) or environmental toxins (Chapter 5).

In Chapter 3, we first sought to assess emerging inflammatory (LPS-driven) and environmental (rotenone-driven) single-insult models and to compare these with a classical neurotoxin model (6-OHDA-driven) in terms of their behavioural and neuropathological profile. This allowed us to determine the impact of these factors when administered to the brain as a single insult. Once this had been determined, we then used LPS and rotenone as subsequent challenges with AAV-α-synuclein in the dual exposure studies.

In Chapter 4, we wanted to determine the impact of dual exposure to AAV-α-synuclein and LPS in order to determine if exposing rats with a high nigrostriatal burden of α-synuclein to bacterial-like neuroinflammation would exacerbate their Parkinsonism. In these experiments, α-synuclein was overexpressed in the rodent brain using AAV vectors and this was followed several weeks later by LPS administration to the striatum. This combination of genetic and inflammatory insults allowed us to assess whether this genetic-inflammatory system was a valid approach to modelling Parkinson's disease.

In Chapter 5, we wanted to determine the impact of dual exposure to AAV-α-synuclein and rotenone in order to determine if exposing rats with a high nigrostriatal burden of α-synuclein to environmental pesticides would exacerbate their Parkinsonism. In these experiments, α-synuclein was overexpressed in the rodent brain using AAV vectors and this was followed several weeks later by rotenone administration to the striatum. This combination of genetic and environmental insults allowed us to assess whether this genetic-environmental system was a valid approach to modelling Parkinson's disease.

The specific *in vitro*, *in vivo* and *ex vivo* methodologies used in this project are detailed below.

2.5 AAV preparation

2.5.1 GFP and α-synuclein plasmid preparation

Plasmids carrying GFP (pTRUF-SEW plasmid, Fig. 2.1) or normal human α-synuclein (pAAV2-SnaSW plasmid, Fig 2.2) transgenes under the control of the human synapsin promoter were kindly donated by Prof. Deniz Kirik, Lund University, Sweden.

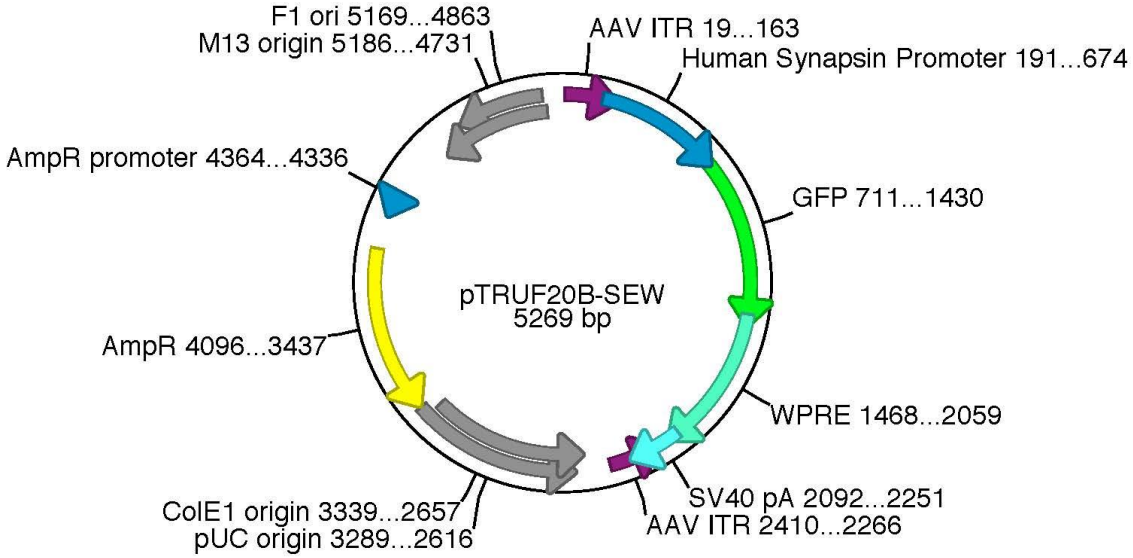


Fig 2.1: GFP plasmid. The map of the GFP plasmid used to produce our $AAV_{2/5}$-GFP viral vectors under the control of the synapsin promoter and containing a WPRE (Woodchuck Hepatitis Posttranscriptional regulatory Element) region which increases transgene expression of our region of interest.

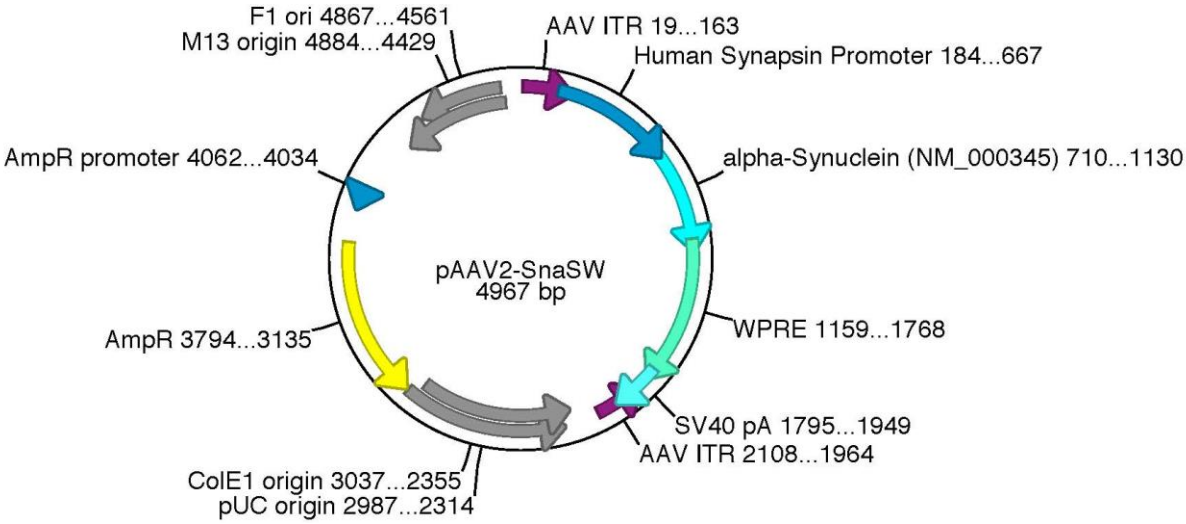


Fig 2.2: α-synuclein plasmid. The map of the α-synuclein plasmid used to produce our $AAV_{2/5}$-α-synuclein controls under the control of the synapsin promoter and containing a WPRE region, or the Woodchuck Hepatitis Posttranscriptional regulatory Element.

2.5.2 HEK 293T cell culture

Transformed human embryonic kidney 293 (HEK 293T) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated horse serum and 1% penicillin/streptomycin at 37ºC in a humidified 5% CO_2 atmosphere. Cells were passaged every 2-3 days using 0.025% trypsin-EDTA to detach the adherent cells from the flask. After 5 min at 37ºC, twice the volume of medium was added to inactivate the trypsin-EDTA. Suspended and detached cells were pelleted at 300 x g for 5 min. The supernatent was removed and the cells were resuspended with 10 ml of fresh medium. Cell number was counted using a haemocytometer and cells were seeded at a density of 2 x 10^4 cells cm^{-2} in a T175 flask. For viral transfection, HEK 293T cells were grown and passaged until a total of 40 T175 flasks had reached 70-80% confluency.

2.5.3 Preparation of plasmid DNA

Competent Dh5 alpha *E. coli* bacteria were transformed with the individual plasmids, conferring ampicillin resistance to the plasmid containing *E.Coli*. Glycerol stocks were then generated and stored at -80ºC (Appendix 1.1). Preparation of glycerol stocks of bacteria allows for long-term storage at -80ºC without compromising the viability of cells by reducing the harmful effects of ice crystals on bacteria which can damage cells by dehydration leading to denaturation. Frozen Dh5alpha *E.Coli* glycerol stocks for GFP and α-synuclein were streaked on separate agar plates containing ampicillin at 50 mg ml^{-1} and incubated overnight at 37ºC. Single colonies were picked and added to 2 ml nutritionally rich lysogeny broth (LB) containing 50

mg ml^{-1} ampicillin and incubated overnight at 37ºC shaking at 180 rpm. A sample 500 µl ml^{-1} of GFP or α-synuclein starter culture was added to multiple 1 L flasks of LB broth and incubated for 12-16 hours at 34ºC while shaking. Bacterial cells were then harvested by centrifugation at 6000 x g for 15 min at 4ºC. Plasmid isolation was performed using QIAGEN® Plasmid Gigaprep Kit (QIAGEN) according to the manufacturers' instructions. The pellet was air-dried for 30 min and dissolved in 10 ml sterile TE buffer.

2.5.4 Plasmid quantification

Plasmid DNA quantification was determined by spectrophotometric measurement of the absorption at 260nm using a NanoDrop® sample quantification system. Plasmids were stored at -20ºC (Appendix 1.2).

2.5.5 Transfection, harvesting and concentration of AAV

The AAV-α-synuclein and AAV-GFP viral vectors were produced by co-transfecting HEK 293T cells with the relevant AAV plasmid and pDG-5 as described previously (Grimm et al., 1998) for 48 hours by jetPEI® (Appendix 1.3). Briefly, T-175 cell culture flasks, 70-80% confluent with HEK 293T cells, were split into 15 cm round cell culture dishes (one flask was split into two dishes) the day before transfection. Media was drawn off the dishes and the cells were transfected with a total of 500 µg of plasmid DNA per 10 dishes by jetPEI®. Cells were incubated under standard conditions for 48 hours. For harvesting, 3-5 ml of media was drawn off the dishes and the cells were scraped into 50 ml falcon tubes. The tubes were then

centrifuged (5000 x g for 10 min), supernatent discarded and lysis buffer added to lyse cells. To augment the lysing process, cells went through three freeze thaw cycles. Viral vectors were purified by the treatment of the transfected cell pellet with a DNA endonuclease to degrade any non-encapsulated DNA at 37ºC for 1 hour. For purification, the virus was concentrated using iodixanol gradient ultracentrifugation (350,000 x g for 90 min). Further concentration of the virus via centrifugal filter units resulted in a final viral volume of 400-600 µl. Viral titers were established using real time PCR expressed as vector genomes (Hammerum et al.) μl^{-1}. Viruses were aliquoted and stored at -80ºC.

2.6 Surgery

2.6.1 Stereotaxic surgery

Stereotaxic surgery was conducted under isofluorane anaesthesia (5% in oxygen for induction; 2% in oxygen for maintenance) in a stereotaxic frame with the nose bar set at -2.3 mm. An incision was made through the skin over the skull and the skull was exposed. Following location of bregma, the stereotaxic arm holding the stainless steel injection cannula was adjusted to the coordinates of the target injection site and a drill was used to expose dura over the injection site. The injection cannula (30 G) was connected to a Hamilton syringe with polyethene tubing (0.28 mm ID) filled with sterile saline. A micro-infusion pump was used to depress the Hamilton syringe and allowed the appropriate volume of solution to be delivered. All solutions were kept on ice until immediately prior to administration. The substantia nigra was targeted at 1 site using stereotaxic coordinates AP -5.3, ML ± 2.0 (from bregma) and DV -7.2 (below dura), while the striatum was targeted at 4 sites using stereotaxic

coordinates AP + 1.3, ML ± 2.7; AP + 0.4, ML ± 3.1; AP -1.3, ML ± 4.7 (from bregma) and DV -5.0 (below dura); at 2 sites using stereotaxic co-ordinates AP + 0.4, ML ± 3.1; AP – 0.4, ML ± 1.2 (from bregma) and DV -5.0 (below dura); or at 1 site using stereotaxic coordinates AP ± 0.0, ML ± 3.7 (from bregma) and DV -5.0 (below dura) (see Fig. 2.3). All toxin injections or their corresponding vehicle infusions were 3 µl in volume at an infusion rate of 1 µl min^{-1} and allowed 2 mins for diffusion, except for single site intrastriatal LPS which was in a final volume of 2 µl. Following infusions, the incision was sutured, a topical anaesthetic was applied and animals were allowed recover before being returned to their home cage.

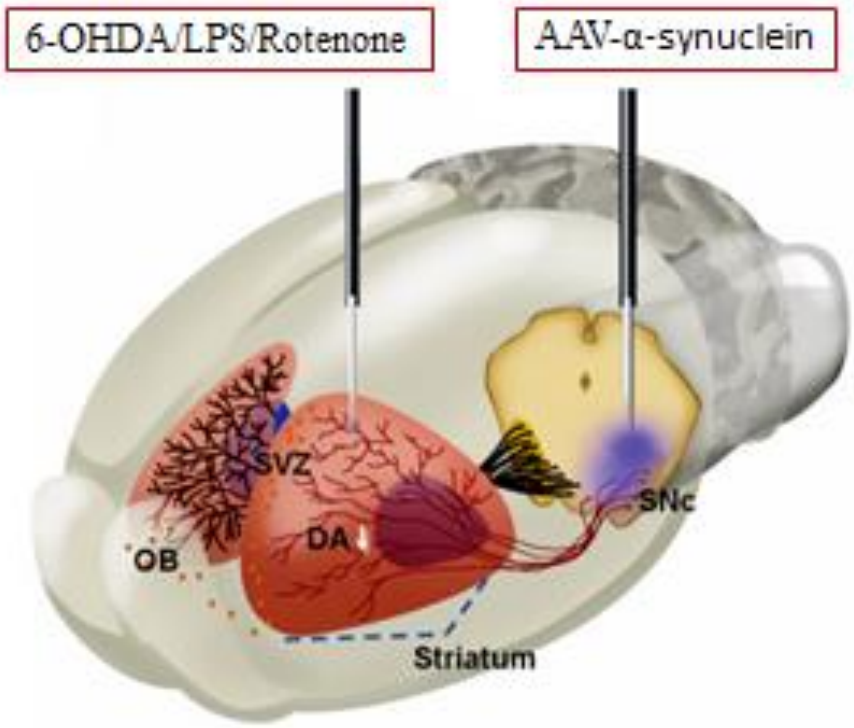


Fig. 2.3: Stereotaxic striatal and nigral sites: Location of nigral and striatal injection sites highlighting the nigrostriatal pathway and showing the site of unilateral intrastriatal and intranigral administration. The striatum was lesioned at one, two or four sites depending on the extent of the lesion required. OB: Olfactory Bulb; DA: Dopamine; SNc: Substantia Nigra; SVZ: Subventricular Zone. Image modified from Arias-Carrion (2008).

2.7 Behavioural Tests of Motor Function

2.7.1 Corridor test

The Corridor Test (Dowd et al., 2005, Fitzsimmons et al., 2006) (Fig. 2.4A) relies on the phenomenon of sensorimotor contralateral neglect where rats with unilateral dopaminergic lesions develop a bias to their ipsilateral side and almost exclusively ignore their contralateral side. The apparatus consisted of a long narrow corridor (length = 150 cm, height = 24.5 cm and width = 7 cm) into which lids (1 cm diameter) with CocoPops® are placed in adjacent pairs every 10 cm. After first habituating to the apparatus, rats were then allowed to freely explore the corridor and retrieve CocoPops® from their left and right sides. The numbers of ipsilateral or contralateral retrievals were counted until a total of 20 retrievals were made or until the 5 min test time had elapsed. The retrievals made from the ipsilateral and contralateral sides were expressed as a percentage of the total retrievals made.

2.7.2 Stepping test

The stepping test (Olsson et al., 1995) (Fig. 2.4B) is a measure of forelimb akinesia which is impaired on the side of the body contralateral to a unilateral dopaminergic lesion. In this test, after first habituating to the hold required, the rat was held with its hindlimbs and 1 forelimb gently restrained. The rat was then allowed to make sideways adjusting steps with the free forelimb along the edge of the table over a distance of 90 cm covered in approximately 5 seconds. The number of adjusting steps made in each direction (forehand and backhand) with each forepaw was counted. In this task, it was essential to habituate both the rat and the experimenter

thoroughly so that the environment in which the testing was conducted was calm (as a stressful environment could lead to the rat making steps in an attempt to escape which would be deemed as a false positive result).

2.7.3 Whisker Test

The vibrissae-elicited forelimb placement test (Schallert et al., 2000) measures the rat's sensorimotor integration which is impaired on the side of the body contralateral to the unilateral dopaminergic lesion (Fig. 2.4C). In this test, after first habituating to the hold required, the rat was held with its hindlimbs and one forelimb gently restrained. The rat was then brought close to the corner of a table and its vibrissae (whiskers) were allowed to brush against the corner of the table causing the rat to reach forward and place the unrestrained forepaw on the table edge. This was then repeated for 10 times with each forepaw. The numbers of forepaw placings on both the ipsilateral and contralateral sides of the rat were counted. In line with the stepping test, the experimenter was required to habituate the rat thoroughly to the handling in this test to allow for accurate recording and interpretation of the results.

2.7.4 Cylinder Test

The Cylinder Test is used as a measure of forelimb asymmetry and was completed as previously described (Schallert et al., 2000) (Fig. 2.4D). This test was designed to evaluate limb use asymmetry in unilateral lesioned animals whereby rats were placed into a clear cylinder and allowed to freely explore using either or both forelimbs to support themselves against the wall while exploring in the vertical direction. The

number of forepaw placings using the ipsilateral and contralateral paws was counted until a total of 20 forelimb contacts were recorded or until the 5 min test time had elapsed. The number of ipsilateral and contralateral forelimb placings was expressed as a percentage of the total forelimb contacts made.

2.7.5 Amphetamine induced rotations

Dopaminergic asymmetry was assessed via drug-induced circling behaviour as previously described by Ungerstedt and colleagues (Ungerstedt and Arbuthnott, 1970) (Fig. 2.4E). Rats were removed from their home cage and placed into a basin containing standard bedding and allowed to habituate to their new environment for 10 mins. Once the habituation period was complete, animals were then administered $_{D}$-amphetamine (2.5 mg/kg^{-1} i.p.) Full body ipsiversive and contraversive rotations were counted for 60 mins in 10 x 1 min time bins. Data was expressed as net ipsilateral turns min^{-1}.

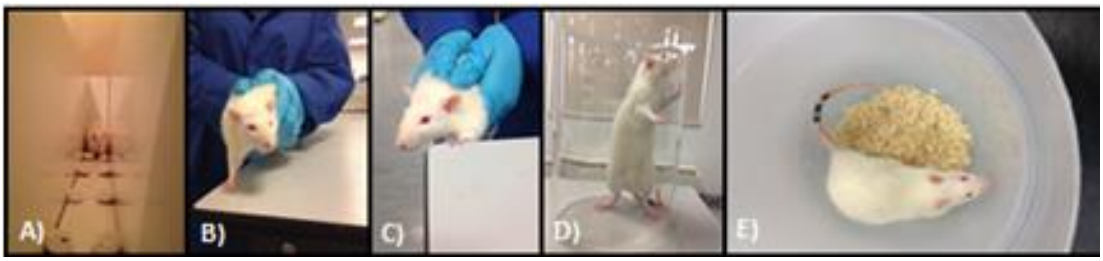


Fig. 2.4: Photographs of rats performing the Corridor, Stepping, Whisker, Cylinder and Amphetamine Rotation Tests. In the Corridor Test, the rat is allowed to freely retrieve Coco Pops® from pots placed at intervals (A). In the Stepping Test, one forepaw is gently restrained while the other is allowed to make adjusting steps along the table (B). In the Whisker Test, the rat is gently restrained with one forelimb remaining free, and vibrissae-elicited forepaw placings with the free forepaw were counted (C). During the Cylinder Test of forelimb asymmetry, the rat was placed into a clear cylinder for 5 mins and the number of times either forelimb touched the side walls was counted (D). Rotational behaviour induced by a single injection of $_{D}$-amphetamine (2.5 mg/kg i.p.) and the number of ipsiversive and contraversive turns per min were counted for 60 mins in 10 x 1 min time bins (E).

2.7.6 Open field test

The open field test was also used to measure exploratory behaviour and general locomotor activity (Hall, 1932, Hall, 1934) (Fig. 2.5). Testing was carried out in two specially constructed white circular arenas (diameter: 75 cm; height 50 cm). In all experiments, the arena was illuminated by four 60 watt bulbs which provided constant light intensity of 180 ± 10 lux at floor level of the arena. A camera positioned above the arena fed live images to a DVD recorder for subsequent analysis using a computerised video tracking system (EthoVision®, Versions 3.1 and 8 Noldus Information Technology, Wageningen, Netherlands). Rats were placed in the centre of the arena and their locomotor activity was recorded for a 5 min trial

period. When the trial was finished, the animal was placed back in their home cage and the arena was cleaned with 70% Industrial Methylated Spirits (IMS) for the next trial.

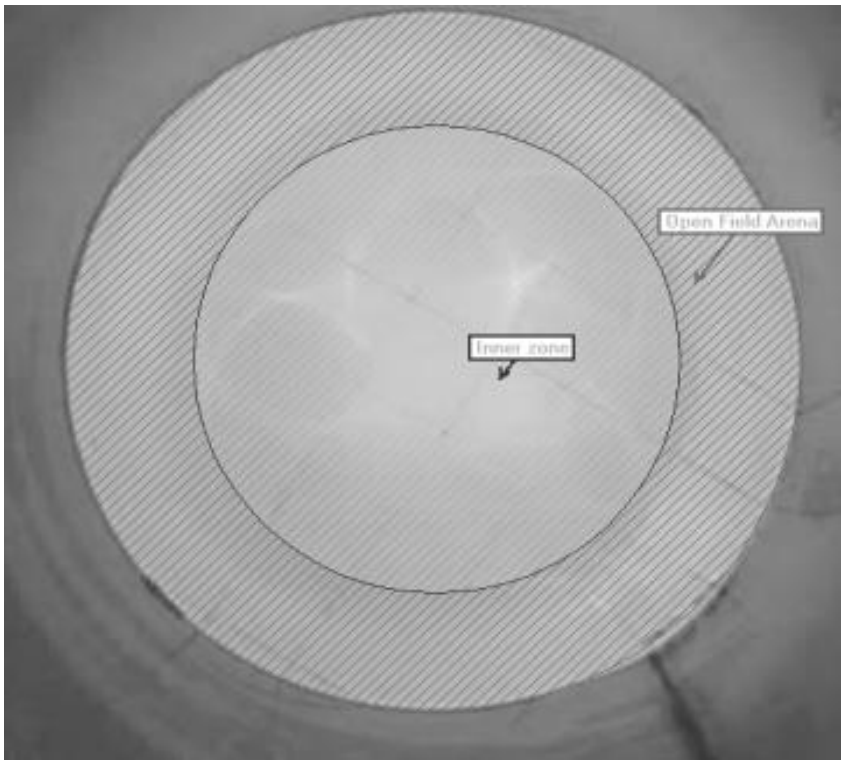


Fig. 2.5: Open Field Arena. Open field arena (75 cm) showing inner zone (50 cm). Briefly, the rat was placed in the arena for a 5 min trial and subsequently assessed using a computerised video tracking system.

2.8 Behavioural Tests of Cognitive Function

2.8.1 Novel Object Recognition Test

The novel object recognition test is based on the natural tendency of the animal to spend more time exploring new, rather than previously encountered, objects (Berlyne, 1950), and was first described as a measure of recognition memory by (Ennaceur and Delacour, 1988, Ennaceur et al., 2008). The procedure used herein

was based on a number of protocols described previously (Bevins and Besheer, 2006, King et al., 2004) with some modifications. Testing was conducted in the same arena as the open field test. The objects used included cans of Coca-Cola® (diameter: 6.5 cm; height: 9.5 cm) and a carton of Ribena® (length: 5.5 cm; width: 5 cm; height: 9 cm). In all cases, the objects had no apparent natural significance to the rats and were secured to the base of the arena with white tack so they were not easily displaced. These objects were thoroughly wiped down before and after each rat were placed into the arena. Animals were habituated to the arena in the absence of objects for 20 mins. The test day comprised three stages: (A) Habituation, (B) Exposure 1 and (C) Exposure 2. Rats were introduced to the arena for a 3 min habituation period on the test day and then returned to their home cage for 7 mins. During Exposure 1, two identical objects were placed in the arena 16 cm from the perimeter of the circular arena. The rat was allowed to freely explore the arena and the identical objects (in this case cans of Coca-Cola®) for a 3 min period after which the animal was removed from the arena and placed back into its home cage for an interval of 3 mins. Prior to Exposure 2, one of the identical objects was replaced with a novel object (in this case a carton of Ribena®). The animal was then placed back into the arena and was once again allowed to freely explore the arena and the objects for a period of 3 mins and then returned to its home cage. Representative images from Exposure 1 and Exposure 2 are shown in Fig. 2.4. The arena was cleaned with 70% IMS between rats in order to remove odours and olfactory cues, and faecal pellets were removed between exposures. Objects were also cleaned thoroughly with 70% IMS. Exploration of an object was defined as sniffing the object and rearing against the object. Exploratory behaviour and general behaviours of sniffing, rearing and

grooming were manually rated from the DVD recordings of each of the three test stages with the aid of Ethovision® behavioural tracking system. The proportion of time spent exploring the object was assessed by calculating a discrimination ratio as follows: total time spent exploring either object/total time spent exploring both objects.

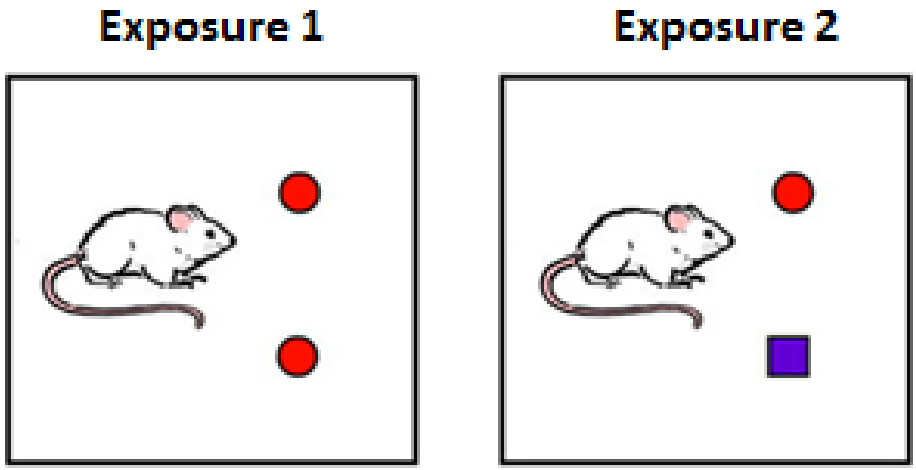


Fig. 2.6: Novel Object Recognition Test: Briefly, on the test day the animal is introduced into the arena for 3 mins and then removed to their home cage for 7 mins. They are then introduced into the arena again with two identical objects for 3 mins, termed Exposure 1, after which they are removed from the arena into their home cage. Finally, they are reintroduced into the arena with one of the familiar objects replaced with a novel object for another 3 mins, termed Exposure 2. Trials were subsequently assessed using the EthoVision® computerised video tracking system.

2.9 Immunohistochemistry

2.9.1 Tissue processing

Rats were deeply anaesthetised with sodium pentobarbital (50 mg/kg^{-1} i.p., Vetoquinol, Dublin) and transcardially perfused with 100 ml of ice cold heparinised saline (5000 units litre^{-1}, Wockhardt, Wrexham, UK) followed by 150 ml of 4% paraformaldehyde (pH 7.4) (Appendix 1.6). Their brains were removed and placed into 4% paraformaldehyde for post fixation for 24 hours prior to their transfer to 25% sucrose plus 0.1% sodium azide solution. After a minimum of 48 hours equilibrium in sucrose solution, 30-40 µm coronal serial sections of the fixed brains were cut using a freezing sledge microtome (Bright, Cambridgeshire, UK) and collected in a series of 12. A 1:12 or 1:6 (where indicated) series of sections were used for all quantitative immunohistochemistry.

2.9.2 Immunohistochemistry

Free floating immunohistochemistry was carried out as described previously (Mulcahy et al., 2012, Naughton et al., 2016a) (Appendix 1.7 and 1.8). All incubations were carried out in a volume of 1 ml. For immunoperoxidase-based staining, following quenching of endogenous peroxidase activity for 5 mins (using a solution of 3% hydrogen peroxide/10% methanol in distilled water), sections were washed 3 x 5 mins using tris-buffered saline (TBS) followed by blocking of non-specific secondary antibody binding for 1 hour (using 3% normal serum in TBS containing 0.2% Triton-X 100, pH 7.4). Sections were then incubated overnight at room temperature in the appropriate primary antibody (in normal serum and TBS

containing 0.2% Triton-X, pH 7.4). The sections were then washed 3 x 10 mins washes using TBS and then incubated with the appropriate biotinylated secondary antibody for 3 hours (in 1% normal serum and tris-buffered saline, pH 7.4). The sections were then washed 3 x 10 min TBS washes before incubating in a streptavidin-biotin-horseradish peroxidase solution for 2 hours (Vector, Peterborough, UK). Further washes with TBS (3 x 10 mins) and tris-non saline (TNS) (3 x 5 mins) were carried out before immunolabelling was revealed by incubating the sections in a 0.5% solution of diaminobenzidine tetrahydochloride in TNS containing 30% hydrogen peroxide. Sections were mounted on gelatine coated microscope slides, dehydrated in an ascending series of alcohols (5 mins in each of 50%, 70%, 100% and 100% ethanol), cleared twice in xylene (5 mins in each), coverslipped using DPX mountant (BDH chemicals, Yorkshire, UK) and allowed to air-dry overnight. All primary and secondary antibodies used in the present project are detailed in Table 2.1 below.

Primary Antibody	Target	Source and Catalogue number	Host	Dilution
Tyrosine hydroxylase (TH)	Catecholaminergic neurons	Millipore (MAB318)	Mouse	1:1000
α-synuclein	α-synuclein protein	Millipore (Syn211)	Mouse	1:10000
OX-42/CD11b	Microglia	Chemicon (CBL1512)	Mouse	1:400

Secondary Antibody	Source and Catalogue number	Host	Reactivity	Dilution
Biotinylated	Vector (BA2001)	Horse	Mouse	1:200

Table 2.1 A list of the primary and secondary antibodies used in this thesis.

2.10 Histological quantification

All image analysis was carried out using ImageJ software. All analyses were carried out on intact and lesioned sides of the brain and, in most experiments, quantitative immunohistochemical data on the lesioned side is expressed as a percentage of the intact side.

2.10.1 Substantia nigra quantifications

To quantify the survival of dopaminergic neurons in the substantia nigra after toxic insult, tyrosine hydroxylase immunopositive cells were counted in three coronal photomicrographs throughout the structure (Fig. 2.7). For each animal, photomicrographs of the region were obtained using a Nikon DXM1200C digital camera mounted onto a Nikon dissecting microscope. The numbers of tyrosine hydroxylase-positive cell bodies in the substantia nigra were counted on both the

ipsilateral and contralateral sides according to distinct boundaries as previously defined (Kirik et al., 1998). Briefly, the area was defined to include the tyrosine hydroxylase immunoreactive cell bodies of the substantia nigra but to exclude the tyrosine hydroxylase immunoreactive cell bodies of the ventral tegmental area (VTA). In most cases, data was expressed as a percentage of the intact side.

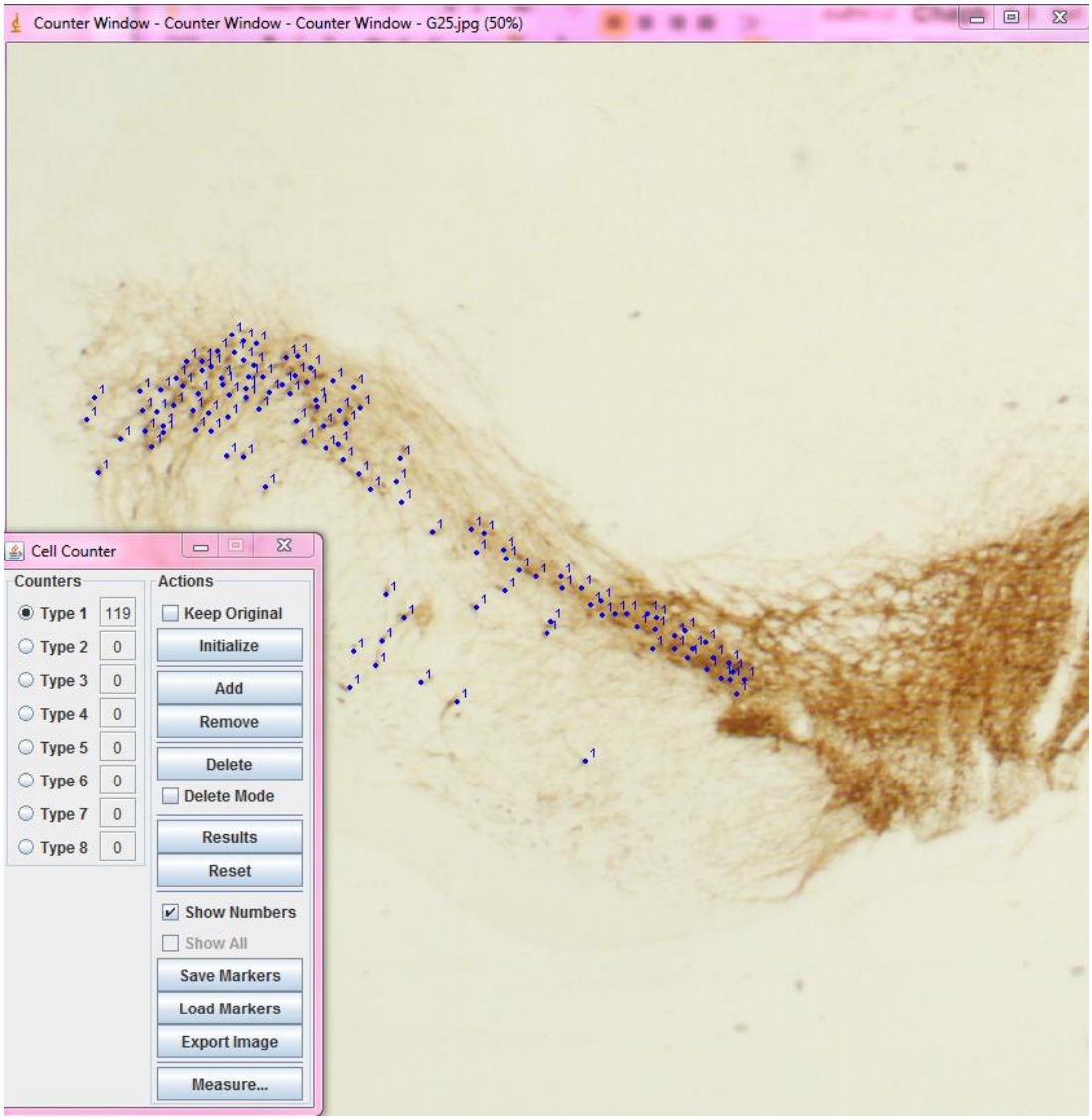


Fig 2.7: Screen grab of ImageJ software used to determine tyrosine hydroxylase positive cell bodies in the substantia nigra. Blue counter dots on the image indicate the location of cell bodies. For each section, counts were performed on both the left and right side of the substantia nigra.

2.10.2 Striatal quantifications

Tyrosine hydroxylase-positive nerve terminals of the dorsal striatum in three coronal representative photomicrographs throughout the striatum were analysed using ImageJ software (Fig. 2.8). For each animal, photomicrographs were obtained using a DXM1200C digital camera mounted onto a Nikon dissecting microscope. For all optical density analyses, images were converted to 16-bit black and white image within ImageJ, and the mean grey value of both the intact and lesioned striata was measured together with the mean background grey value of the adjacent (unstained) corpus callosum in each section. These were then converted to optical densities by applying the conversion formula in ImageJ (optical density = $\log_{10}$(255/mean grey value). Final optical densities were then calculated as the difference in staining between an immunostained striata and unstained corpus callosum. Measurements were taken on both the ipsilateral and contralateral sides of the brain, and, in most cases, data were expressed as a percentage of the intact side. In addition to optical density measurements, the number of tyrosine hydroxylase-positive dystrophic neurites in the striatum were counted using ImageJ (Fig. 2.9). For each animal, photomicrographs were taken from one representative striatal section using an Olympus microscope BX40 with an Olympus C-5060 digital camera. The number of dystrophic neurites in the striatum ipsilateral to the lesion was counted. For quantification of AAV-α-synuclein mediated expression of α-synuclein in the striatum, the optical density of the α-synuclein immunoreactivity in the striatum was quantified in a single coronal section and optical density was calculated as outlined above.

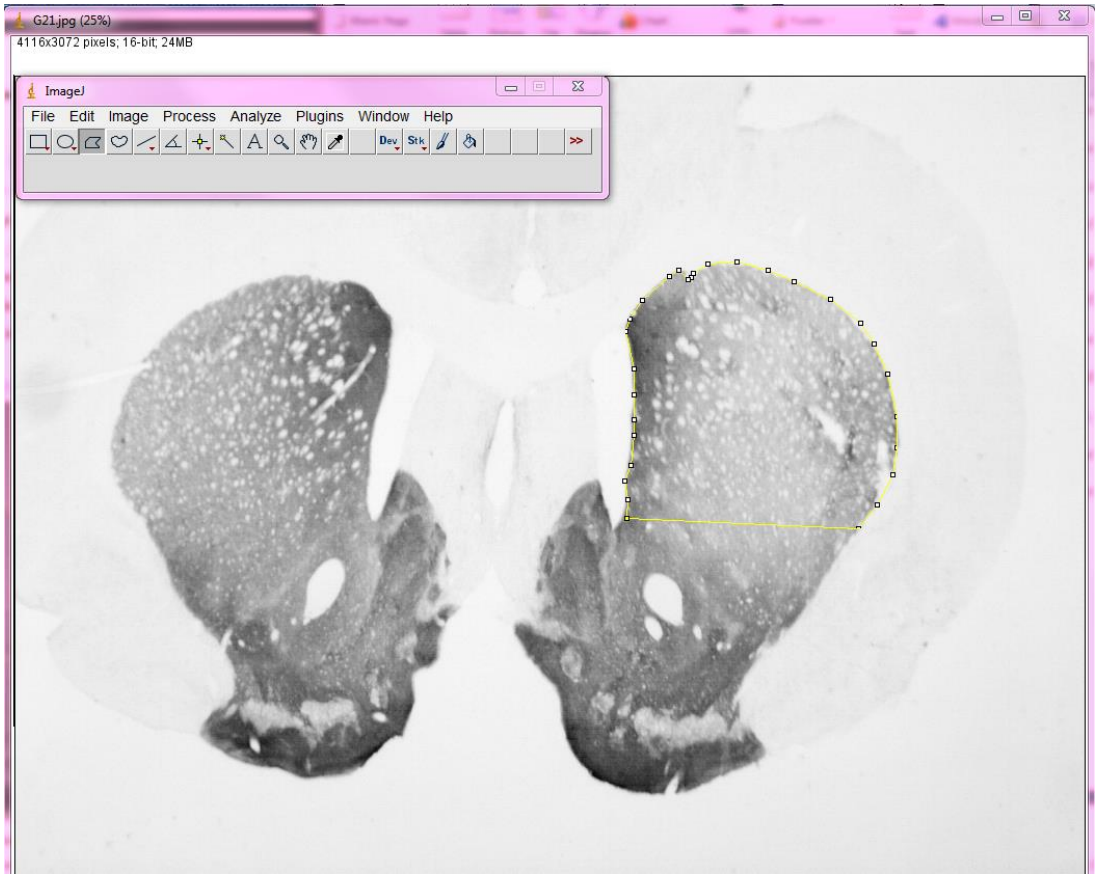


Fig 2.8: Screen grab of the ImageJ software used to quantify optical density of tyrosine hydroxylase positive terminals of the dorsal striatum. Images were converted to greyscale prior to analysis. The yellow border depicts the area to be measured. The border is defined so as to exclude the nucleus accumbens. For each section, measurements were taken of the left and right striatum in addition to a measurement of the non-stained corpus callosum to be used for background measurement.

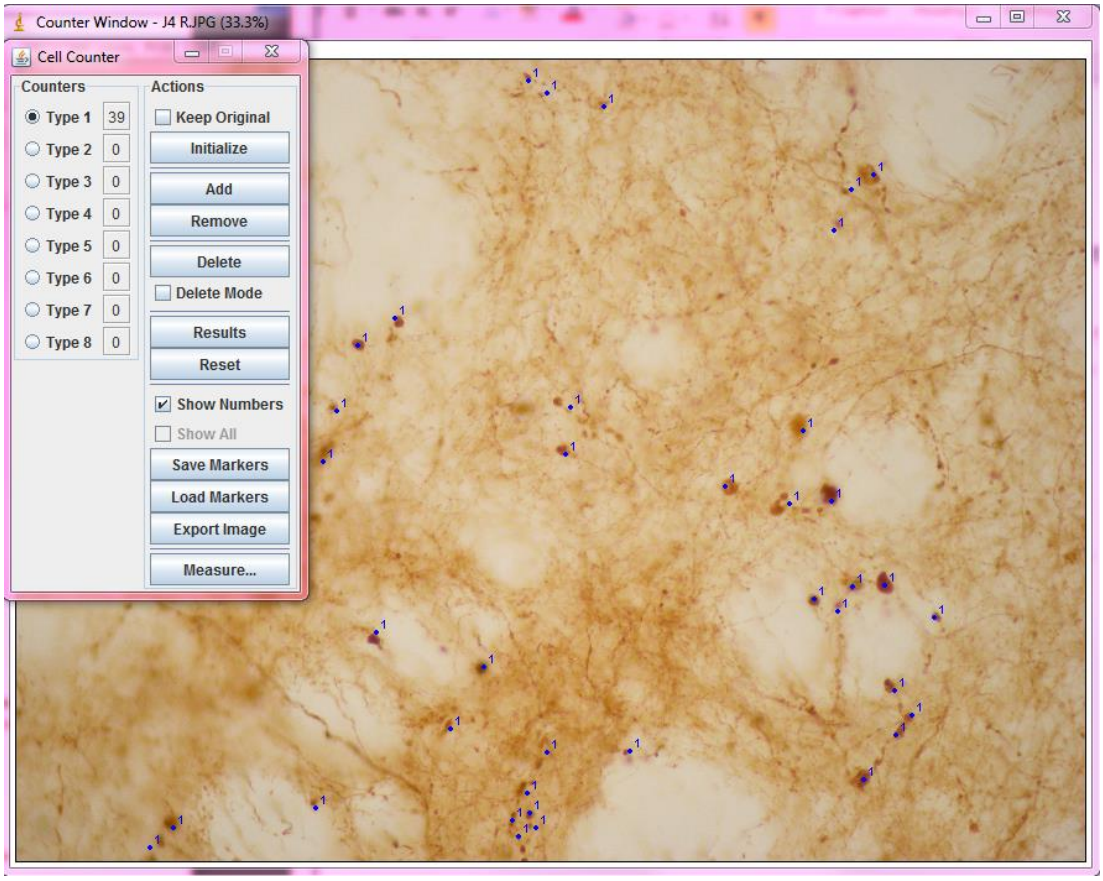


Fig 2.9: Screen grab of ImageJ software used to quantify tyrosine hydroxylase positive dystrophic neurites in the striatum. Blue counter dots on the image indicate the location of dystrophic neurites in the striatum.

2.11 Statistical analysis

All data were assessed for normality and equality of variance using Shapiro-Wilk and Bartlett's tests, respectively and were 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 whereas two-way ANOVA was used to compare the mean of more than two groups on two factors simultaneously. Behavioural data were analysed using two-way repeated measures ANOVA with within subject factor of time and between subject factor of group. *Post-hoc* testing was conducted using the Bonferroni or

Student Newman-Keuls tests where appropriate. Analyses were deemed to be significant at $P<0.05$.

Chapter 3: Comparison of neurotoxic, inflammatory and environmental models of Parkinson's disease

3.1 Introduction

The severe and progressive loss of midbrain dopaminergic neurons from the substantia nigra *pars compacta* is one of the distinguishing neuropathological hallmarks of Parkinson's disease (Eriksen et al., 2005b). The consequent loss of dopamine from the striatum results in the emergence of the cardinal motor syndrome of the disease which includes resting tremor, bradykinesia, rigidity and postural instability (Schapira and Jenner, 2011). In the initial stages of the disease, these motor symptoms can be controlled pharmacologically using several dopaminergic therapies as described in Chapter 1. However, as the disease progresses, the serious side effects and major limitations of these drugs manifest. One factor that has been proposed to underlie the paucity of novel therapies for this condition is the lack of relevant animal models which capture the etiological, neuropathological and behavioural features of the human disease (Linazasoro, 2004, Willis and Kennedy, 2004).

The unilateral 6-hydroxydopamine lesion is used to induce the 'gold standard' model of Parkinson's disease which was first described by Ungerstedt in 1968 (Ungerstedt, 1968). This model has been used extensively as it induces a rapid and pronounced dopaminergic neurodegeneration with associated motor dysfunction (Decressac et al., 2012a). However, there are some limitations of the 6-OHDA model, namely its poor etiological relevance to human Parkinson's disease which is thought to result

from interplay between genetic and environmental factors. As environmental factors are now believed to influence the risk of Parkinson's disease, exposure to exogenous factors such as pesticides (Betarbet et al., 2000, Sherer et al., 2003, Tanner et al., 2011) and infectious/inflammatory agents (Castano et al., 1998, Liu and Bing, 2011) are now underpinning the drive to develop more etiologically relevant models of Parkinson's disease.

As previously iterated, rotenone is an organic pesticide which has been causally linked to Parkinson's disease (Tanner et al., 2011). The neurotoxic effects of exposure to this agritoxin are mediated through Complex I inhibition subsequently impairing mitochondrial function (Li et al., 2003, Schapira et al., 1990). Infection and the resulting inflammatory response (modelled using LPS) also cause dopaminergic cell death through oxidative stress albeit indirectly through the production of proinflammatory cytokines and reactive oxygen species from immune cells such as microglia (Liu and Bing, 2011). Although systemic administration of rotenone has been shown to induce motor dysfunction (Alam and Schmidt, 2002, Zhu et al., 2004) and neurodegeneration (Sherer et al., 2003), it is limited due to the peripheral organ toxicity (Ferrante et al., 1997, Lapointe et al., 2004), body weight loss (Alam et al., 2004, Greene et al., 2009) and high mortality rates (Ferrante et al., 1997, Antkiewicz-Michaluk et al., 2003). Similarly, systemic LPS has also been associated with 'sickness behaviour' (Hart, 1988). Therefore, for these reasons, models have been developed in which rotenone (Mulcahy et al., 2012) or LPS (Hoban et al., 2013) are injected directly into the rat nigrostriatal pathway.

Since these emerging intracerebral rotenone and LPS models have different inductive mechanisms compared to the 6-OHDA model, and this may have consequences for neuropathology and motor function, we first sought to characterise these models in their own right relative to the gold standard 6-OHDA model, before taking them forward to subsequent dual exposure studies in combination with AAV-α-synuclein. Therefore, the aim of this chapter was to assess the behavioural profiles induced by direct intra-cerebral LPS and rotenone in comparison to that induced by direct intracerebral 6-OHDA.

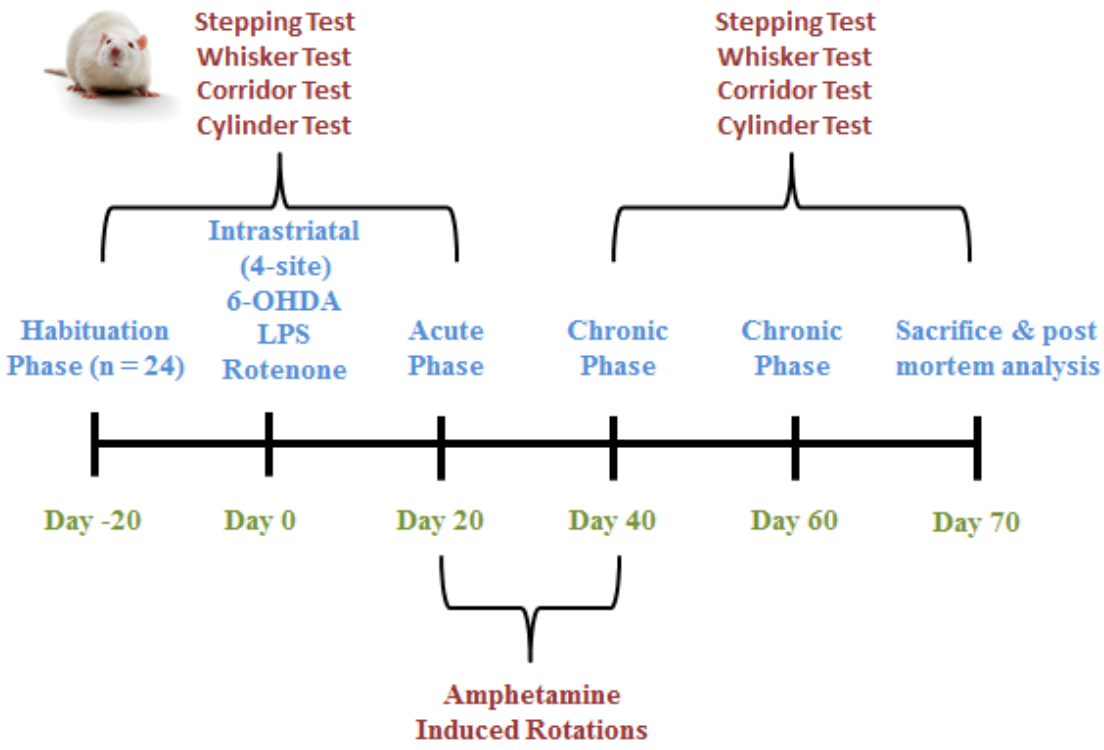
3.2 Methods

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

3.3 Experimental design

Twenty-four young adult male Sprague Dawley rats were used in this experiment (weighing 250 ± 10 g at start of baseline testing). All rats underwent habituation to the Corridor, Stepping and Whisker tests. They were then performance-matched to receive unilateral, 4 site, intrastriatal infusion of 6-OHDA (4 x 7 μg; n=8), LPS (4 x 5 μg; n=8) or rotenone (4 x 0.9 μg; n=8). Doses were chosen to induce a similar level of nigrostriatal neurodegeneration based on our previous studies (Mulcahy et al., 2011, Hoban et al., 2013, Walsh et al., 2011). This was to allow for objective comparison of the behavioural profiles of the different models. Behavioural testing resumed the day after lesion surgery and continued for 10 weeks during which time amphetamine-induced rotation was also assessed (at 3 weeks post-lesion). The animals were then sacrificed via transcardial perfusion-fixation and their brains were

used for *post mortem* assessment of nigrostriatal neurodegeneration via quantitative tyrosine hydroxylase immunohistochemistry. Below is a schematic representation of the timeline of habituation, surgery, testing and sacrifice.



3.4 Results

3.4.1 Body weight after 6-OHDA, LPS or Rotenone

In order to maintain the general health and well-being of the animals, their weights were consistently monitored. Unilateral intrastriatal administration of 6-OHDA, LPS or rotenone did not cause any mortality or ill health in the animals over the course of the study. Apart from an initial transient post-operative slowing of weight gain, unilateral 4-site intrastriatal infusion of either neurotoxin did not induce any ill health in the animals, and they all continued to gain weight during the testing period (Fig. 3.1; Time, $F_{(24,504)} = 206.00$, $P<0.0001$). Importantly, there was no difference in weight gain between the three groups (Group, $F_{(2,21)} = 0.69$; $P>0.05$) indicating that direct intra-cerebral administration the non-selective toxins (LPS and rotenone) was not significantly more toxic (in general terms) than injection of the catecholaminergic toxin 6-OHDA.

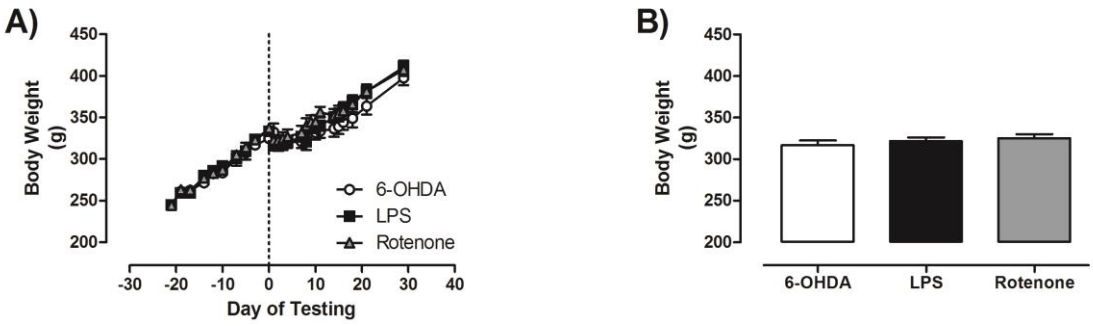


Fig. 3.1: Unilateral intrastriatal infusion of 6-OHDA, LPS or rotenone does not affect body weight gain. Unilateral intrastriatal four site administration of 6-OHDA, LPS or rotenone does not adversely affect rat's general health over the time course of the study where, apart from a slight decline in the immediate post-operative phase, their body weights continued to increase. The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data. Vertical dotted line indicates the day of surgery. Data are shown as mean ± SEM.

3.4.2 Nigrostriatal Integrity after 6-OHDA, LPS or Rotenone

The doses of 6-OHDA, LPS and rotenone selected for this study were chosen specifically to induce a similar level of nigrostriatal neurodegeneration and this was based on our previous published and unpublished studies. To confirm that all neurotoxins led to an equivalent level of nigrostriatal neurodegeneration, the number of tyrosine hydroxylase immunopositive cell bodies in the substantia nigra and the density of tyrosine hydroxylase immunopositive terminals in the striatum were quantified on the ipsilateral and contralateral sides (Fig. 3.2). All neurotoxins led to a significant loss of dopaminergic cell bodies (Side, $F_{(1,19)}$=51.12; P<0.0001) and terminals (Side, $F_{(1,19)}$ = 52.53; P<0.0001) and we confirmed that there was no

significant difference in the extent of the lesion between groups (Cell bodies: Group, $F_{(2,19)}$=1.06; P>0.05; Terminals: Group, $F_{(2,19)}$=0.10; P>0.05).

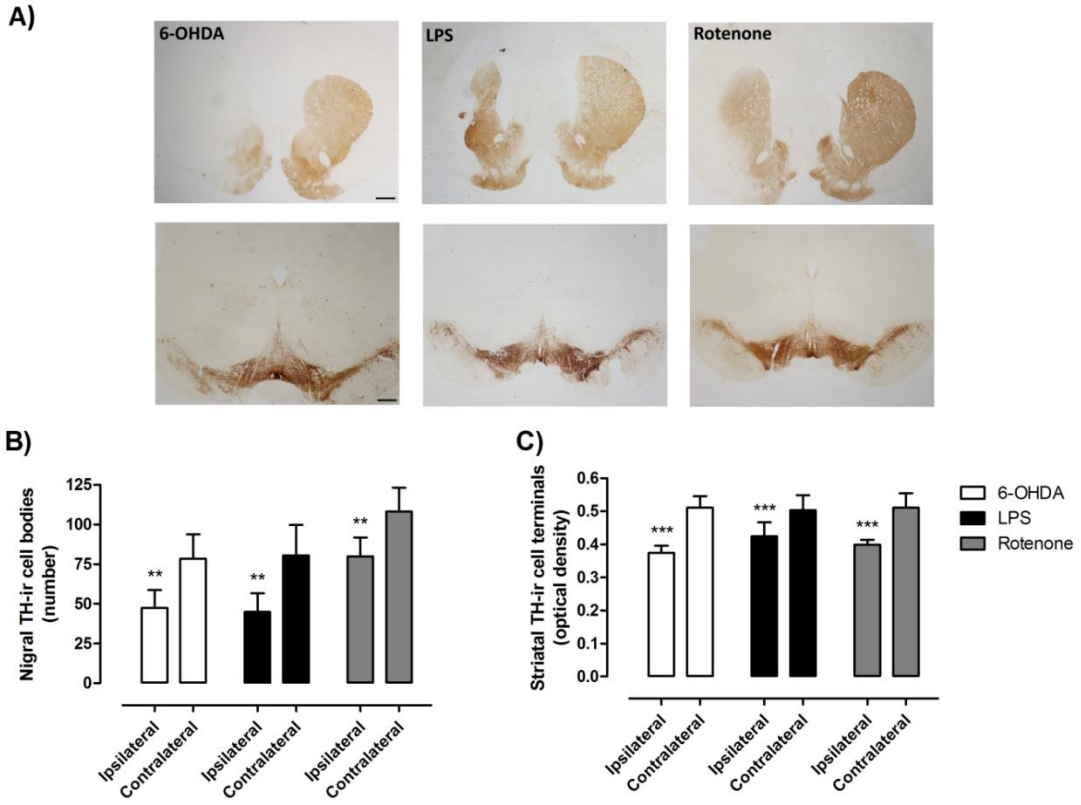


Fig. 3.2: Confirmation of similar level of nigrostriatal neurodegeneration between models. Tyrosine hydroxylase immunohistochemical analysis confirmed that unilateral intrastriatal administration of each neurotoxin induced a significant neurodegeneration at the level of the nigrostriatal cell bodies and terminals. Representative photomicrographs (A) and quantitative histology (B and C) are shown. ***P<0.001, **P<0.01 *vs*. contralateral side by two-way repeated measures ANOVA with *post-hoc* Bonferroni. Scale bar in striatal images represents 2 mm and in nigral images represents 1 mm.

3.4.3 Amphetamine-Induced Rotation after 6-OHDA, LPS or Rotenone

Unilateral nigrostriatal lesions are classically associated with amphetamine-induced turning behaviour caused by the dopaminergic asymmetry between the two striata (Ungerstedt and Arbuthnott, 1970). Therefore, at 3 weeks post-lesion, we sought to determine if there were any differences between the models in this "gold-standard" probe. Interestingly, at this time-point, despite an equivalent level of nigrostriatal neurodegeneration and a profound impairment in some of the spontaneous tests (see below), neither LPS nor rotenone-lesioned animals exhibited any rotational behaviour after injection of amphetamine, and were significantly different to the established 6-OHDA model in response to this dopamine-releasing drug (Fig. 3.3: $F_{(2,21)}$=7.42; P<0.01; differences with 6-OHDA model were confirmed by *post-hoc* Bonferroni).

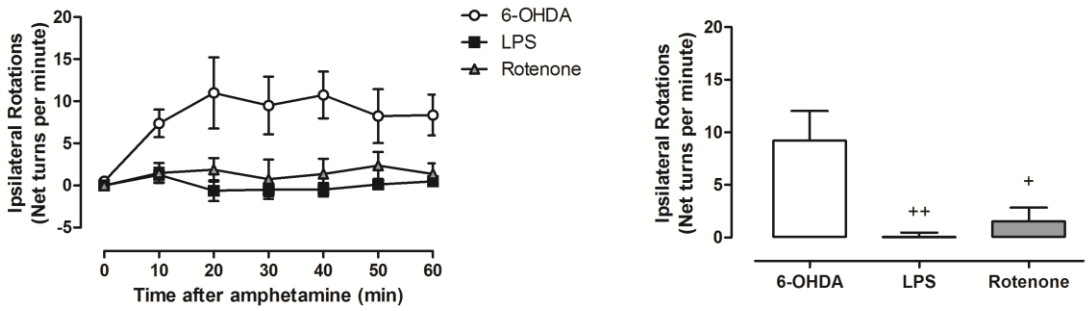


Fig. 3.3: Intrastriatal infusion of 6-OHDA, but not LPS or rotenone, is associated with amphetamine-induced rotational behaviour. Neither LPS nor rotenone-lesioned animals exhibited any rotational behaviour after injection of amphetamine, and were significantly different to 6-OHDA-lesioned animals which were driven to rotate in the ipsilateral direction after injection of this dopaminergic drug. Data are shown as ± SEM. $^{++}P<0.01$, $^{+}P<0.05$ *vs.* 6-OHDA by 1-way ANOVA and *post-hoc* Bonferroni.

3.4.4 Ipsilateral Motor Function after 6-OHDA, LPS or Rotenone

A key feature of any unilateral model of Parkinson's disease is the emergence of a bradykinetic syndrome on the side of the body opposite to that in which the neuropathology develops. Therefore, in order to assess the impact of each neurotoxin on ipsilateral and contralateral motor function, the animals were subjected to a battery of lateralised motor tests each of which assesses different aspects of the bradykinetic syndrome. On the ipsilateral side, neither 6-OHDA, LPS nor rotenone induced any impairment in the Stepping, Whisker, Corridor or Cylinder Tests (Fig. 3.4). This confirms that any behavioural changes observed on the contralateral side are due to the neurodegenerative effects of each neurotoxin on the injected side of the brain and not due to any other indirect effect of the toxins (such as LPS-induced sickness behaviour which is known to alter locomotor activity (Hart, 1988). Due to their contralateral impairment (Fig. 3.7B), 6-OHDA-injected animals developed a bias towards the ipsilateral side in the Corridor Test and they preferentially retrieved CocoPops® from this side over the course of testing (Group x Time, $F_{(44,462)}$=3.30, P<0.0001). Similarly, due to their contralateral impairment all groups of animals developed a bias towards the ipsilateral side in the Cylinder Test (Fig. 3.8B) and they preferentially supported themselves using this paw in the cylinder over the course of testing (Time, $F_{(7,147)}$=13.33, P<0.0001).

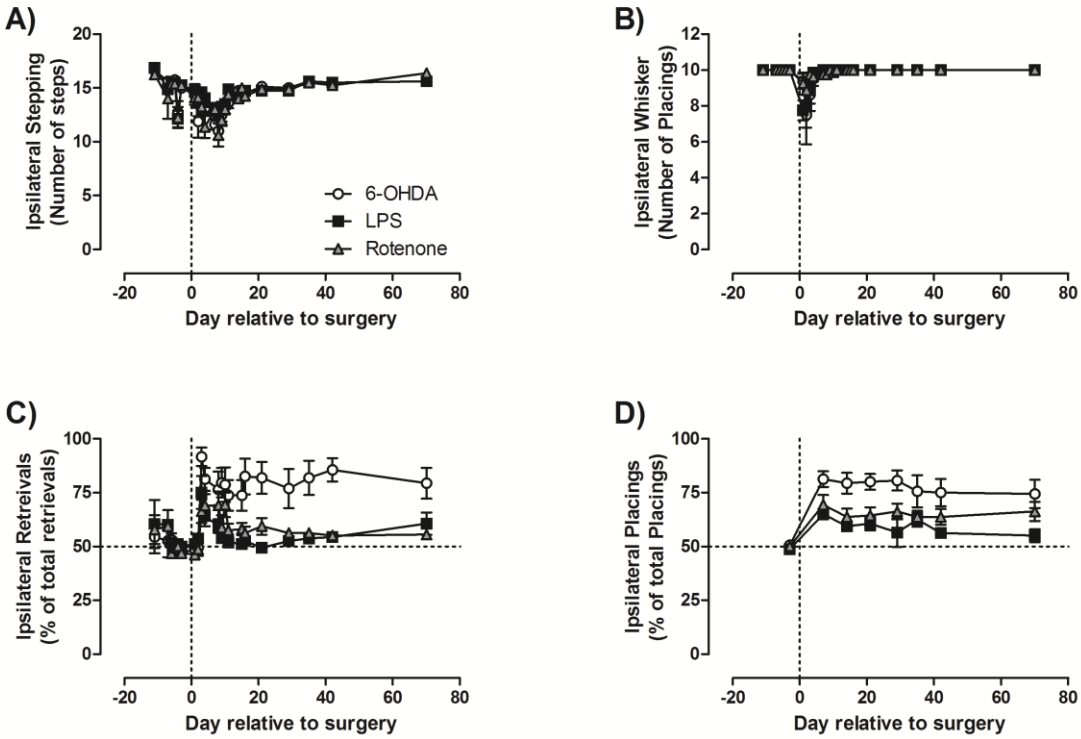


Fig. 3.4: Intrastriatal infusion of 6-OHDA, LPS or rotenone does not impair ipsilateral motor function. Unilateral intrastriatal administration of the neurotoxins did not cause any motor dysfunction on the ipsilateral side in the Stepping (A), Whisker (B), Corridor (C) or Cylinder (D). Due to their significant contralateral motor impairments (see Fig. 3.5-3.8), 6-OHDA-injected animals developed a bias towards their ipsilateral side in the Corridor Test (C) and all groups developed a bias towards the ipsilateral side in the Cylinder test (D). Vertical dotted lines indicate the day of surgery, while the horizontal dotted line indicates no lateralised bias. Data are shown as ± SEM.

3.4.5 Contralateral Motor Function after 6-OHDA, LPS or Rotenone

In contrast to the ipsilateral side, unilateral injection of 6-OHDA, LPS or rotenone induced significant motor impairments on the contralateral side of the body in most behavioural challenges (Fig. 3.5-3.8; Stepping Test: Time, $F_{(22,462)}$=158.80, P<0.0001; Whisker Test: Time, $F_{(22,462)}$=76.94, P<0.0001; Corridor Test: Time, $F_{(22,462)}$=9.03, P<0.0001; Cylinder Test: Time, $F_{(7,147)}$=13.33, P<0.0001). However, for each behavioural challenge, the pattern and duration of impairment varied significantly between lesion models. In order to facilitate analysis of these differences, the behavioural data generated was divided into three phases of testing, namely the baseline phase, an acute postoperative phase (Day 0-14) and a chronic post-operative phase (Day 15-70). We were particularly interested in; 1) whether there was a significant impairment in the acute and chronic phases of testing (relative to baseline), 2) whether there was any significant spontaneous recovery in the chronic phase (relative to the acute phase), and 3) whether there were any significant difference in the level of impairment between the emerging models (LPS and rotenone) and the established model (6-OHDA).

In the Stepping Test of forelimb kinesis, injection of 6-OHDA, LPS or rotenone led to a profound loss of ability to make adjusting steps on the contralateral side of the rats' bodies from the day after lesion surgery (Time, $F_{(2,42)}$ = 237.90, P<0.0001). Relative to baseline performance, this impairment was evident in both the acute and chronic phases of testing (confirmed by *post-hoc* Bonferroni with levels of significance indicated by asterisk (*) symbols on Fig. 3.5B). However, for all models, the impairments in stepping performance underwent some spontaneous

recovery with partial restoration of the ability to make adjusting steps in the chronic phase of testing (confirmed by *post-hoc* Bonferroni with levels of significance indicated by the hash (#) symbols on Fig. 3.5B). In both the acute and chronic phases of testing, the level of impairment induced by the inflammatory and environmental neurotoxins did not differ significantly from that induced by 6-OHDA (confirmed by *post-hoc* Bonferroni).

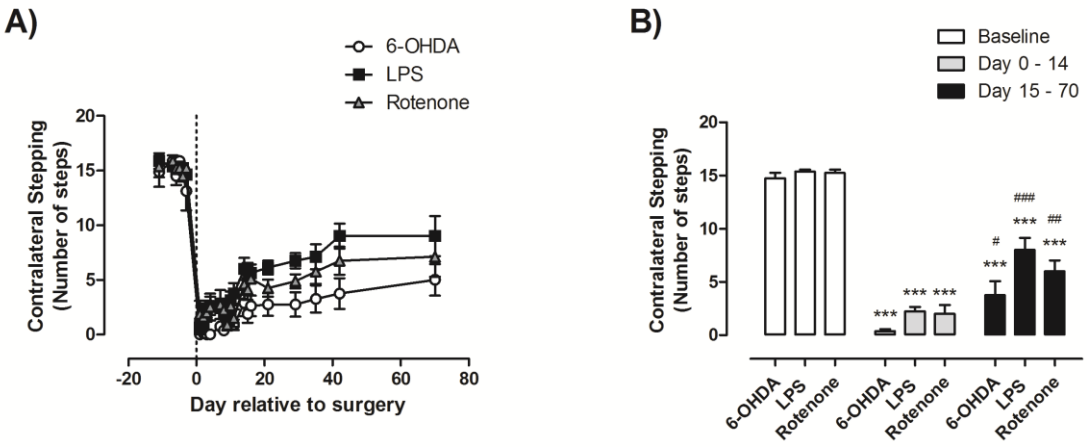


Fig. 3.5: Intrastriatal infusion of 6-OHDA, LPS or rotenone leads to different patterns of contralateral motor dysfunction in the Stepping Test of forelimb akinesia. Unilateral intrastriatal administraiton of the neurotoxins caused significant motor dysfunction on the contralateral side in the Stepping Test. The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data from the baseline, acute and chronic phase of testing. Vertical dotted line indicates the day of surgery, while the horizonal line indicates no lateralised bias. Data are shown as ± SEM. ***P<0.001 *vs.* relative baseline; $^{###}P$<0.001, $^{##}P$<0.01, $^{#}P$<0.05 *vs*. acute phase by 2-way repeated measures ANOVA and *post-hoc* Bonferroni.

In the Whisker Test of sensorimotor integration, injection of 6-OHDA, LPS or rotenone led to a profound loss of ability to make vibrissae-elicited forelimb placings on the contralateral side of the rats' bodies from the day after lesion surgery (Time, $F_{(2,42)} = 152.20$; $P<0.0001$). Relative to baseline performance, this impairment was evident in both the acute and chronic phases of testing (confirmed by *post-hoc* Bonferroni with levels of significance indicated by asterisk (*) symbols on Fig. 3.6B). Interestingly, only the LPS model underwent spontaneous recovery with partial restoration of the ability to make vibrissae-elicited forelimb placings in the chronic phase of testing in this group only (confirmed by *post-hoc* Bonferroni with levels of significance indicated by the hash (#) symbols on Fig. 3.6B). In both the acute and chronic phases of testing, the level of impairment induced by the LPS and rotenone did not differ significantly from that induced by 6-OHDA (confirmed by *post-hoc* Bonferroni).

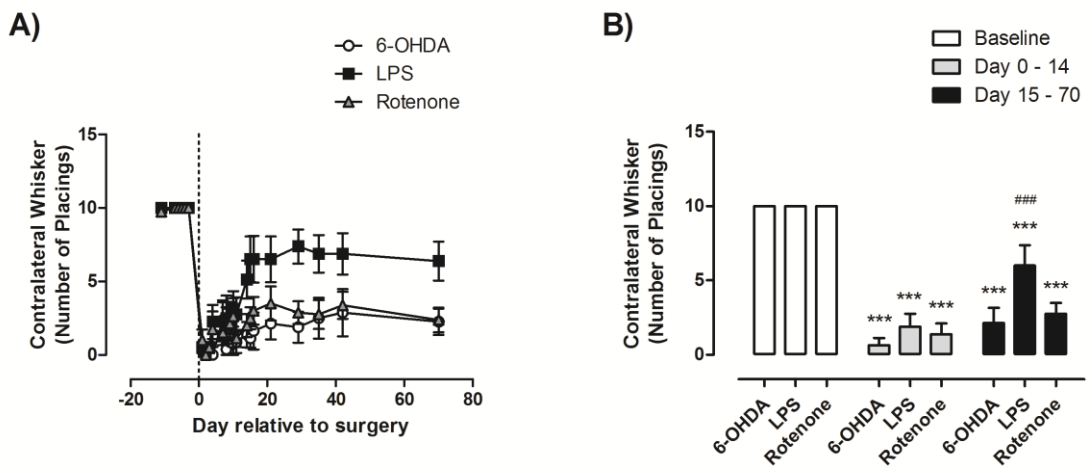


Fig. 3.6: Intrastriatal infusion of 6-OHDA, LPS or rotenone leads to different patterns of contralateral motor dysfunction in the Whisker Test of sensorimotor neglect. Unilateral intrastriatal administraiton of the neurotoxins caused significant motor dysfunction on the contralateral side in the Whisker Test. The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data from the baseline, acute and chronic phase of testing. Vertical dotted line indicates the day of surgery, while the horizonal line indicates no lateralised bias. Data are shown as ± SEM. ***P<0.001 *vs.* relative baseline; $^{###}$P<0.001 *vs*. acute phase by 2-way repeated measures ANOVA and *post-hoc* Bonferroni.

In the Corridor Test of lateralised neglect, injection of 6-OHDA or rotenone, but not LPS, led to neglect of food on the contralateral side of the rats' bodies from the day after lesion surgery (Time, $F_{(2,42)}$ = 15.58; P<0.0001). Relative to baseline performance, this impairment was only sustained though the acute and chronic phases of testing in the 6-OHDA group (confirmed by *post-hoc* Bonferroni with levels of significance indicated by asterisk (*) symbols on Fig. 3.7B). Interestingly, neither the LPS nor rotenone group showed any evidence of impairment in this task

in the chronic phase of testing, and their performance was significantly different from that of the established 6-OHDA model over this post-operative stage (confirmed by *post-hoc* Bonferroni with levels of significance indicated by plus (+) symbols on Fig. 3.7B).

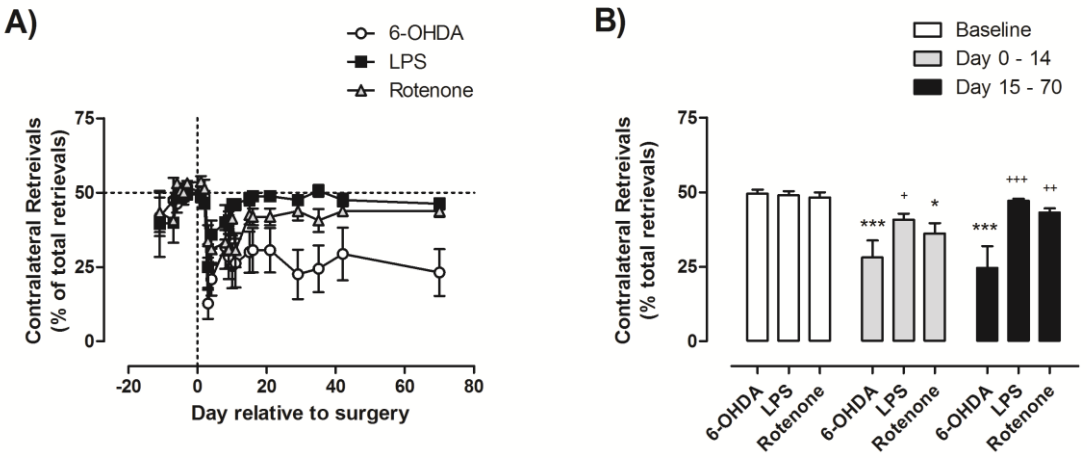


Fig. 3.7: Intrastriatal infusion of 6-OHDA, LPS or rotenone leads to different patterns of contralateral motor dysfunction in the Corridor Test of contralateral neglect. Unilateral intra-striatal administraiton of the neurotoxins caused significant motor dysfunction on the contralateral side in the Corridor Test. The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data from the baseline, acute and chronic phase of testing. Vertical dotted line indicates the day of surgery, while the horizonal line indicates no lateralised bias. Data are shown as ± SEM. ***P<0.001, *P<0.05 *vs.* relative baseline; ^{+++}P<0.001, ^{++}P<0.01, ^{+}P<0.05 *vs.* 6-OHDA by 2-way repeated measures ANOVA and *post-hoc* Bonferroni.

In the Cylinder Test of forepaw use, injection of all neurotoxins led to neglect of the contralateral paw for support while exploring the cylinder from the first time point of testing post lesion surgery (Time, $F_{(2,42)} = 57.75$; $P<0.0001$). Relative to baseline performance, this impairment was evident in both the acute and chronic phases of testing (confirmed by *post-hoc* Bonferroni with levels of significance indicated by asterisk (*) symbols on Fig. 3.8B) and there was no evidence of spontaneous recovery in any of the models on this task. Interestingly, the impairment in Cylinder Test performance in the LPS and rotenone models was significantly less pronounced than that in the 6-OHDA model in both the acute and chronic phases of testing (confirmed by *post-hoc* Bonferroni with levels of significance indicated by plus (+) symbols on Fig. 3.8B).

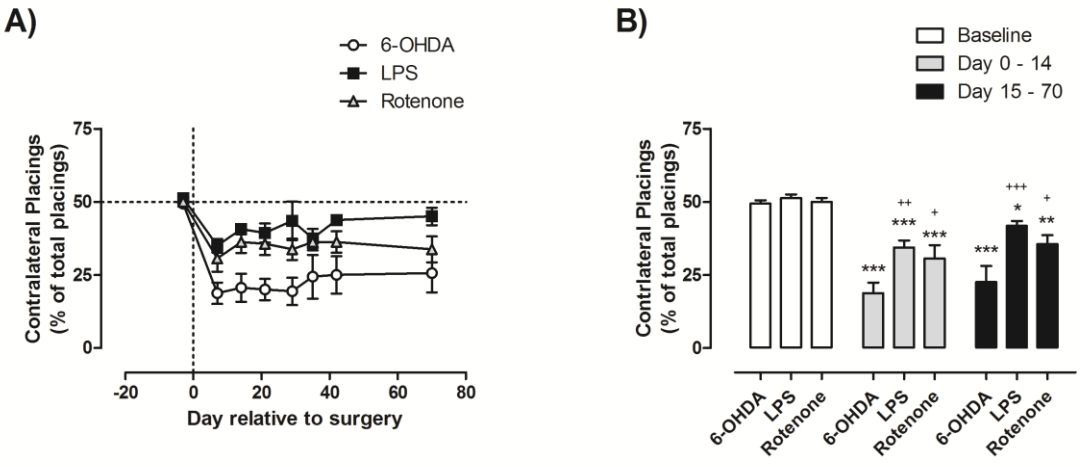


Fig. 3.8: Intrastriatal infusion of 6-OHDA, LPS or rotenone leads to different patterns of contralateral motor dysfunction in the Cylinder Test of forelimb akinesia. Unilateral intra-striatal administraiton of the neurotoxins caused significant motor dysfunction on the contralateral side in the Cylinder Test. The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data from the baseline, acute and chronic phase of testing. Vertical dotted line indicates the day of surgery, while the horizonal line indicates no lateralised bias. Data are shown as ± SEM. ***P<0.001, **P<0.01, *P<0.05, *vs.* relative baseline; ^{+++}P<0.001, ^{++}P<0.01, ^{+}P<0.05 *vs.* 6-OHDA by 2-way repeated measures ANOVA and *post-hoc* Bonferroni.

3.5 Discussion

The overarching aim of this study was to characterise the profile of motor impairments resulting from unilateral intrastriatal infusion of the inflammagen, LPS, and the Parkinson's disease associated pesticide, rotenone, and to compare these with the widely-used unilateral intrastriatal 6-OHDA model. To our knowledge, this is the first time that these newer models have been assessed in terms of their behavioural pattern and compared to the 6-OHDA model which induces a profound and stable motor dysfunction. As expected, we found that administration of 6-OHDA led to significant amphetamine-induced rotational behaviour, and also caused pronounced and stable contralateral motor impairments in the Stepping, Whisker, Corridor and Cylinder Tests. This lateralised motor dysfunction was underpinned by significant neurodegeneration of the nigrostriatal pathway. In contrast to the 6-OHDA model, despite a similar level of nigrostriatal neurodegeneration throughout all experimental groups, neither the LPS nor the rotenone models were associated with any amphetamine-induced rotation and they induced distinctly different patterns of motor disability in the spontaneous tasks.

This study confirmed many previous studies showing that unilateral intrastriatal infusion of 6-OHDA causes partial nigrostriatal neurodegeneration with consequent contralateral motor dysfunction in rats (Kirik et al., 1998, Grealish et al., 2008). Infusion of 6-OHDA into the striatum initiates a progressive and retrograde degeneration of catecholaminergic neurons due to the combined effect of the production of reactive oxygen species and quinones (Cohen, 1984). The intrastriatal 6-OHDA model of Parkinson's disease is considered a more etiologically relevant

model (than cell body or axonal infusion) as the condition is now considered to be a retrograde die-back disease in which impaired terminals retrogradely degenerate resulting in nigral cell body death (Cheng et al., 2010, Dauer and Przedborski, 2003). One of the most valuable 6-OHDA-related experimental paradigms is the evaluation of dopaminergic asymmetry via rotational behaviour in rats bearing unilateral lesions of the nigrostriatal pathway. In rats with unilateral lesions, amphetamine stimulates dopamine release from nigrostriatal terminals on the intact side which then drives rotational behaviour through activation of dopamine receptors expressed on GABAergic striatopallidal/striatonigral output neurons. Indeed, in line with expectations, 6-OHDA-lesioned animals rotated significantly in response to amphetamine and also displayed pronounced and stable contralateral motor dysfunction from the day after lesion surgery in all of the spontaneous behavioural tests (Stepping, Whisker, Corridor and Cylinder Test). Despite this robust motor syndrome, the drawback of the 6-OHDA model is the fact that it does not mimic all of the pathological features of the disease and is typically induced using a single insult which is far removed from the etiology of the disease.

We then sought to compare the effects of LPS and rotenone to these well-established effects of 6-OHDA, and several intriguing differences emerged. Strikingly, despite a similar level of nigrostriatal neurodegeneration and dopaminergic deafferentation of the striatum, neither animals injected with LPS, nor animals injected with rotenone, displayed any rotational asymmetry after systemic challenge with the dopamine-releasing drug, amphetamine. Given that there was a similar level of nigrostriatal neurodegeneration in the LPS and rotenone lesioned rats as there was in the 6-

OHDA-lesioned rats, this indicates that some other effect of the inflammatory and environmental toxins must have prevented dopamine-driven rotational behaviour. It is conceivable that this was due to functional impairment of the GABAergic striatopallidal/striatonigral output neurons that are essential for driving rotational behaviour because, unlike 6-OHDA which is selective for dopaminergic neurons, neither LPS nor rotenone are dopamine selective. Indeed, LPS is a Toll-like receptor agonist which causes non-selective neuronal dysfunction/death secondary to activation of neuroimmune cells such as microglia (reviewed in Liu and Bing, 2011) while rotenone is a highly lipid soluble complex I inhibitor which causes non-selective neuronal dysfunction/death through inhibition of the mitochondrial electron transport chain (Li et al., 2003, Schapira et al., 1990). Thus, it is probable that LPS and rotenone, but not 6-OHDA, could have caused damage to the intrinsic GABAergic neurons which prevented the nigrostriatal dopaminergic asymmetry from manifesting as rotational behaviour after amphetamine challenge.

In addition to the differences in rotational behaviour, we also observed intriguing differences between the models in their performance of the more clinically-relevant spontaneous behavioural tests. As indicated above, we focused on determining the pattern and stability of motor dysfunction manifest in each model in each test. This was important because each behavioural test probes different aspects of the human Parkinson's disease motor condition (i.e. bradykinesia, sensorimotor integration, sensorimotor neglect etc.) and information on any differences in behavioural profile caused by neurotoxic, inflammatory and environmental neurotoxins could be

essential in informing the appropriate experimental design of future preclinical Parkinson's disease studies.

In comparison to the 6-OHDA lesion model, some key differences in motor dysfunction emerged in the inflammation-driven and the pesticide-driven models in the spontaneous tests used. Perhaps most strikingly is the failure of LPS-induced or rotenone-induced nigrostriatal neurodegeneration to induce a sustained impairment in the Corridor Test of sensorimotor neglect, despite a pronounced and significant impairment in the other behavioural tests. This finding is in line with our previous report which indicated that unilateral LPS injection into the striatum did not induce any contralateral impairment in this task (Hoban et al., 2013). The reason for this is not known, however given that the level of nigrostriatal neurodegeneration between the different models was similar, then the failure to exhibit sensorimotor neglect after LPS or rotenone cannot be attributed to the lack of a functionally-relevant lesion. Rather it must be something related to the non-selective neuropathology caused by these agents and the consequent sensorimotor neglect probed by the Corridor Test. Perhaps the neuronal systems underlying this behaviour are less sensitive to the neuropathology caused by inflammatory or environmental stimuli, rather than the neuropathology caused by directly neurotoxic stimuli.

Despite a failure to induce a measurable impairment in sensorimotor neglect, LPS and rotenone did cause profound and significant impairments in contralateral forelimb kinesis (Stepping Test), sensorimotor integration (Whisker Test) and forepaw use (Cylinder Test), although these tended to be significantly less

pronounced and stable than the 6-OHDA-induced deficit on some behavioural tests. 6-OHDA-treated rats exhibited a stable motor deficit in the Whisker, Corridor and Cylinder Tests which was maintained throughout the 10 weeks of testing (although there was a slight recovery in the Stepping Test). In comparison, LPS, and to a lesser extent, rotenone, exhibited spontaneous recovery in the latter 8 weeks of testing. Compensatory action and functional recovery is a well-known feature of the nigrostriatal system and can be attributed to increased tyrosine hydroxylase synthesis, increased dopamine release, increased dopamine turnover (Robinson et al., 1990, Robinson et al., 1994, Zhang et al., 1988, Zigmond et al., 1989, Zigmond et al., 1990c) or even sprouting of the remaining dopaminergic fibres (Hansen et al., 1995). The precise mechanism(s) underlying functional recovery in the LPS and rotenone models in the present study is not known, but it should be borne in mind that stable motor dysfunction is a highly desirable feature of Parkinson's disease models as spontaneous recovery of motor function can be a confounding factor in long-term studies of experimental anti-Parkinsonian therapies.

One striking feature common to all of the neurotoxins used was the rapidity with which the behavioural impairments manifested after their intra-striatal administration. Where 6-OHDA, LPS and rotenone caused impairment, this was immediately evident from the first time-point after testing (Day 1 post-lesion for the Stepping, Whisker and Corridor Tests). Since Parkinson's disease is an age-progressive neurodegenerative disease, a progressive onset of motor dysfunction is another highly desirable feature of Parkinson's disease models. In line with previous reports, this is evidently not a feature of any of these acute lesion models (Grealish et

al., 2008, Hoban et al., 2013, Kirik et al., 1998, Mulcahy et al., 2011), and this should be borne in mind as a limitation of all of these models.

It is clear that these models are not without their limitations; specifically, 1) their acute degeneration process, 2) the fact that they are induced using a single insult, 3) the behavioural dysfunction does not worsen over time, and finally 4) that there is no associated protein aggregation or α-synuclein pathology. Because of these limitations, further studies in this thesis will attempt to address some of these disadvantages by using these etiologically relevant toxins in dual-exposure model systems with α-synuclein.

Chapter 4: Assessment of dual exposure to intranigral α-synuclein and intrastriatal LPS as a novel approach to modelling Parkinson's disease in the rat

4.1. Introduction

Neurotoxic models of Parkinson's disease have allowed us to gain considerable insight into some of the pathogenic mechanisms underlying the neurodegeneration in Parkinson's disease. The drawback of these models is their acute toxicity which typically reflects end stage Parkinson's disease. Moreover, there is no associated α-synuclein pathology (Tieu, 2011). It is now widely accepted that the disease is multifaceted in nature and no one model can accurately reflect the complex etiology of the disease. For this reason, combining relevant risk factors is a valid approach to modelling the disease (Blesa et al., 2012). It has been previously established that the nigrostriatal pathway is readily able to compensate for the effects of exposure to a unitary environmental or genetic factor acting alone (Agid et al., 1973, Anglade et al., 1995, Zigmond et al., 1990c). Therefore, exposing the delicate circuitry of the nigrostriatal pathway to multiple factors may result in sustained or continuing degeneration which is more reflective of the progressive nature of the disease.

As Parkinson's disease is thought to arise from a complex interaction between genetics and exogenous factors, the drive to develop genetic animal models of the disease has gained considerable momentum in the last two decades. Parkinson's disease-associated mutations have been investigated extensively, and to date, the most relevant model of the disease is the viral-mediated over-expression of the

Parkinson's disease protein – α-synuclein (Lindgren et al., 2012, Kirik et al., 2003, Kirik et al., 2002). However, this model is limited by its unpredictability as it induces a mild phenotype characterised by development of α-synuclein pathology and variable nigrostriatal cell loss which precipitates a highly variable degree of motor decline (Kirik et al., 2002, Lauwers et al., 2007). Thus, this chapter focusses on overcoming some of the limitations associated with this model by incorporating a second, etiologically-relevant "hit", specifically exposure to the bacterial endotoxin, lipopolysaccharide (LPS).

Infection and the resulting inflammatory response (modelled in animals using LPS) cause dopaminergic cell death indirectly through the production of pro-inflammatory cytokines and reactive oxygen species from immune cells such as microglia (reviewed in Liu and Bing, 2011). Moreover, microglial activation was reported in the nigrostriatal system of *post mortem* Parkinsonian brains (Mcgeer et al., 1988b). The first report of an inflammation-driven preclinical model of the disease came from two independent laboratories in 1998 who utilised the bacterial endotoxin LPS and administered it directly into the nigrostriatal pathway which resulted in microglial activation and dopaminergic degeneration (Bing et al., 1998, Castano et al., 1998). Since then a number *of in vitro* and *in vivo* studies have confirmed the neurotoxic effects of LPS administration (Liu et al., 2000, Bronstein et al., 1995, Qin et al., 2004, Lee et al., 1993, Lu et al., 2000, Herrera et al., 2000, Hoban et al., 2013). As viral-mediated transfer of α-synuclein is associated with robust α-synuclein expression but only variable neurodegeneration and motor impairments (Kirik et al., 2002), and intracerebral LPS is associated with inflammation-driven

neurodegeneration and motor impairments without synucleinopathy (Hoban et al., 2013, Concannon et al., 2015), we considered that the combination of both of these approaches could be a valid and novel approach to modelling Parkinson's disease.

Therefore, the aim of this chapter was to determine whether direct intrastriatal administration of the bacterial inflammagen LPS to rats with a high α-synuclein burden could result in a more etiologically relevant animal model of the disease than either insult alone.

4.2. Methods

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

4.3. Experimental design

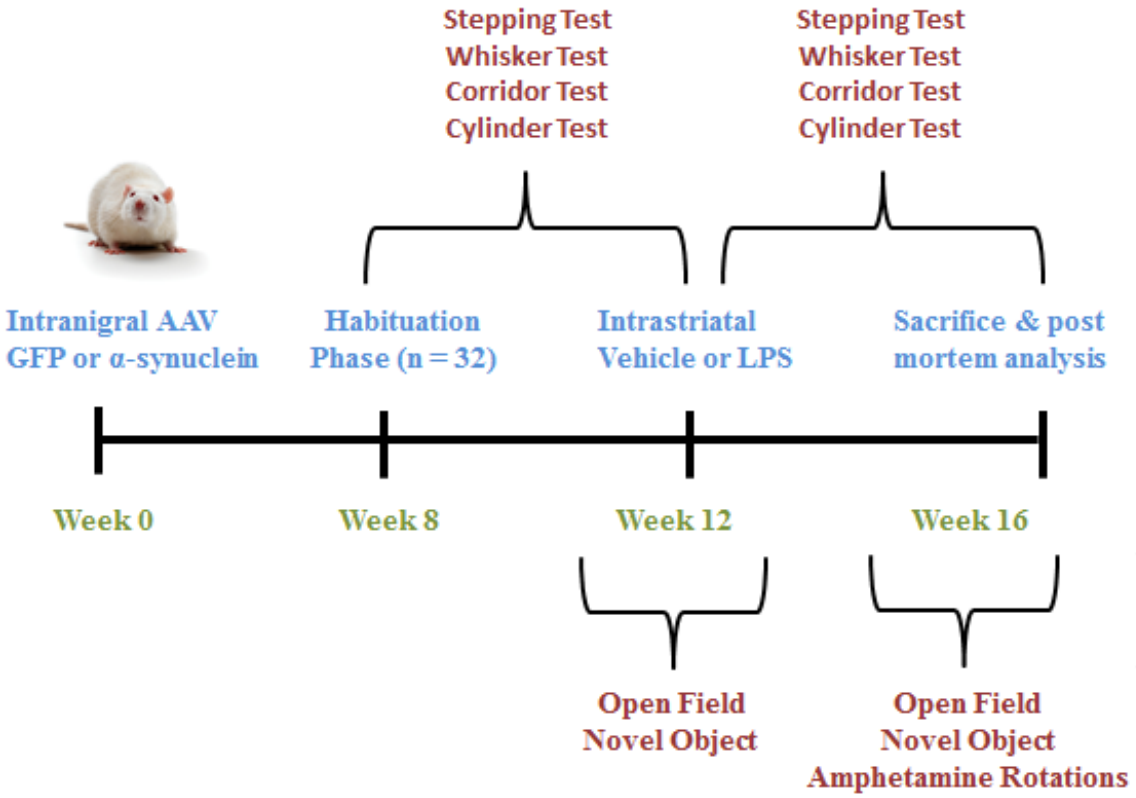
Thirty-two young adult male Sprague Dawley rats were used in this experiment. Rats were assigned to four groups: 1) Control, 2) LPS, 3) $AAV_{2/5}$-α-synuclein and 4) $AAV_{2/5}$-α-synuclein and LPS. According to their groups, animals received unilateral intranigral administration of either 2 µl of $AAV_{2/5}$-α-synuclein (viral titre 1 x 10^{10} vg/µl) or its corresponding $AAV_{2/5}$-GFP control (viral titre 8 x 10^{7} vg/µl), followed 12 weeks later by intrastriatal administration of either LPS (10 µg in 2 µl) or its corresponding vehicle (2 µl of sterile saline). Animals were habituated to the Corridor, Stepping and Whisker tests at 9 weeks post-AAV infusion for testing at week 10, 11 and 12 and for a further 4 weeks post LPS administration. Animals also underwent testing for general ambulatory behaviour in the open field at weeks 12 and 17, cognitive function in the novel object recognition test at weeks 12 and 17 and

amphetamine-induced rotation at week 16. Once behavioural testing was complete, rats were sacrificed via transcardial perfusion-fixation and their brains were processed for *post mortem* quantitative assessment of α-synuclein expression (via α-synuclein immunohistochemistry), nigrostriatal neurodegeneration (via tyrosine hydroxylase immunohistochemistry) and neuroinflammation (via OX-42 immunohistochemistry).

Group	**Virus injection**	**LPS injection**	***n***
Control	$AAV_{2/5}$-GFP	Vehicle	8
LPS	$AAV_{2/5}$-GFP	LPS	8
α-Synuclein	$AAV_{2/5}$-α-synuclein	Vehicle	8
Combined	$AAV_{2/5}$-α-synuclein	LPS	8

Table 4.1. Groups used in this study:. At week 0, rats were randomly divided into two groups to receive either $AAV_{2/5}$-GFP or $AAV_{2/5}$-α-synuclein. Twelve weeks later, they were performance matched to receive either LPS or its corresponding vehicle to yield 4 final groups as shown.

Below is a schematic representation of the timeline of habituation, surgery, testing and sacrifice.



4.4. Results

4.4.1. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS is not detrimental to the rats general health

In order to assess the impact of exposing rats to intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS, their weights were monitored over the course of the study. Unilateral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS did not induce any ill health in the animals and they all continued to gain weight during the testing period (Weights: Group, $F_{(3,31)}$ = 1.75, P=0.18). This indicates that the combined administration of intranigral infusion of $AAV_{2/5}$-α-synuclein and intrastriatal LPS infusion was not significantly more toxic (in general terms) than injection of either insult alone (Fig. 4.1).

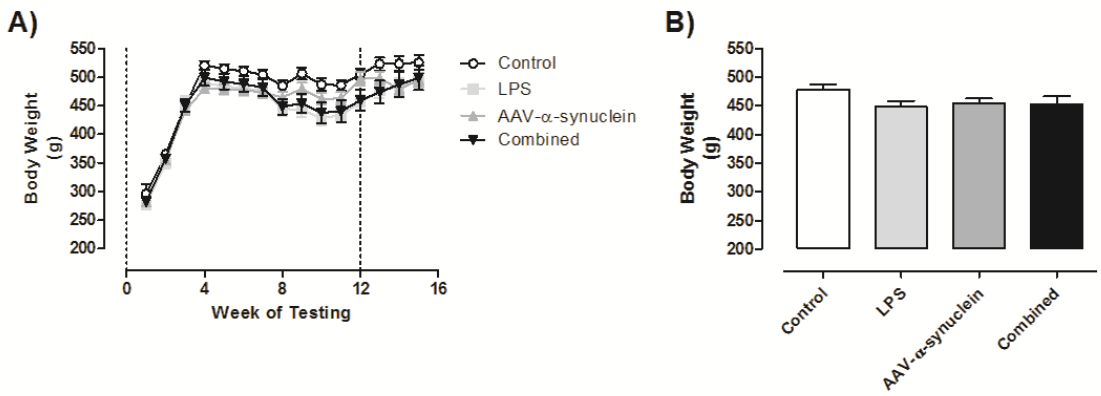


Fig. 4.1: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS did not affect the ability of the rats to gain weight over the course of the study. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS did not adversely affect the rat's general health over the time course of the study and they continued to gain weight. The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. $P>0.05$ by one-way ANOVA and *post-hoc* Newman-Keuls.

4.4.2. Unilateral intranigral $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS does not impair ipsilateral motor function

An important feature of any unilateral model of Parkinson's disease is the emergence of a bradykinetic syndrome on the side of the body contralateral to that in which the neuropathology develops. Therefore, in order to assess the impact of unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS on ipsilateral and contralateral motor function, the animals were subjected to a battery of lateralised motor tests each of which assesses different aspects of any emerging bradykinetic syndrome.

On the ipsilateral side, neither $AAV_{2/5}$-α-synuclein nor intrastriatal LPS, alone or in combination, induced any impairment in the Stepping, Whisker, Corridor or Cylinder tests (Fig 4.2 and Fig 4.3). This confirms that any of the behavioural changes that we observed on the contralateral side are due to the neurodegenerative effects of the neurotoxins rather than any other indirect effect of the toxin. This was also further substantiated by performing the open field test as there was no difference between the groups in their general ambulatory behaviour indicating that the motor dysfunction that we observed was specific for the lateralised motor tasks incorporated into this testing paradigm (Fig 4.4).

Due to a transient contralateral motor dysfunction in the Corridor and Cylinder test (Fig 4.6), animals who received the combination of $AAV_{2/5}$-α-synuclein and LPS developed an ipsilateral bias preferentially retrieving Cocopops® from this side of their body (Fig 4.3 A and B: Group $F_{(3,31)} = 3.85$, $P<0.05$; *post-hoc* Newman-Keuls confirmed a significant increase in their ipsilateral retrievals relative to the control group). Similarly, in line with these results, LPS lesioned and combined $AAV_{2/5}$-α-synuclein and LPS lesioned groups of animals also developed a bias towards their ipsilateral side in the Cylinder test and they preferentially supported themselves using their ipsilateral paw in the cylinder (Fig 4.3 C and D: Group, $F_{(3,31)} = 9.43$, $P<0.001$; LPS and combined group *vs.* control group. $P<0.001$, $P<0.05$; LPS and combined *vs.* α-synuclein confirmed with *post-hoc* Newman-Keuls).

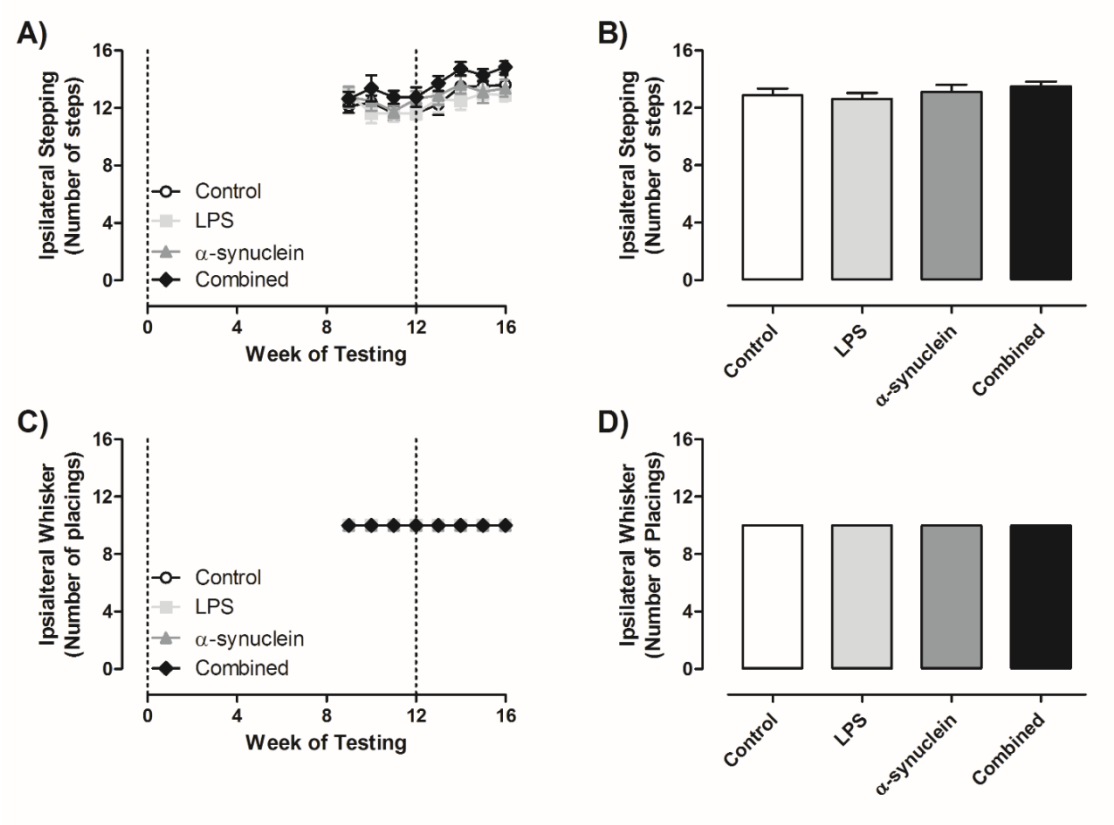


Fig. 4.2: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS does not impair ipsilateral motor function in the Stepping and Whisker Test. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS did not cause any motor dysfunction on the ipsilateral side of the Stepping (A and B) or the Whisker tests (C and D). The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. $P>0.05$ by one-way ANOVA and *post-hoc* Newman-Keuls.

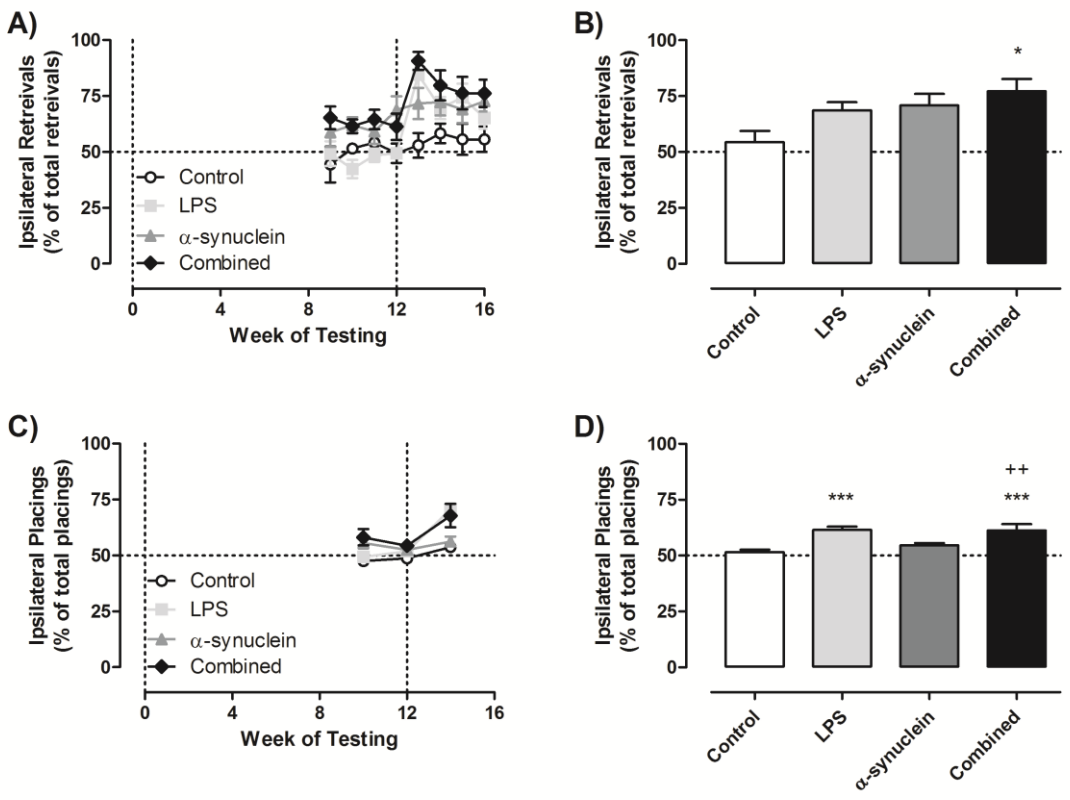


Fig. 4.3: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS does not impair ipsilateral motor function in the Corridor and Cylinder tests. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS did not cause any motor dysfunction on the ipsilateral side of the Corridor (A and B) or the Cylinder tests (C and D). The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001, *P<0.05 *vs.* control; ^{++}P<0.01 *vs.* α-synuclein by one-way ANOVA with *post-hoc* Newman-Keuls.

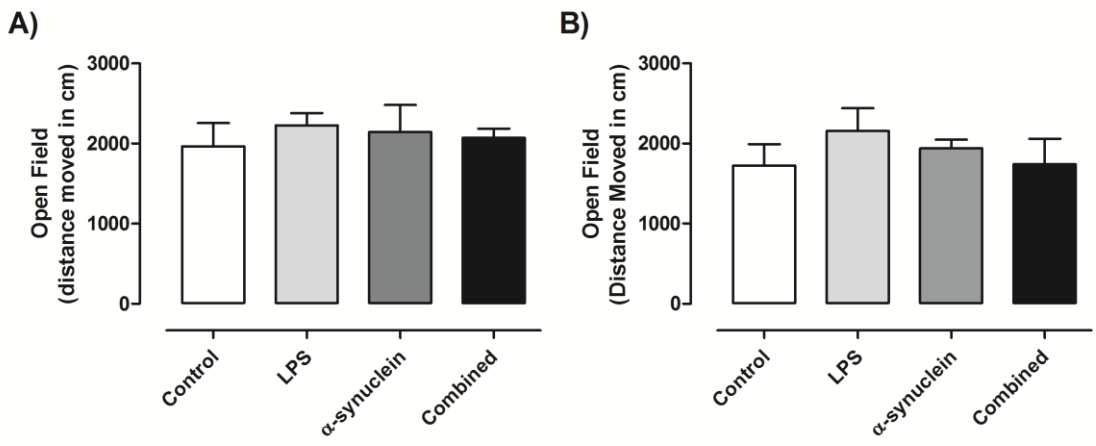


Fig. 4.4: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS does not adversely affect the rats general locomotor activity. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS did not cause any general motor dysfunction in the Open Field test at 12 (A) or 17 (B) weeks post viral surgery. Data are shown as mean ± SEM. P>0.05 by one-way ANOVA with *post-hoc* Newman-Keuls.

4.4.3. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS induces a variable pattern of contralateral motor dysfunction

In contrast to the ipsilateral side, the unilateral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS induced a modest motor impairment in the battery of behavioural tasks employed in this study. In the Stepping test of forelimb kinesis, infusion of LPS alone, but not α-synuclein alone, led to a significant reduction in the ability of animals to make adjusting steps on their contralateral side (Fig 4.5B: Stepping: Group, $F_{(3,31)}$ = 2.69, P<0.05). Importantly however, there was no significant additive effect of the two challenges in the combined group. In the

Whisker test of sensorimotor integration, the ability of animals to make vibrissae-evoked contralateral forelimb placings was significantly reduced after infusion with LPS or the combination of LPS and α-synuclein (Fig 4.5D; Whisker: Group, $F_{(3,31)}$ = 7.55, P<0.05). Again, the combined group indicated that there was no significant additive effect of the two challenges.

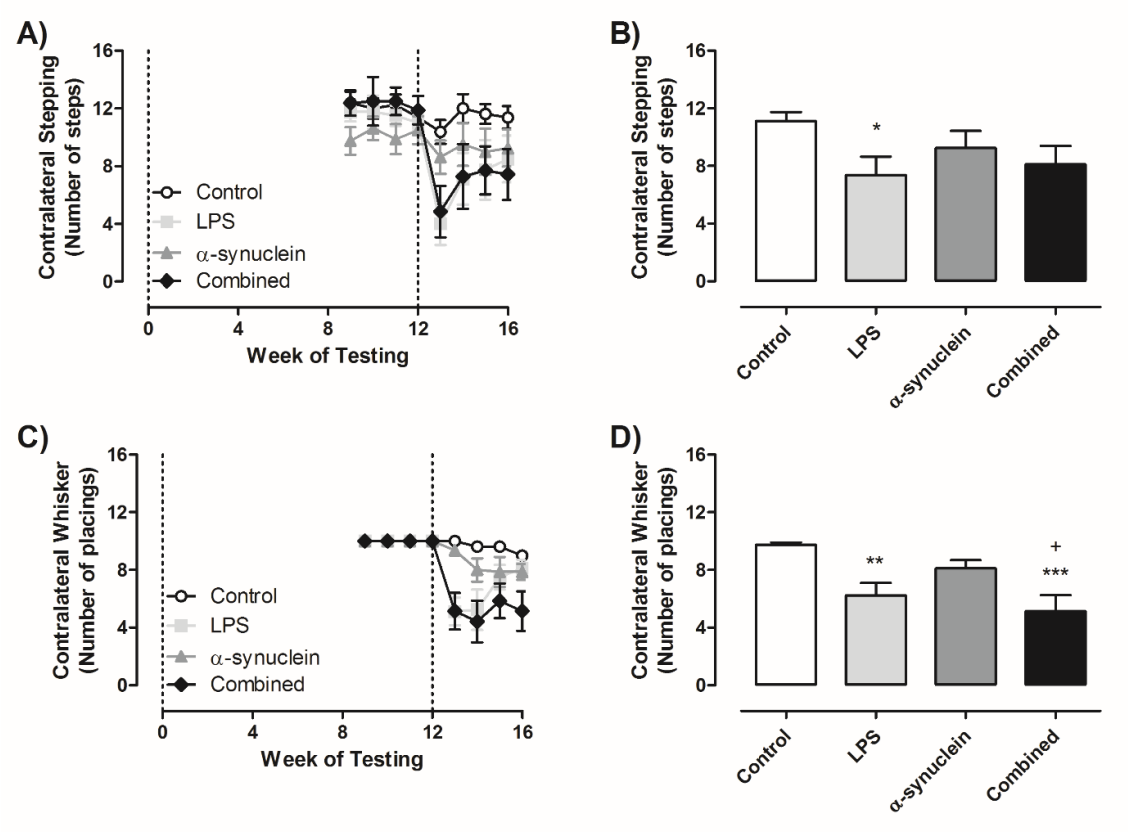


Fig. 4.5: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS results in the emergence of a variable motor dysfunction in the Stepping and Whisker tests. In the Stepping test (A and B), the ability of animals to make adjusting steps in the LPS lesioned group was significantly impaired. This trend also emerged in the Whisker test (C and D) whereby animals who received LPS and the combination of LPS and α-synuclein showed a significant decrease in the number of contralateral forelimb placings. However there was no additive effect of administering both of these neurotoxins. The XY plot on the left depict the data collected over the course of the study whereas the bar charts on the right represent collapsed data. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001, **P<0.01 *P<0.05, *vs.* control; ^{+}P<0.05 *vs* α-synuclein by one-way ANOVA with *post-hoc* Newman-Keuls.

In the Corridor test of lateralised neglect, infusion of either LPS or α-synuclein alone led to a strong, but not statistically significant, trend for contralateral neglect (Fig 4.6B). When these were sequentially administered however, this led to a significant reduction in food retrieval from the contralateral side (Corridor: Group, $F_{(3,31)} = 3.84$, $P<0.05$).

In the Cylinder test of forepaw use, animals infused with LPS alone and in combination with α-synuclein neglected to use their contralateral forepaw for support while exploring the cylinder, preferentially supporting themselves with their ipsilateral forelimb instead (Fig 4.6D; Cylinder: Group, $F_{(3,31)} = 10.11$; $^{***}P<0.001$). Importantly however, there was again no significant additive effect of the two challenges in the combined group.

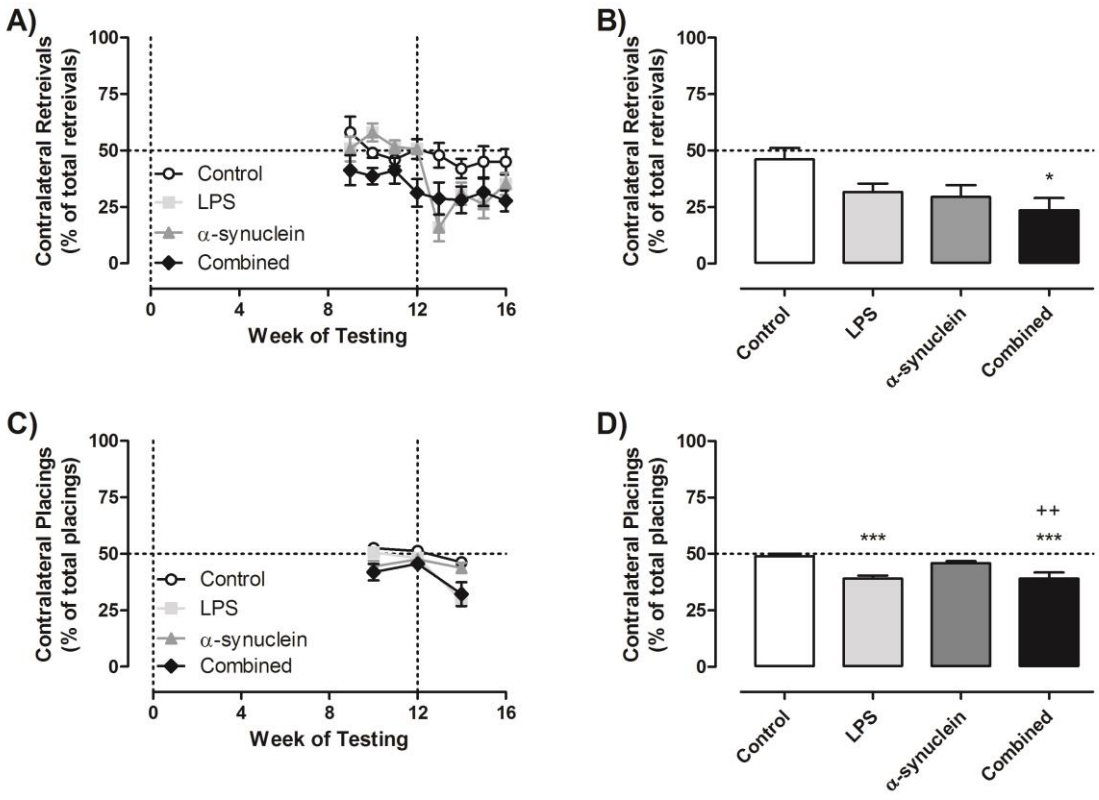


Fig. 4.6: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS results in the emergence of a variable motor dysfunction in the Corridor and Cylinder tests. In the Corridor (A and B) and Cylinder (C and D) tests of contralateral neglect, there was a significant impairment in the ability of the rats to make contralateral food retrievals or forepaw placings in the LPS and/or Combined group. However there was no additive effect of the neurotoxins. The XY plots on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001, *P<0.05 *vs.* control; ^{++}P<0.01 *vs.* α-synuclein by one-way ANOVA with *post-hoc* Newman-Keuls.

Classically, unilateral nigrostriatal lesions are associated with amphetamine-induced rotational behaviour which is caused by the dopaminergic asymmetry between the two striata. As this is the classic test for neurotoxic lesions to the nigrostriatal

pathway, we sought to determine if there were any differences between the groups at the later timepoint of 17 weeks post-viral vector infusion (5 weeks post LPS). Interestingly, all infused groups exhibited a small, but statistically significant, increase in ipsilateral turning after amphetamine injection (Fig. 4.7; Group, $F_{(3,31)}$ = 4.46, **P<0.01). However there was no additive effect of sequentially administering the genetic and inflammatory challenges.

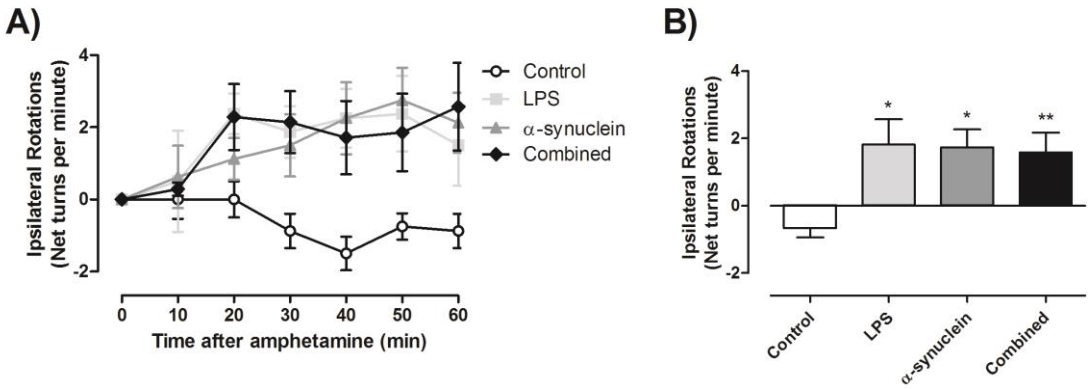


Fig 4.7: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS is associated with amphetamine-induced rotational behaviour. All experimental groups were associated with amphetamine-induced rotational behaviour at 17 weeks post viral infusion but there was no additive effect of administering both of these neurotoxins to the nigrostriatal pathway. Data are shown are mean ± SEM. **P<0.01, *P<0.05 *vs*. control by one-way ANOVA and *post-hoc* Newman-Keuls.

4.4.4. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS results in cognitive disturbances

In addition to the motor tests described above, we also wanted to assess whether the genetic and inflammatory challenges, alone or in combination, could cause any impairment in cognitive function, and this was examined through the novel object recognition test at 12 weeks post viral infusion and 5 weeks post LPS infusion. During the habituation phase we found no difference between the groups in the amount time spent grooming, sniffing or rearing (Fig. 4.8; Week 12: Group, $F_{(3,112)}$ = 625.0, P>0.05; Week 17: Group, $F_{(3,112)}$ = 208.2, P>0.05). However, during the test phase at week 17, we found that rats injected with α-synuclein, alone or in combination with LPS, spent more time exploring the familiar object and less time exploring the novel object (Week 17; Group, $F_{(3,112)}$ = 15.42; P<0.05) indicating a memory deficit in these groups. However, importantly, there was again no additive effect in the dual exposure model.

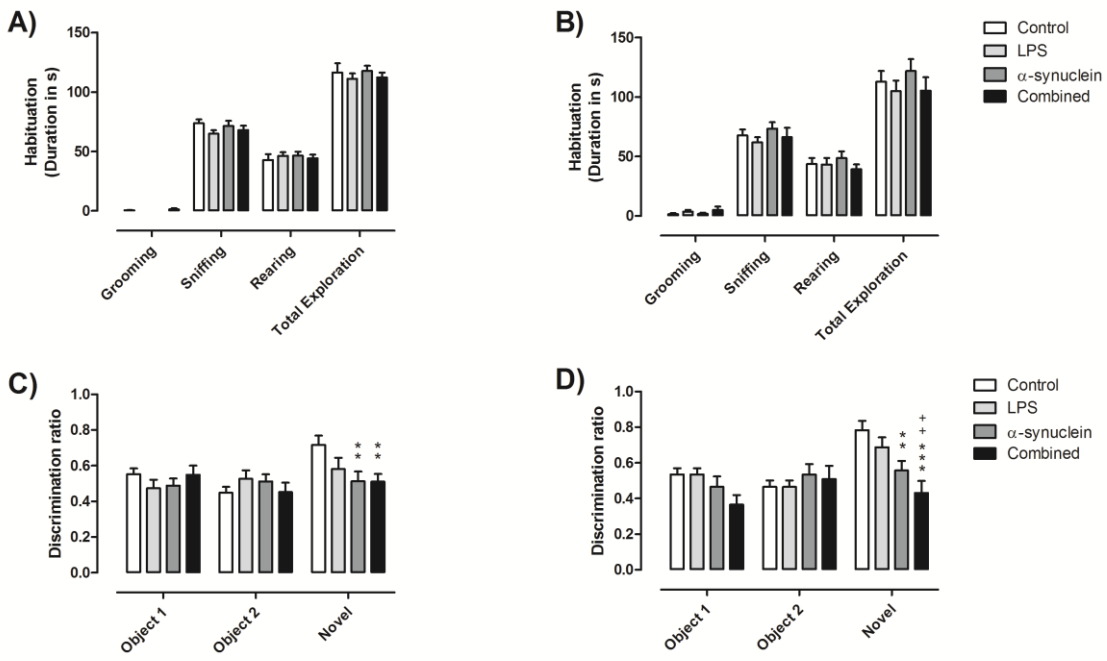


Fig 4.8: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein but not intrastriatal LPS induces a cognitive impairment. During habituation at week 12 (A) and 17 (B), administration of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS did not result in any significant spontaneous behavioural dysfunction as determined by the amount of time animals from each experimental group spent grooming, sniffing and rearing. In contrast, during testing at weeks 12 (C) and 17 (D), animals infused with α-synuclein display some cognitive deficits but this was not exacerbated by subsequent infusion of LPS. Data are represented as mean ± SEM. ***P<0.001, **P<0.001 *vs.* control; ^{++}P<0.01 *vs.* LPS by two-way ANOVA and confirmed via *post-hoc* Bonferroni.

4.4.5. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS impairs nigrostriatal integrity

In order to assess the impact that administration of α-synuclein and/or intrastriatal LPS had on the integrity of the nigrostriatal pathway, the density of tyrosine hydroxylase immunopositive terminals in the striatum and the number of tyrosine hydroxylase immunopositive cell bodies in the substantia nigra were quantified on the ipsilateral and contralateral sides (Fig. 4.9). Although there was a reduction in the density of tyrosine hydroxylase immunopositive terminals in the striatum across all experimental groups, this was not statistically significant (Group, $F_{(3,30)}$ = 1.60, P>0.05 by one-way ANOVA). In contrast, there was a significant loss of dopaminergic cell bodies from the substantia nigra in the groups infused with α-synuclein (Group, $F_{(3,30)}$ = 4.26, P<0.05) but this was not exacerbated after LPS infusion.

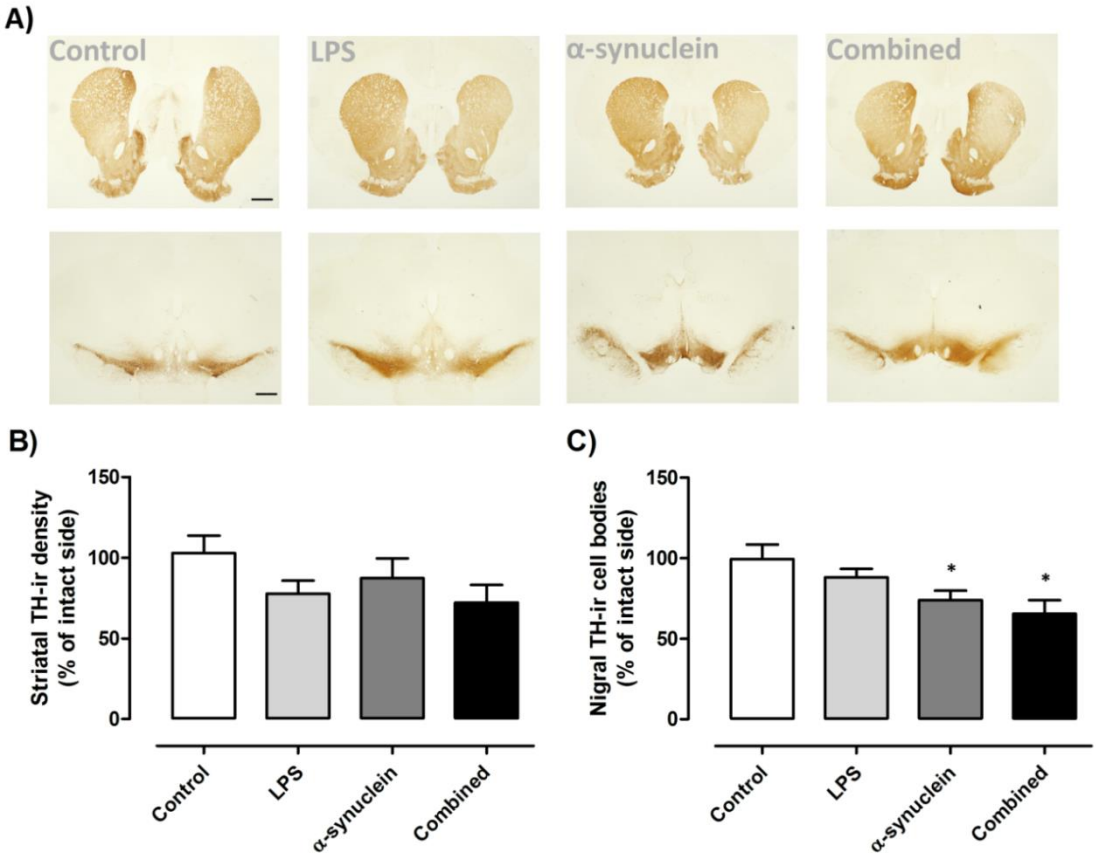


Fig. 4.9: Nigrostriatal integrity following $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein induced a significant level of neurodegeneration at the level of nigrostriatal cell bodies in both infused groups. However, LPS did not exacerbate this. Representative photomicrographs in (A) and quantitative histology (B & C) are shown. Data are represented as mean ± SEM. *P<0.05 *vs.* control by one-way ANOVA with *post-hoc* Newman-Keuls. Scale bar in striatal represents 2 mm and in nigral images represents 1 mm. TH-ir – tyrosine hydroxylase immunoreactivity.

4.4.6. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein results in significant overexpression of the α-synuclein in the nigrostriatal pathway

Central to Parkinson's disease neuropathology is pathological expression and aggregation of the protein α-synuclein. Therefore, we sought to determine the impact of the single or combined insults on α-synuclein expression in the nigrostriatal pathway using α-synuclein immunohistochemistry (Fig. 4.10). As expected, unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein caused a significant increase in the density of human α-synuclein in the substantia nigra and the striatum (Substantia nigra: Group, $F_{(3,25)} = 47.22$, $P<0.001$; Striatum: Group, $F_{(3,25)} = 50.01$, $P<0.001$). Intriguingly, the density of α-synuclein immunostaining in both regions was significantly enhanced by subsequent intrastriatal infusion of LPS. This robust overexpression of α-synuclein was also sufficient enough to induce dystrophic neurites (swollen varicosities in dopaminergic neurons) in the nigrostriatal terminals of the striatum (Fig. 4.11) with a strong, but not statistically significant, trend for LPS-induced enhancement of this α-synucleinopathy.

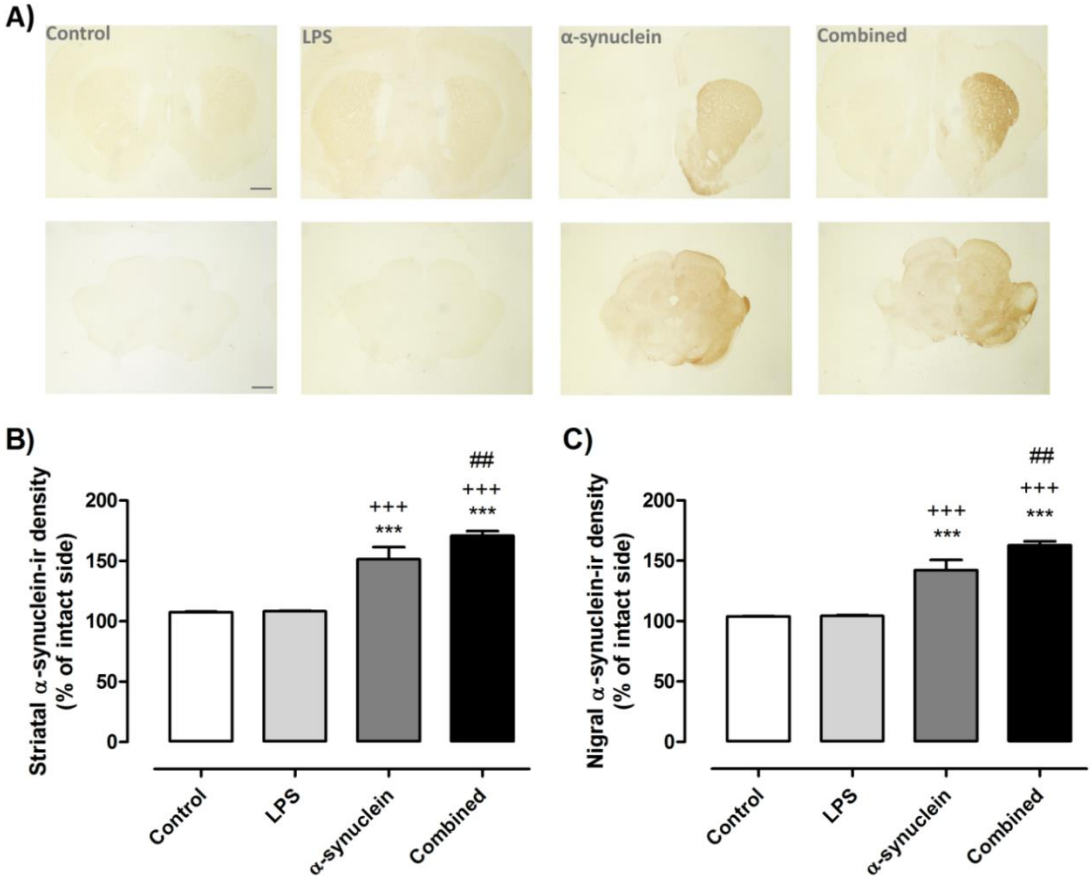


Fig. 4.10: Unilateral intranigral infusion of α-synuclein induces significant overexpression of α-synuclein in the nigrostriatal pathway. Unilateral administration of $AAV_{2/5}$-α-synuclein resulted in significant overexpression of α-synuclein in the nigrostriatal pathway at the levels of the terminals in the striatum (B) as well as in the cell bodies of the substantia nigra (C) with representative photomicrographs in (A). Interestingly, when the genetic and neuroinflammatory challenges were sequentially infused, the magnitude of α-synuclein expression was significantly enhanced. Data are represented as mean ± SEM. ***P<0.001 *vs.* control; ^{+++}P<0.001 *vs.* LPS; $^{\#\#}P$<0.01 *vs.* α-synuclein by one-way ANOVA with *post-hoc* Newman-Keuls. α-synuclen-ir – α-synuclein immunoreactivity. Scale bar in striatal images represents 2 mm and in nigral images represents 1 mm.

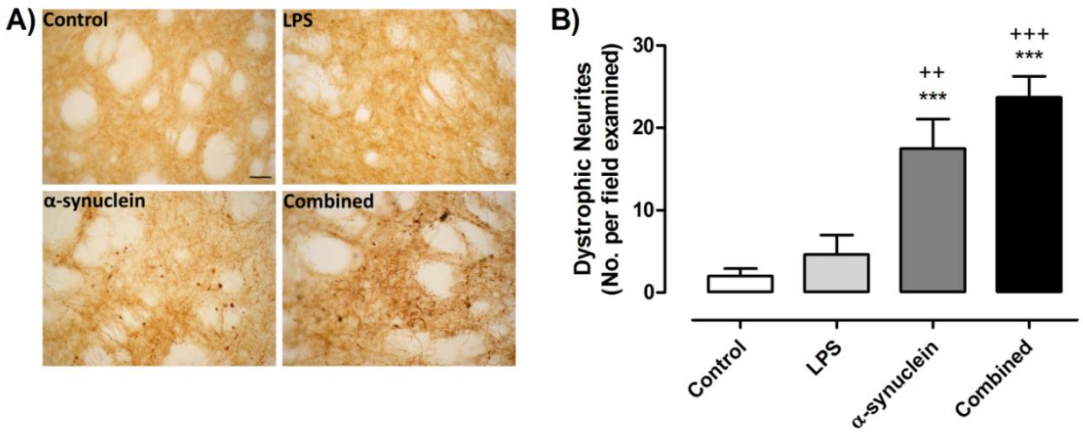


Fig. 4.11: Overexpression of α-synuclein induces neuronal dystrophy in tyrosine hydroxylase immunopositive nigrostriatal terminals of the striatum. Unilateral administration of $AAV_{2/5}$-α-synuclein to the nigrostriatal pathway resulted in neuronal dystrophy as assessed quantitatively in (A) and qualitatively in (B). Data are represented as mean ± SEM. ***P<0.001 *vs.* control and LPS; ^{+++}P<0.001, ^{++}P<0.01 *vs.* LPS by one-way ANOVA and *post-hoc* Newman-Keuls. Scale bar represents 0.2 mm.

4.4.7. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS does not induce a significant microgliosis

We also assessed the ability of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS to activate microglia in the striatum and/or the substantia nigra. We found that unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal LPS did not cause any statistically significant activation of microglia at the levels of the terminals in the striatum when assessed at this protracted time-point after infusion (Fig. 4.12; Striatum: Group, $F_{(3,27)}$ = 2.58, P>0.05, Substantia nigra: Group, $F_{(3,27)}$ = 2.89, P>0.05).

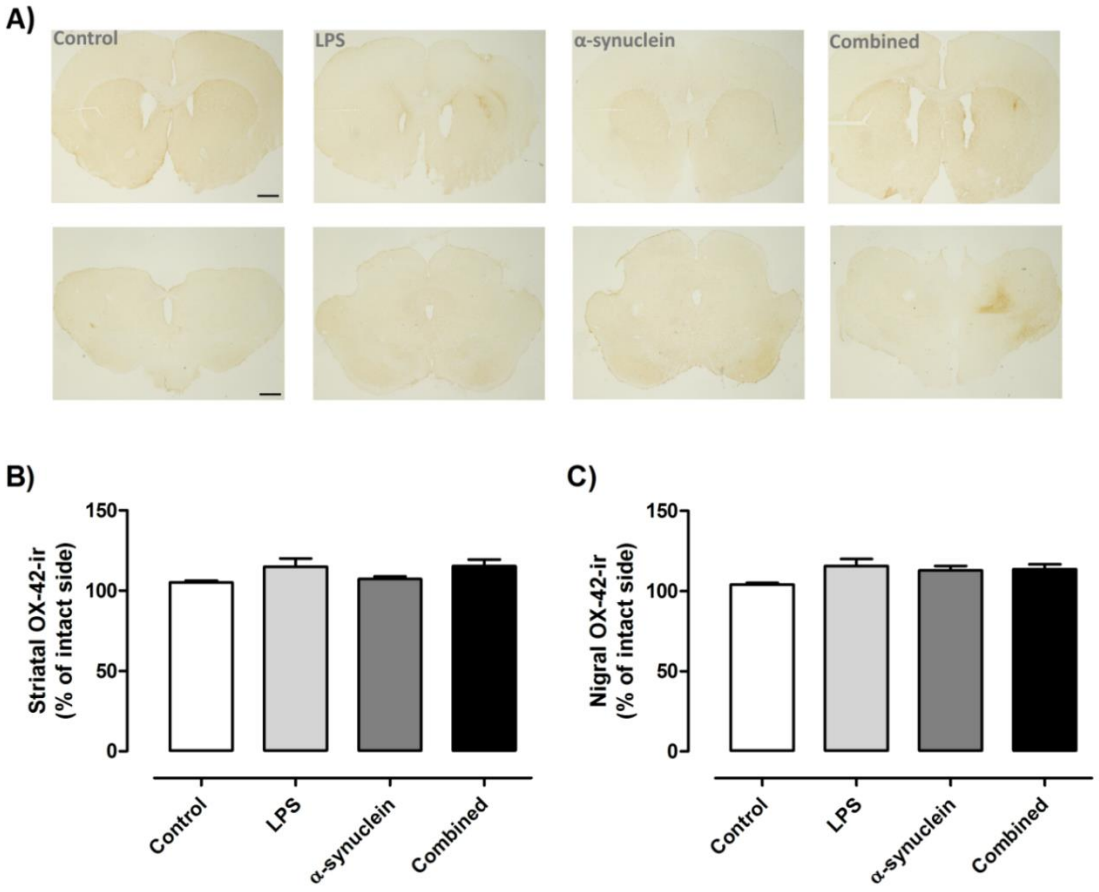


Fig. 4.12: Unilateral intrastriatal LPS administration does not induce microgliosis. OX-42 immunohistochemical staining revealed that there was no significant microgliosis in the nigrostriatal pathway at 17 weeks after injection of $AAV_{2/5}$-α-synuclein and/or 5 weeks post intrastriatal LPS injection into the striatum. Representative photomicrographs in (A), striatal optical density quantitative analysis in (B) and substantia nigra quantitative analysis in (C). Data are represented as mean ± SEM. P>0.05 by one-way ANOVA and *post-hoc* Newman-Keuls. Scale bar in striatal represents 2 mm and in nigral images represents 1 mm. OX-42-ir – OX-42 imunoreactivity.

4.5. Discussion

As Parkinson's disease is thought to arise as a result of interaction between genetic and exogenous factors such as bacterial infections, combining such relevant risk factors should not only provide information on gene-environment interactions in the etiology of Parkinson's disease, but should also result in the generation of a more novel etiologically relevant animal model for testing any potentially disease-modifying therapies for this disease. In this chapter, we were specifically interested in the effect of exposing the nigrostriatal terminals to the bacterial inflammagen LPS in rats who had already developed an α-synuclein pathology. Thus, in order to facilitate investigation into the interaction between α-synuclein and a subsequent inflammatory insult, α-synuclein was delivered to the substantia nigra using AAV vectors and this was then followed 12 weeks later with intrastriatal LPS. We found that sequential exposure to α-synuclein and LPS did not exacerbate any of the behavioural impairments caused by either challenge alone. Similarly, dual exposure to these genetic and neuroinflammatory challenges did not drive further nigrostriatal neurodegeneration. Intriguingly however, exposing rats to a terminal neuroinflammatory challenge significantly increased the density of human α-synuclein expression in the nigrostriatal pathway in rats that had received AAV-α-synuclein, indicating some interaction between these two challenges. Taken together, these data indicate that sequential exposure to α-synuclein and LPS does not provide a more robust or reliable model than either approach in its own right.

The central role that α-synuclein plays in the pathogenesis of Parkinson's disease has been highlighted by the fact that point mutations in the gene encoding α-synuclein, the *SNCA* gene, result in an autosomal dominant form of the disease (Polymeropoulos et al., 1997, Zarranz et al., 2004, Krüger et al., 1998). Moreover, triplications and even duplications of the normal wildtype gene are sufficient to cause the disease (Mutez et al., 2011, Singleton et al., 2003). In addition to this, one of the neuropathological hallmarks of both genetic and idiopathic Parkinson's disease are intracellular protein inclusion bodies known as Lewy bodies, and the primary constituent of Lewy bodies is α-synuclein (Spillantini et al., 1997). In this study, as expected, $AAV_{2/5}$-α-synuclein led to pronounced and widespread α-synuclein overexpression in the midbrain and striatum. This led to nigrostriatal dystrophy and partial loss of nigral cell bodies and terminals. Despite this α-synuclein pathology and consequent nigrostriatal degeneration, animals only developed a mild behavioural impairment which was not sufficient to produce a statistically significant overall impairment. This was not entirely surprising given the well-known mildness and variability of the AAV-α-synuclein-induced phenotype which is known to be the main limitation of this model (Kirik et al., 2002). Interestingly however, this is not unlike the human condition where α-synuclein pathology and even Lewy body formation have been found in presymptomatic individuals without any evidence of a movement disorder (Mikolaenko et al., 2005, Markesbery et al., 2009).

Neuroinflammation also plays a central role in driving the pathogenesis of Parkinson's disease and many other neurodegenerative diseases including

Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Akiyama et al., 2000, McGeer and McGeer, 2003, McGeer and McGeer, 2002b, McGeer and McGeer, 2004, Thomas et al., 2003). Microglia are the resident immune cells of the CNS and have long been implicated in the pathology and progression of many neurodegenerative disease however, in the normal healthy brain they maintain a resting or dormant state (Tamashiro et al., 2012). The primary role of microglia is that they perform innate immune functions by maintaining homeostasis by identifying any signals that require an immune response. In response to toxic stimuli, microglia undergo a morphological change and adopt an amoeboid morphology shifting from a resting to an activated state (reviewed in Kreutzberg, 1996). This dramatic alteration in their morphology and phenotype also results in the release of pro- or anti-inflammatory factors amongst many other changes (Hurley et al., 1999). Toll-like receptors, which are expressed on microglia, play a critical role in the first line of defence against viral and/or bacterial infections. TLR4 is the receptor activated by LPS and TLR4-LPS represents one of the first and best characterised receptor-ligand combinations of the innate immune system (Doyle and O'Neill, 2006). LPS binds to TLR4 on microglia, and upon their activation, microglia release numerous pro-inflammatory cytokines including TNFα, IL-1β, IL-6, proteinases and reactive nitrogen and oxygen species (reviewed in Dutta et al., 2008). These pro-inflammatory cytokines have been implicated in dopaminergic cell death as they express the required cytokine receptors in the production of reactive nitrogen and oxygen species which results in DNA damage, lipid peroxidation and oxidative stress with subsequent cell death (McCoy et al., 2006, Mosley et al., 2006, Koprich et al., 2008). In this study, LPS caused a moderate contralateral impairment in most of the

motor tasks incorporated into this study, but this was not underpinned by a significant nigrostriatal lesion. This failure of intrastriatal LPS to induce a significant lesion in its own right was not entirely surprising as we have previously shown that multiple infusions of LPS across the rostro-caudal axis of the striatum are required to induce nigrostriatal neurodegeneration (Hoban et al., 2013, Naughton et al., 2016a). However, we opted for a milder injection regime because we were concerned that the significant overexpression of α-synuclein could increase the sensitivity of the striatum to LPS possibly resulting in widespread destruction of striatal tissue in the dual exposure group.

The most important question of the present study was whether or not dual exposure to the genetic and environmental challenges could precipitate an enhanced behavioural and neuropathological phenotype relative to each in their own right. Rather disappointingly however, exposing rats with a pathological burden of α-synuclein to a subsequent terminal neuroinflammatory challenge did not exacerbate the effects of the single toxin exposures. Thus, sequential administration of α-synuclein and LPS did not have any additive or synergistic effect on motor function and nor did it drive an exaggerated neurodegenerative phenotype. Interestingly however, exposure to LPS did significantly increase expression of the human α-synuclein protein in both the nigrostriatal cell bodies and terminals. The reason for this is not known although it is becoming increasingly apparent that a complex relationship exists between α-synuclein expression and neuroinflammation. Intriguingly, activated microglia are known to phagocytose aggregated α-synuclein (Sacino et al., 2014) and they also secrete several enzymes that degrade the protein

such as plasminogen (Nakajima et al., 1995) and metalloproteinases (Sung et al., 2005, Tatebe et al., 2010). Thus, intuitively one would expect less α-synuclein expression in a dual α-synuclein-neuroinflammatory model. However, one recent study has shown that exposure to α-synuclein acts as a "priming factor" for microglia which strongly affects their subsequent phagocytic responses to α-synuclein after TLR activation (Roodveldt et al., 2013) leading the authors to conclude that this could drive α-synucleinopathy and progression of disease. Moreover, it was confirmed recently by Sanchez-Guajardo and colleagues, that microglia acquire distinct profiles and in the initial stages of α-synuclein pathology, microglia adopt an M2 phenotype and the primary purpose of M2 macrophages is to produce anti-inflammatory cytokines (Sanchez-Guajardo et al., 2010). Therefore, it is possible that the overexpression of α-synuclein attenuates the microglial response which reduces α-synuclein phagocytosis leading to an increased α-synuclein expression in our dual exposure animals.

Taken together, although sequential exposure to α-synuclein and LPS increased nigrostriatal α-synuclein expression, this was not sufficient to enhance nigrostriatal neurodegeneration or to precipitate a more robust behavioural phenotype. For this reason further studies in this thesis will move to using the Parkinson's disease-associated pesticide, rotenone, in an attempt to develop dual-exposure model systems with α-synuclein.

Chapter 5: Development and characterisation of a novel rat model of Parkinson's disease induced by intrastriatal rotenone and intranigral α-synuclein

5.1 Introduction

The low penetrance of some Parkinson's disease associated mutations and the fact that the underlying etiology is multifaceted suggests that a multiple hit paradigm may be the most valid approach to generate a relevant animal model of the disease (Sulzer, 2007). As described earlier in more detail, animals that have been used to date in preclinical research are typically modelled using single neurotoxic insults. However, because of the ability of the nigrostriatal pathway to readily compensate for the effects of exposure to unitary insults, the multiple hit hypothesis has gained further momentum as it may result in sustained or continuing degeneration which is more reflective of the complex disease etiology (Goldman et al., 2006, Gorell et al., 1999, Imaizumi, 1995, Ross and Petrovitch, 2001, Sato and Hattori, 2011, Svenson et al., 1993, Tanner et al., 2011, Van Den Eeden et al., 2003). Moreover, the paucity of etiologically and neuropathologically relevant models has been suggested as one of the reasons underlying the lack of correlation between preclinically-effective experimental Parkinsonian therapies and their translatability into a clinical setting (Biglan and Ravina, 2007, Kieburtz and Ravina, 2007, Meredith et al., 2008). With this is mind, in this chapter, we further investigate gene-environment animal models of Parkinson's disease using genetic and/or environmental factors relevant to the human condition – specifically α-synuclein and the environmental pesticide and complex I inhibitor, rotenone.

Interestingly, α-synuclein pathology, and even Lewy body formation, is not in itself sufficient to induce an overtly clinical syndrome with both having being identified in the normal aged human brain (Mikolaenko et al., 2005, Markesbery et al., 2009). However, in the Markesbery *et al*. (2009) study, the "normal" patients with α-synuclein pathology were suggested to represent a population with preclinical or presymptomatic Parkinsonism. Given that Parkinson's disease is thought to emerge after interaction between genetic and environmental risk factors, it follows that sufficient exposure of such presymptomatic individuals to environmental risk factors (or conversely, insufficient exposure to environmental protective factors) could result clinically in Parkinson's disease.

The first reporting of a mitochondrial Complex I defect in Parkinson's disease was in 1989 by Schapira and colleagues (Schapira et al., 1989). Moreover, following this pioneering discovery, it was also determined that there was a significant loss of reduced glutathione in the substantia nigra of Parkinsonian patients and in patients with Lewy Body disease (Jenner et al., 1992). The Complex I defect in Parkinson's disease appears to be systemic affecting not only dopaminergic neurons but other tissues outside the brain with numerous studies reporting the reduced activity in the platelets of people with Parkinson's disease (Parker et al., 1989, Krige et al., 1992, Yoshino et al., 1992). Moreover, epidemiological studies and biochemical studies of *post-mortem* Parkinsonian brains and peripheral tissues have confirmed a modest systemic complex I defect further supporting its relevance to disease pathogenesis (Greenamyre et al., 2001). When MPTP was reported to induce all of the clinical and neuropathological features of advanced Parkinsonism this underpinned the search for

other Complex I inhibitors. The systemic rotenone model has been shown to induce relatively selective dopaminergic degeneration and motor dysfunction (Uversky et al., 2001, Sherer et al., 2003, Alam et al., 2004). However, despite this, the model is also associated with significant peripheral organ toxicity, body weight loss and high mortality rates (Ferrante et al., 1997, Greene et al., 2009, Antkiewicz-Michaluk et al., 2003). Therefore, in order to circumvent these major drawbacks, studies have investigated intracerebral administration of rotenone (Heikkila et al., 1985, Antkiewicz-Michaluk et al., 2004, Sindhu et al., 2005, Ravenstijn et al., 2008, Mulcahy et al., 2011).

Taking these approaches together, in this chapter we embarked on a series of experiments to investigate the interactions between α-synuclein and rotenone, and whether the interaction of these Parkinson's disease-associated risk factors could provide a more robust and relevant model of the condition. Moreover, this would also provide a novel platform with which to test any potential neuroprotective and disease-modifying therapies for this chronic disabling condition.

5.2 Methods

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

5.3 Experimental Design

This chapter will detail results obtained from three separate studies all of which combined intra-nigral infusion of AAV-α-synuclein with intra-striatal infusion of rotenone. However each study differed with respect to the viral serotype, viral titre

and/or rotenone injection regime which led to differences in the "clinical" manifestation (i.e. behavioral manifestation) of a motor disorder in this model. Studies 1 and 2 were performed in order to assess the behavioural impact of exposing rats with a clinical load of α-synuclein to a clinical dose of rotenone, but differed with respect to the viral serotypes used (Study 1 used $AAV_{2/5}$ while Study 2 used $AAV_{2/6)}$. Study 3 was performed in order to investigate the impact of exposing rats with a subclinical load of α-synuclein (using $AAV_{2/5}$ vectors) to a subclinical dose of rotenone. The specific design of each study will be detailed below but the main differences are summarised Table 5.1.

Study	Viral serotype & α-synuclein titre	Rotenone dose & injection regime
Study 1	$AAV_{2/5}$ @ 3.5 x 10^8 vg/µl	0.9 µg @ 4 sites
Study 2	$AAV_{2/6}$ @ 5.0 x 10^{10} vg/µl	0.9 µg @ 4 sites
Study 3	$AAV_{2/5}$ @ 1.0 x 10^{10} vg/µl	1.8 µg @ 2 sites

Table 5.1: A summary of the viral serotypes and titres, rotenone doses and administration regimes used in these studies.

5.3.1 Study 1: The effect of clinical intranigral $AAV_{2/5}$-α-synuclein followed by clinical intrastriatal rotenone infusion

Male Sprague Dawley rats were used in this experiment (weighing 225-250 g at the start of the study) and were randomly assigned to two groups for intranigral infusion of $AAV_{2/5}$-GFP (*n*=20) or $AAV_{2/5}$-α-synuclein (*n*=20). Viral titres were 2.6 x 10^7 vg/µl and 3.5 x 10^8 vg/µl for GFP and α-synuclein respectively. Post viral motor function was assessed at 4, 8, 12 and 16 weeks. At 17 weeks post viral vector administration, animals were performance-matched to receive a subsequent intrastriatal challenge of rotenone (4 x 0.9 µg in 3 µl) or its corresponding vehicle (1:1:18 combination of DMSO, Cremophor® and saline respectively) infused at 4 sites along the rostro-caudal axis of the striatum to yield four final groups (see Table 5.2). Behavioural testing resumed one week post rotenone surgery and continued weekly thereafter for a further 5 weeks upon which animals were then sacrificed via transcardial perfusion. Unfortunately for technical reasons (i.e. the microtome was faulty and destroyed the tissue), the tissue from this study was unable to be processed for immunohistochemical analyses.

Group	Virus injection	Rotenone injection	*n*
Control	$AAV_{2/5}$-GFP	Vehicle	10
Rotenone	$AAV_{2/5}$-GFP	Rotenone	9
α-Synuclein	$AAV_{2/5}$-α-synuclein	Vehicle	10
Combined	$AAV_{2/5}$-α-synuclein	Rotenone	10

Table 5.2: At week 0, rats were randomly divided into two groups to receive either $AAV_{2/5}$-GFP or $AAV_{2/5}$-α-synuclein. Seventeen weeks later, they were performance matched to receive either rotenone (0.9 μg in 3 μl at 4 points across the rostro-caudal axis of the striatum) or its corresponding vehicle to yield 4 final groups as shown.

5.3.2 Study 2: The effect of clinical intranigral $AAV_{2/6}$-α-synuclein followed by clinical intrastriatal rotenone infusion

Male Sprague Dawley rats were used in this experiment (weighing 225-250 g at the start of the study) and were randomly assigned to two groups for intranigral infusion of $AAV_{2/6}$-GFP (*n*=20) or $AAV_{2/6}$-α-synuclein (*n*=20; kindly gifted by Prof. Aideen Sullivan, University College Cork). The plasmid map which provided the backbone for the synthesis of the $AAV_{2/6}$-α-synuclein virus (the AAV2 plasmid) is the same AAV2 plasmid which was used in both our previous and subsequent $AAV_{2/5}$-α-synuclein studies. This plasmid was packaged using an AAV6 packing plasmid by VectorBio (293 Great Valley Parkway, Malvern, PA 19355) and viral titres provided for this viral serotype were 5.2 x 10^{10} vg/µl and 5.0 x 10^{10} vg/µl for GFP and α-synuclein respectively. Post viral motor function was assessed at one week post-surgery and weekly thereafter until 10 weeks post viral vector administration when animals were performance-matched to receive a subsequent intrastriatal challenge of rotenone (4 x 0.9 µg in 3 µl) or its corresponding vehicle (1:1:18 combination of DMSO, Cremophor® and saline respectively) which was infused at 4 sites along the rostro-caudal axis of the striatum to yield four final groups (see Table 5.3). Behavioural testing resumed one week post rotenone surgery and continued weekly thereafter for a further 4 weeks upon which animals were then sacrificed via transcardial perfusion. Unfortunately for technical reasons (i.e. the microtome was faulty and destroyed the tissue), the tissue from this study was unable to be processed for immunohistochemical analyses.

Group	Virus injection	Rotenone injection	*n*
Control	$AAV_{2/6}$-GFP	Vehicle	9
Rotenone	$AAV_{2/6}$-GFP	Rotenone	10
α-Synuclein	$AAV_{2/6}$-α-synuclein	Vehicle	10
Combined	$AAV_{2/6}$-α-synuclein	Rotenone	10

Table 5.3: At week 0, rats were randomly divided into two groups to receive either $AAV_{2/6}$-GFP or $AAV_{2/6}$-α-synuclein. Ten weeks later, they were performance matched to receive either rotenone (0.9 μg in 3 μl at 4 points across the rostro-caudal axis of the striatum) or its corresponding vehicle to yield 4 final groups as shown.

5.3.3 Study 3: The effect of subclinical intranigral $AAV_{2/5}$-α-synuclein followed by subclinical intrastriatal rotenone infusion

Male Sprague Dawley rats were used in this experiment (weighing 225-250 g at the start of the study) and were randomly assigned to two groups for intranigral infusion of $AAV_{2/5}$-GFP (n=19) or $AAV_{2/5}$-α-synuclein (n=20). Viral titres were 8.0 x 10^7 vg/µl and 1.0 x 10^{10} vg/µl for GFP and α-synuclein respectively. Post viral motor function was assessed at 7, 8, 10 and 12 weeks. At 12 weeks post viral vector administration, animals were performance-matched to receive a subsequent intrastriatal challenge of rotenone (2 x 1.8 µg in 3 µl) or its corresponding vehicle (1:1:18 combination of DMSO, Cremophor® and saline respectively) infused at 2 sites along the rostro-caudal axis of the striatum to yield four final groups (see Table 5.4). Behavioural testing resumed one week post rotenone surgery and continued weekly thereafter for a further 4 weeks upon which animals were then sacrificed via transcardial perfusion and their brains were processed for *post mortem* quantitative assessment of nigrostriatal neurodegeneration (via tyrosine hydroxylase immunohistochemistry) and α-synucleinopathy (via α-synuclein immunohistochemistry).

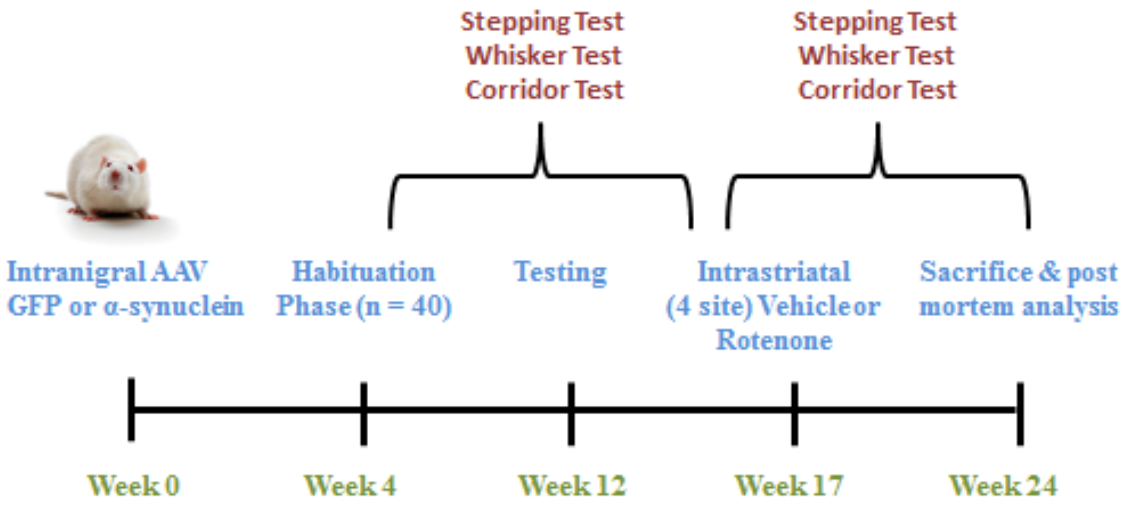
Group	Virus injection	Rotenone injection	*n*
Control	$AAV_{2/5}$-GFP	Vehicle	10
Rotenone	$AAV_{2/5}$-GFP	Rotenone	9
α-Synuclein	$AAV_{2/5}$-α-synuclein	Vehicle	10
Combined	$AAV_{2/5}$-α-synuclein	Rotenone	10

Table 5.4: At week 0, rats were randomly divided into two groups to receive either $AAV_{2/5}$-GFP or $AAV_{2/5}$-α-synuclein. Twelve weeks later, they were performance matched to receive either rotenone (1.8 μg in 3 μl at 2 points across the rostro-caudal axis of the striatum) or its corresponding vehicle to yield 4 final groups as shown.

5.4 Results

5.4.1 Study 1: The effect of clinical intranigral $AAV_{2/5}$-α-synuclein followed by clinical intrastriatal rotenone infusion

In this study, the impact of sequential administration of unilateral intranigral $AAV_{2/5}$-α-synuclein followed 17 weeks later by unilateral intrastriatal rotenone was assessed. Below is a schematic representation of the timeline of habituation, surgery, testing and sacrifice.



5.4.1.1 Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone does not impair ipsilateral motor function

As indicated in previous chapters, the key feature of any unilateral model of Parkinson's disease is the emergence of a bradykinetic syndrome on the side of the body contralateral to that in which the neuropathology develops. Therefore, in order to assess the impact of unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone on ipsilateral and contralateral motor function, the animals were subjected to a battery of lateralised motor tests each of which assesses different aspects of an emerging bradykinetic syndrome.

On the ipsilateral side, neither $AAV_{2/5}$-α-synuclein nor intrastriatal rotenone, alone or in combination, induced any impairment in the Stepping, Whisker, Corridor or Cylinder tests (Fig 5.1 and Fig 5.2). This confirms that any of the behavioural changes that we observed on the contralateral side are due to the effects of the

neurotoxins rather than any other indirect effect of the toxin. Due to a contralateral motor dysfunction in the Corridor and Cylinder test (Fig 5.4) animals who received the combination of $AAV_{2/5}$-α-synuclein and rotenone developed an ipsilateral bias preferentially retrieving Cocopops® from this side of their body (Corridor: Group, $F_{(3,35)} = 48.95$, $P<0.0001$; post-hoc Newman-Keuls confirmed a significant increase in their ipsilateral retrievals relative to the control group). Similarly, in line with these results, rotenone lesioned and combined $AAV_{2/5}$-α-synuclein and rotenone lesioned groups of animals also developed a bias towards their ipsilateral side in the Cylinder test and they preferentially supported themselves using their ipsilateral paw in the cylinder (Cylinder: Group, $F_{(3,35)} = 18.07$, $P<0.0001$; rotenone and combined group *vs.* control group and $P<0.001$ combined *vs.* α-synuclein confirmed with *post-hoc* Newman-Keuls).

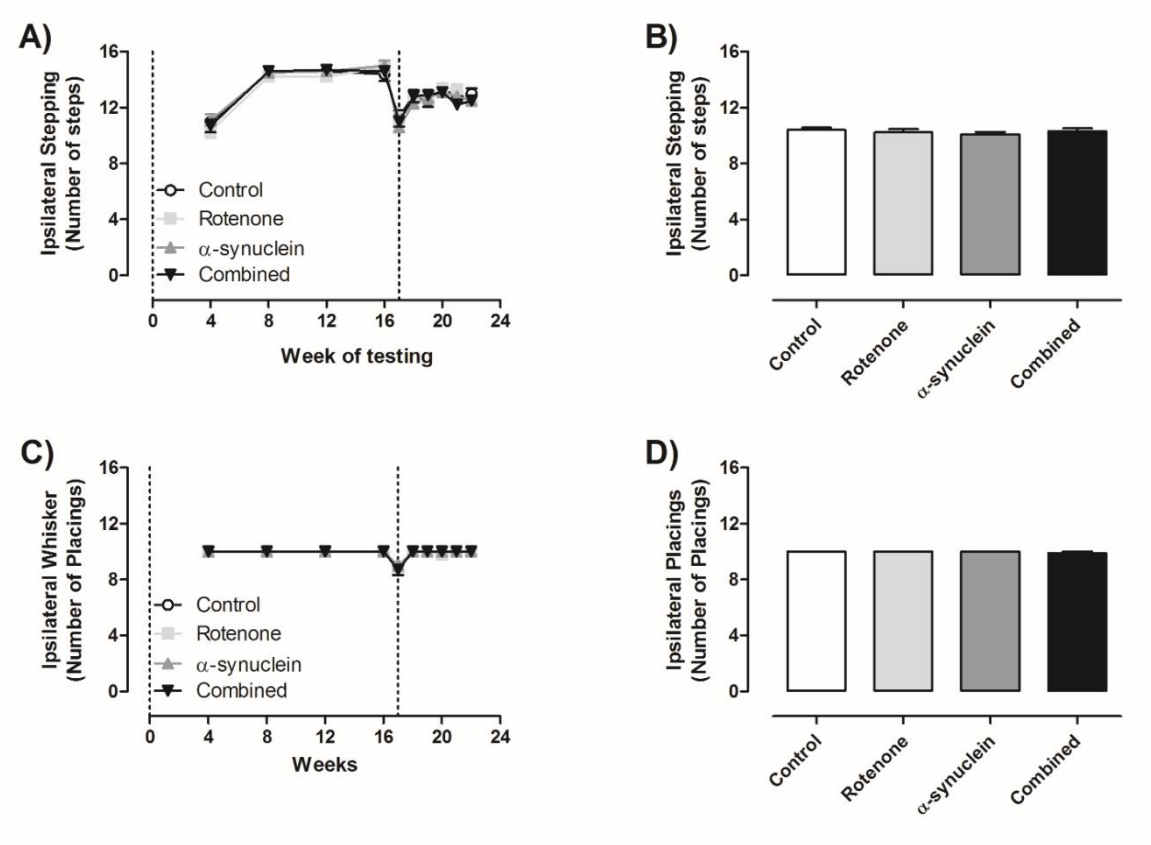


Fig. 5.1: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone does not impair ipsilateral motor function in the Stepping and Whisker Test. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone did not cause any motor dysfunction on the ipsilateral side of the Stepping (A and B) or the Whisker (C and D) tests. The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data from the post-operative period only. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. $P>0.05$ by one-way ANOVA and *post-hoc* Newman-Keuls.

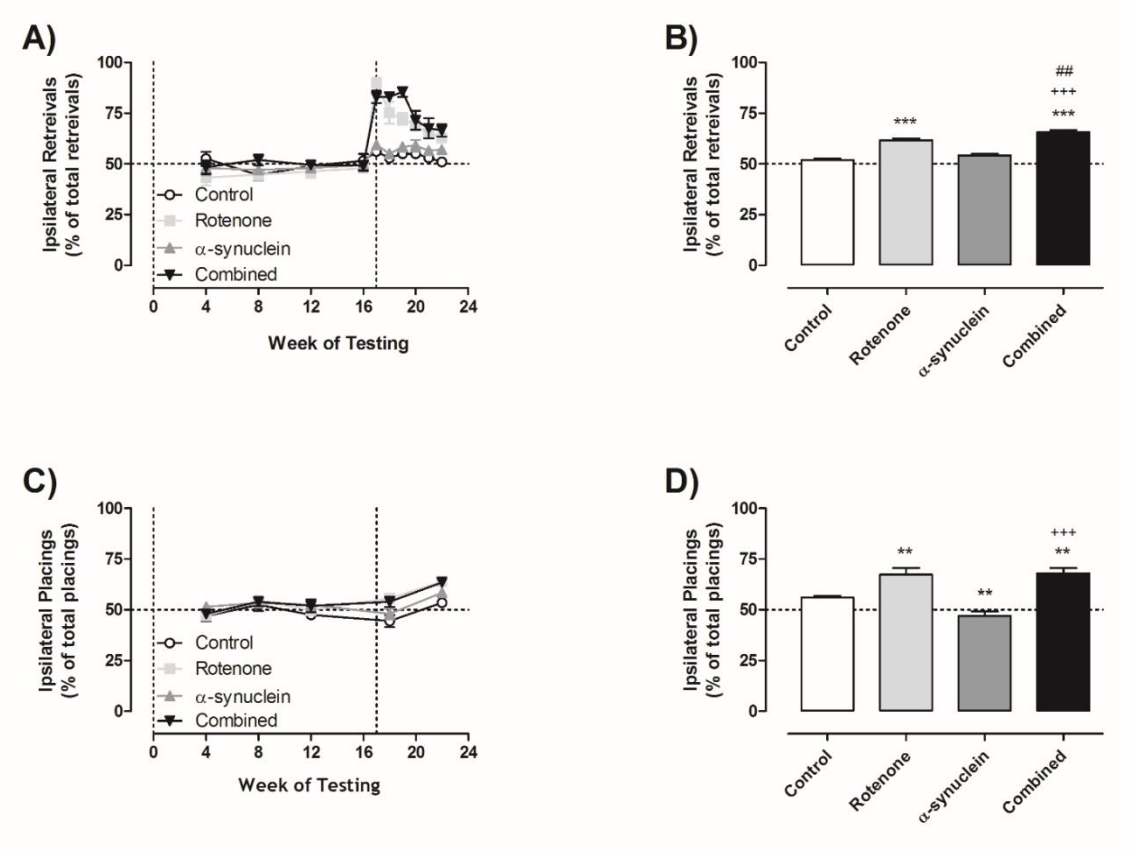


Fig. 5.2: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone does not impair ipsilateral motor function in the Corridor and Cylinder tests. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone did not cause any motor dysfunction on the ipsilateral side of the Corridor (A and B) or the Cylinder (C and D) tests. The XY plot on the left depict the data collected over the course of the study whereas the bar charts on the right represent collapsed data from the post-operative period only. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001, **P<0.01 *vs.* control; ^{+++}P<0.001 *vs.* α-synuclein; $^{\#\#}P$<0.01 *vs*. rotenone by one-way ANOVA with *post-hoc* Newman-Keuls.

5.4.1.2 Unilateral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone induces significant contralateral motor dysfunction

In contrast to the ipsilateral side, on the contralateral side the unilateral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone induced a motor impairment in the battery of behavioural tasks employed in this study, and this was mild after AAV-α-synuclein and pronounced after rotenone.

In the Stepping test of forelimb akinesia, the infusion of rotenone and α-synuclein alone and in combination with each other, led to a significant reduction in the ability of the animals to make adjusting steps on their contralateral sides (Fig. 5.3B: Stepping: Group, $F_{(3,35)}$ = P<0.0001). Importantly, there was a significant additive effect of the two challenges in the combined group (see *post-hoc* outcome on Fig. 5.3B). In the Whisker test of sensorimotor integration, the infusion of rotenone and α-synuclein alone and in combination with each other, also led to a significant reduction in the ability of the animals to make vibrissae-evoked contralateral forelimb placings (Fig. 5.3D: Whisker: Group, $F_{(3,35)}$ = 284.2, P<0.0001). Although, in contrast to the Stepping test where we observed a significant additive effect of the two challenges, in the Whisker test this additive effect was not apparent.

In the Corridor test of lateralised neglect, infusion of rotenone but not α-synuclein, resulted in a significant impairment of the rats to retrieve Cocopops® from the contralateral sides of their bodies (Fig 5.4B: Corridor: Group, $F_{(3,35)}$ = 46.60, P<0.0001). This trend also emerged in the Cylinder test with rotenone lesioned animals neglecting to use their contralateral forepaw for support while exploring the

cylinder, preferentially supporting themselves with their ipsilateral forelimb instead (Fig 5.4D: Cylinder: Group, $F_{(3,35)} = 4.478$, $P<0.05$). Importantly however, there was no additive effect observed with the combination of both α-synuclein and rotenone in either test.

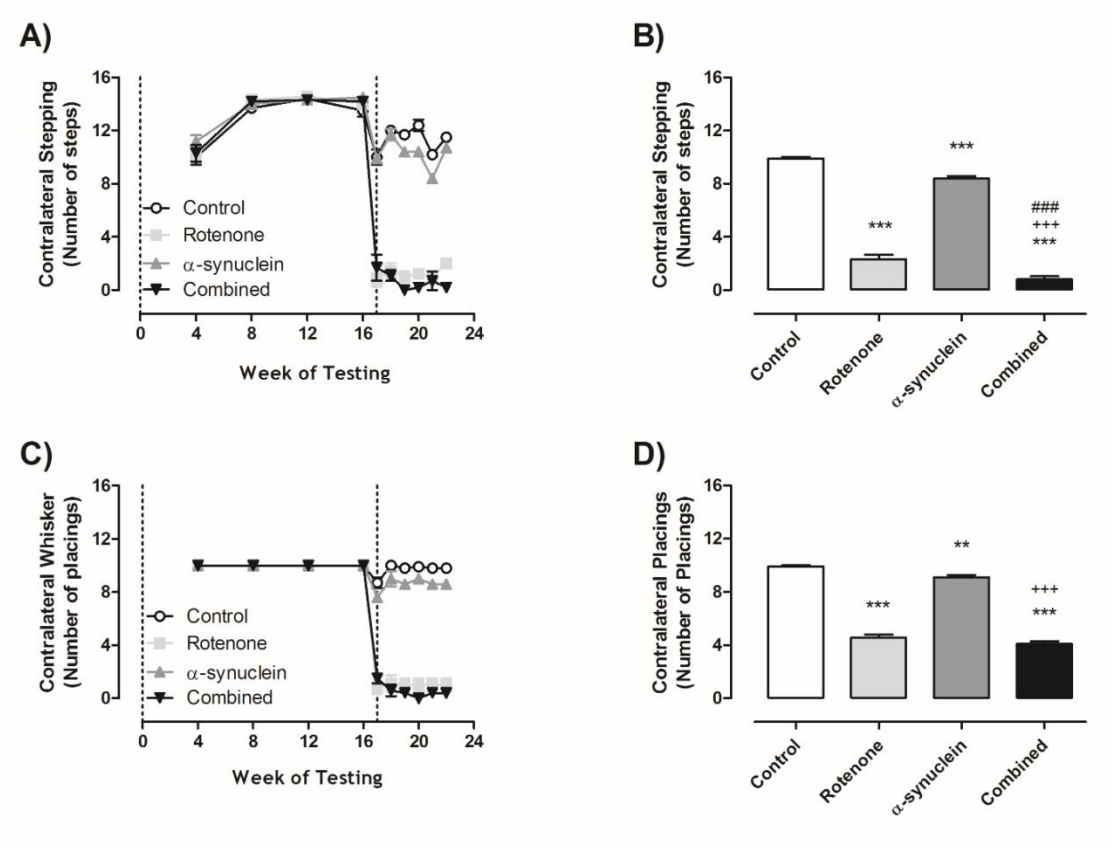


Fig. 5.3: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone results in the emergence of a significant contralateral motor dysfunction in the Stepping and Whisker tests. In the Stepping test, the ability of animals to make adjusting steps in the rotenone and α-synuclein lesioned groups was significantly impaired and when the Parkinsonian challenges were administered sequentially this was enhanced. However there was no additive effect of administering both of these neurotoxins in the Whisker test. The XY plot on the left depict the data collected over the course of the study whereas the bar charts on the right represent collapsed data collected from the post-operative period only. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001, **P<0.01, *vs.* control; ^{+++}P<0.001 *vs* α-synuclein; $^{###}P$<0.001 *vs.* rotenone by one-way ANOVA with *post-hoc* Newman-Keuls.

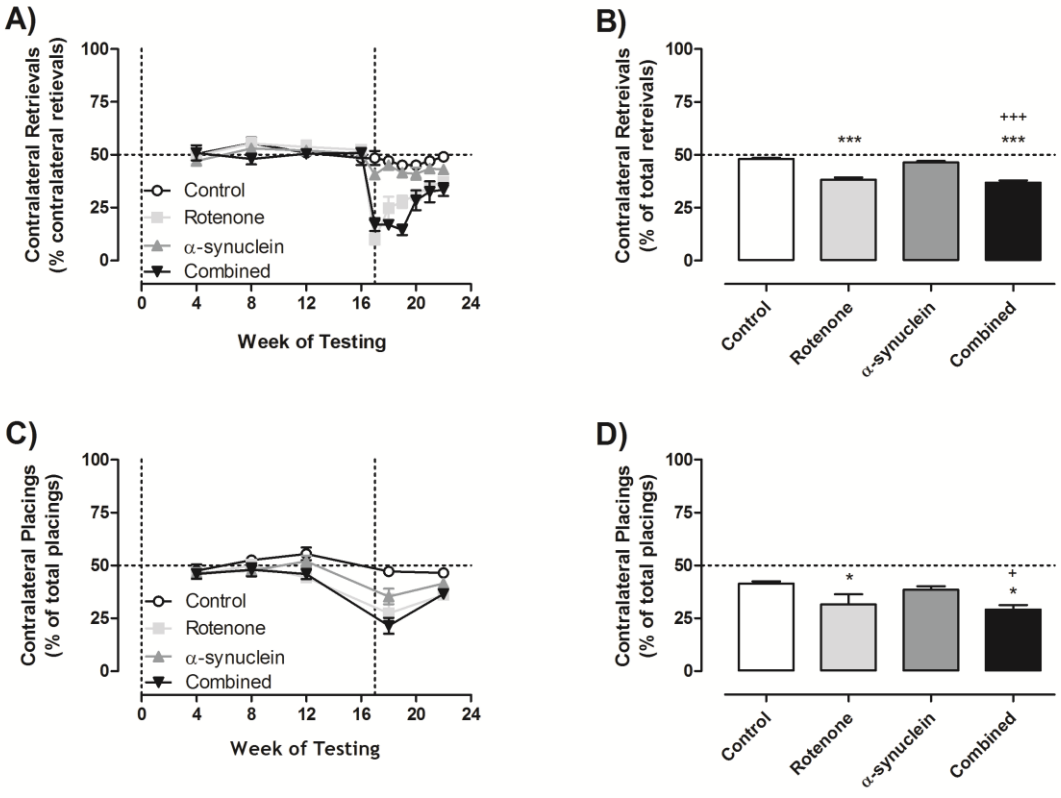
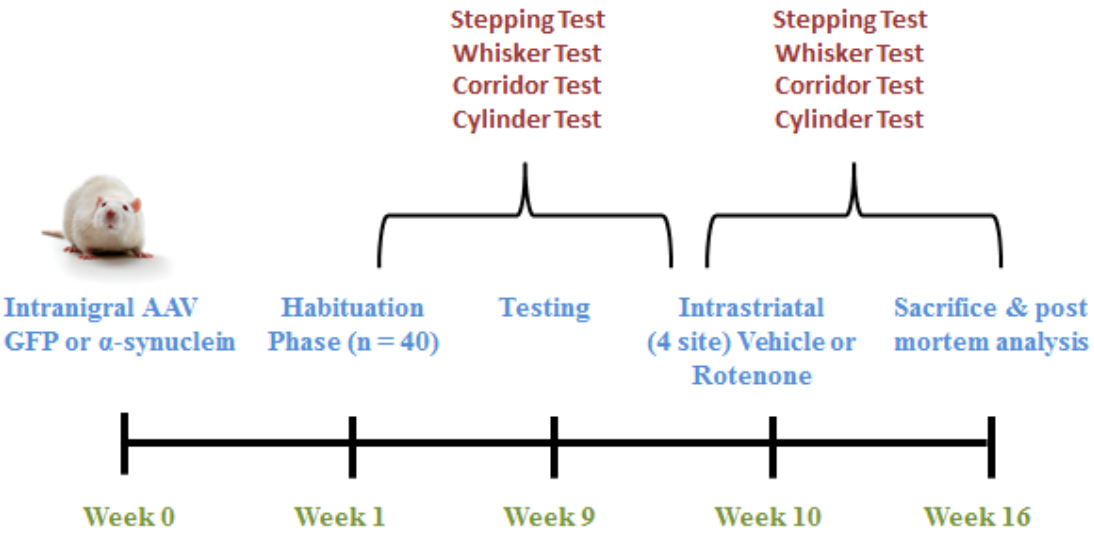


Fig. 5.4: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone results in the emergence of a significant contralateral motor dysfunction in the Corridor and Cylinder tests. In the Corridor (A and B) test, the ability of animals to make contralateral retrievals in the rotenone group and the combined group was significantly impaired. However there was no additive effect of administering both neurotoxins. In the Cylinder (C and D) test, there was no combined effect observed with the administration of both Parkinsonian challenges. The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data collected from the post-operative period only. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001, *P<0.05 *vs.* control; ^{+++}P<0.001, ^{+}P<0.05 *vs* α-synuclein by one-way ANOVA with *post-hoc* Newman-Keuls.

Unfortunately for technical reasons (i.e. the microtome was faulty and destroyed the tissue), the tissue from this study was unable to be processed for immunohistochemical analyses.

5.4.2 Study 2: The effect of clinical intranigral $AAV_{2/6}$-α-synuclein followed by clinical intrastriatal rotenone infusion

Although both $AAV_{2/5}$ (Kirik et al., 2003) and $AAV_{2/6}$ (Decressac et al., 2012b) pseudotyped viral vectors have been used to transfer human α-synuclein to the adult rat brain, the use of the $AAV_{2/6}$ vector may partly be responsible for the improved phenotype in the newer models (Lindgren et al., 2012). Therefore, because $AAV_{2/5}$-α-synuclein led to such a mild phenotype in the previous study, we decided to use $AAV_{2/6}$-α-synuclein in the present study in an attempt to enhance the behavioural and neuropathological effects of pathological α-synuclein expression. Below is a schematic representation of the timeline of habituation, surgery, testing and sacrifice.



5.4.2.1 Unilateral intranigral $AAV_{2/6}$-α-synuclein and/or intrastriatal rotenone does not impair ipsilateral motor dysfunction

As expected, on the ipsilateral side of the body, neither rotenone nor $AAV_{2/6}$-α-synuclein, alone or in combination with each other, induced any significant motor dysfunction in the Stepping, Whisker, Corridor or Cylinder Tests (Fig 5.5 and Fig 5.6). This confirms that any of the behavioural changes that we observed on the contralateral side were due to the neurodegenerative effects of the neurotoxins rather than any other indirect effect. Due to a significant contralateral motor dysfunction in the Corridor test (Fig 5.8B), animals who received rotenone and $AAV_{2/6}$-α-synuclein and the combination of both, developed an ipsilateral bias, preferentially retrieving Cocopops® from this side of their body (Corridor: Group, $F_{(3,35)} = 12.20$, $P<0.0001$, *post-hoc* Newman-Keuls confirmed a significant increase in their ipsilateral retrievals relative to the control group).

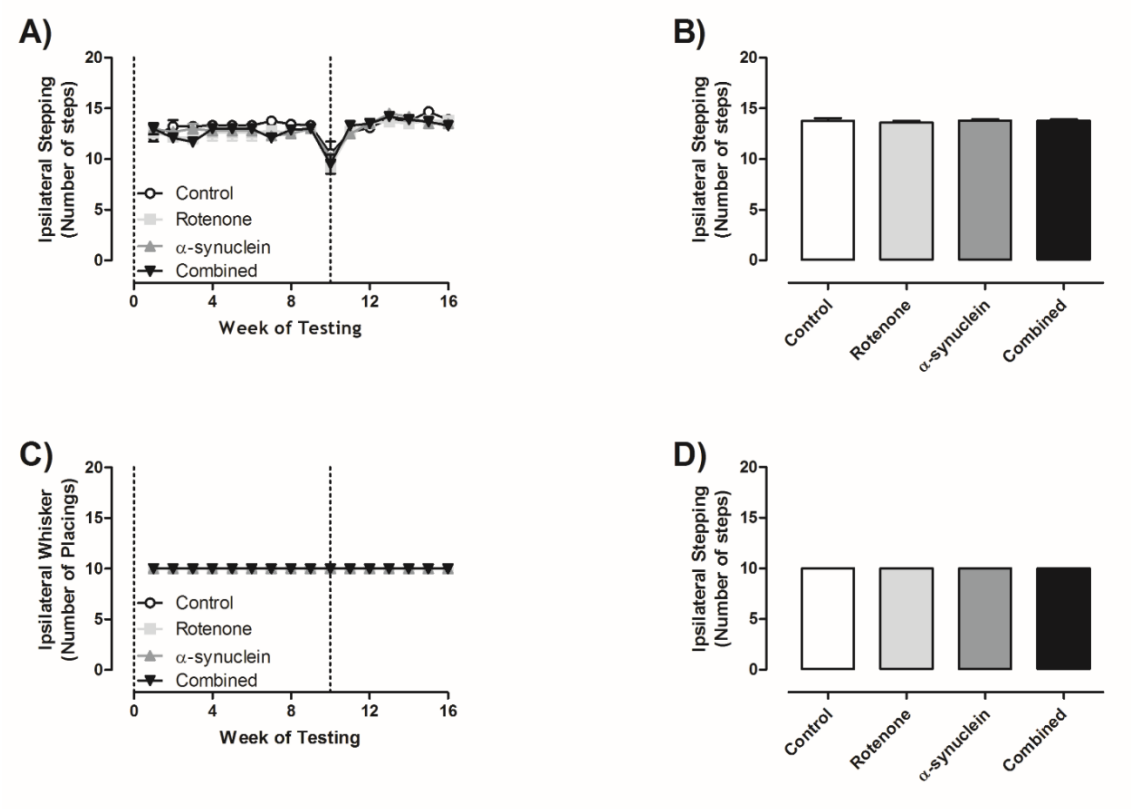


Fig. 5.5: Unilateral intranigral infusion of $AAV_{2/6}$-α-synuclein and/or intrastriatal rotenone does not impair ipsilateral motor function in the Stepping and Whisker Test. Unilateral intranigral infusion of $AAV_{2/6}$-α-synuclein and/or intrastriatal rotenone did not cause any motor dysfunction on the ipsilateral side of the Stepping (A and B) or the Whisker (C and D) tests. The XY plot on the left depict the data collected over the course of the study whereas the bar charts on the right represent collapsed data from the post-operative period only. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. $P>0.05$ by one-way ANOVA and *post-hoc* Newman-Keuls.

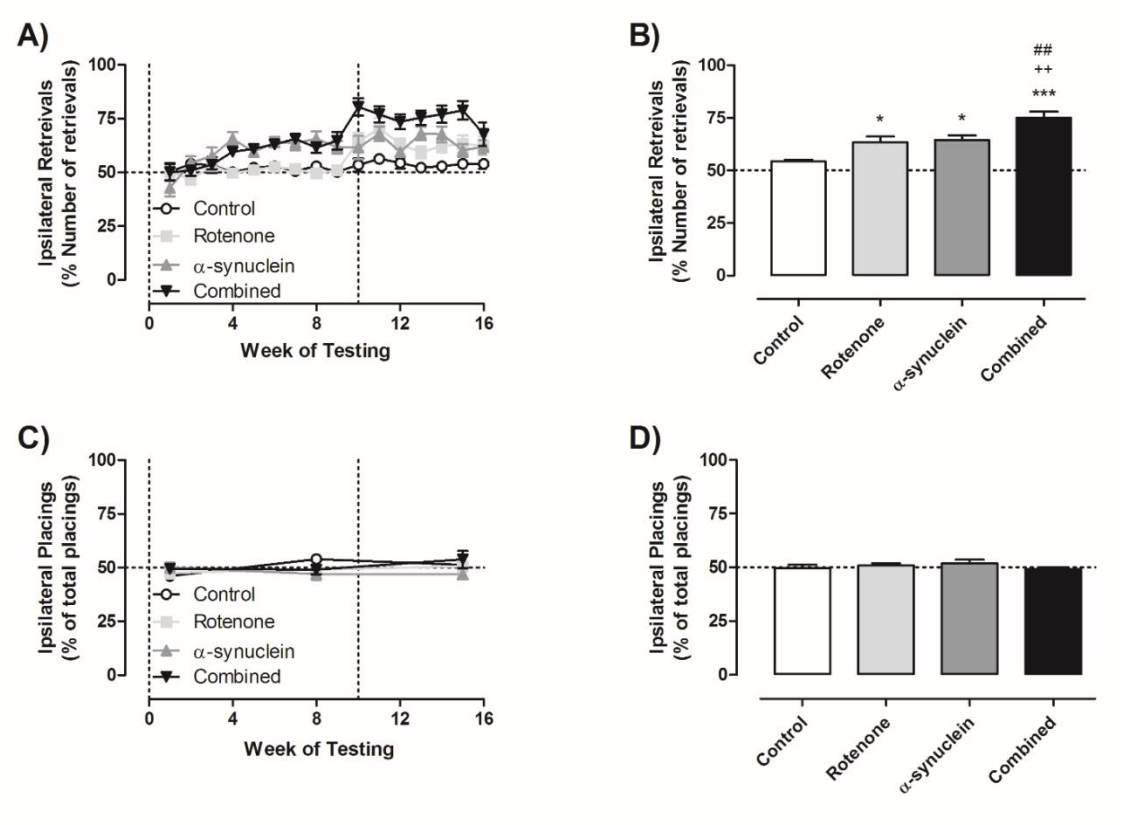


Fig. 5.6: Unilateral intranigral infusion of $AAV_{2/6}$-α-synuclein and/or intrastriatal rotenone does not impair ipsilateral motor function in the Corridor and Cylinder tests. Unilateral intranigral infusion of $AAV_{2/6}$-α-synuclein and/or intrastriatal rotenone did not cause any motor dysfunction on the ipsilateral side of the Corridor (A and B) or the Cylinder (C and D) tests. In the corridor test, animals preferentially retrieved Cocopops® from the ipsilateral sides of their bodies due to their significant contralateral impairment. The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data from the post-operative period. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001, *P<0.05 *vs.* control; ^{++}P<0.01 *vs.* α-synuclein, $^{\#\#}$P<0.01 *vs.* rotenone by one-way ANOVA with *post-hoc* Newman-Keuls.

5.4.2.2 Unilateral intranigral infusion of $AAV_{2/6}$-α-synuclein and/or intrastriatal rotenone induces a significant contralateral motor dysfunction

In contrast to the ipsilateral side, the unilateral infusion of $AAV_{2/6}$-α-synuclein and/or intrastriatal rotenone induced a significant contralateral motor impairment in the battery of behavioural tasks employed in this study.

In the Stepping test of forelimb kinesis, the ability of α-synuclein and rotenone treated animals to make adjusting steps on their contralateral side was significantly reduced (Fig 5.7B: Group, $F_{(3,35)}$ = 8.912, P<0.0001). However, there was no additive effect of the two challenges observed in this task. In the Whisker test of sensorimotor integration, the ability of rotenone treated animals to make vibrissae-evoked contralateral forelimb placings was significantly reduced. This impairment was also apparent in animals who received the combination of $AAV_{2/6}$-α-synuclein and rotenone (Fig 5.7D: Whisker: Group, $F_{(3,35)}$ = 6.521, P<0.0001). Again, in line with results observed in the stepping test, the combined group indicated that there was no significant additive effect of the two challenges.

In the Corridor test of lateralised neglect, infusion of either rotenone or $AAV_{2/6}$-α-synuclein alone led to a contralateral impairment (Fig 5.8B: Corridor: Group $F_{(3,35)}$ = 12.73, P<0.0001). Interestingly, the combination of both $AAV_{2/6}$-α-synuclein and rotenone further reduced the rats' ability to make food retrievals from the contralateral sides of their bodies than with either insult alone. In contrast to this, in

the Cylinder test of forelimb akinesia, animals continued to use both their ipsilateral and contralateral forelimbs to support themselves in the cylinder (Fig 5.8D).

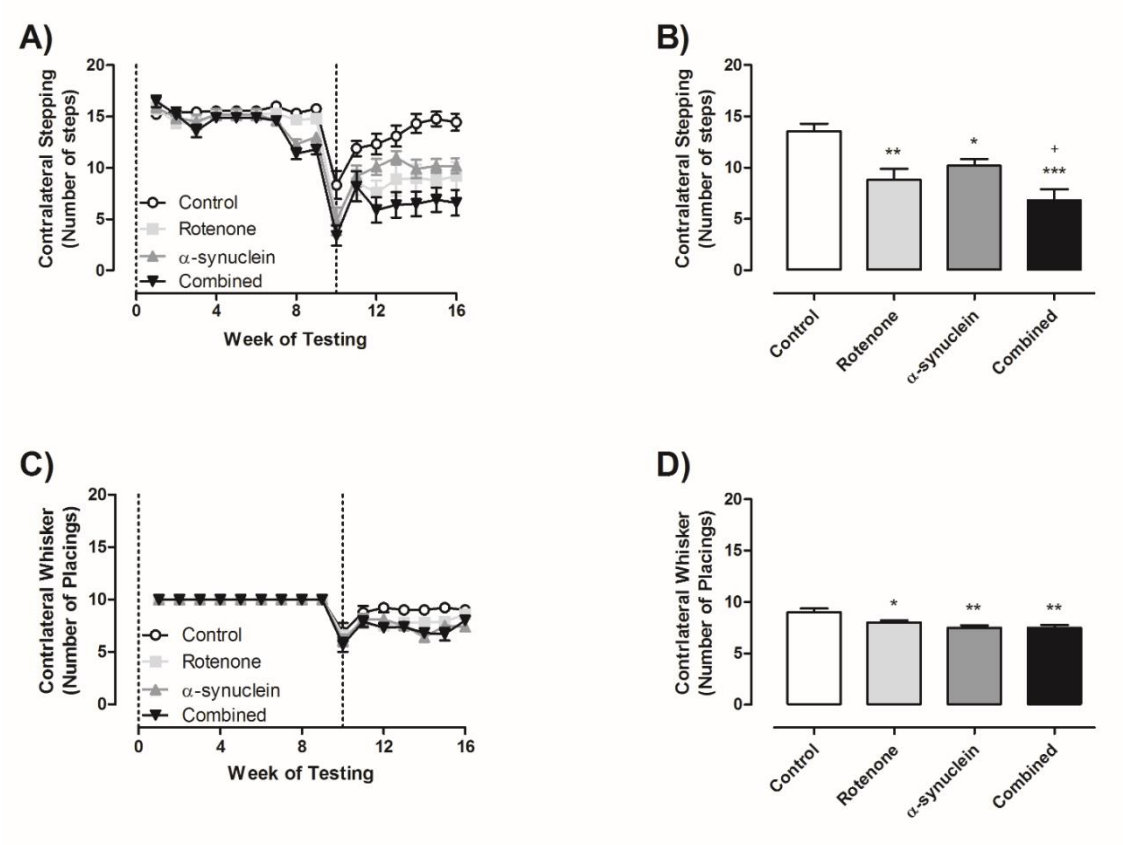


Fig. 5.7: Unilateral intranigral infusion of $AAV_{2/6}$-α-synuclein and/or intrastriatal rotenone causes significant motor dysfunction in the Stepping and Whisker tests. In the Stepping (A and B) and Whisker (C and D) tests, the ability of $AAV_{2/6}$-α-synuclein and rotenone treated animals to make adjusting steps or to make vibrissae-evoked forelimb placings was significantly impaired. However, there was no additive effect of combining these Parkinsonian challenges. The XY plot on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data from the post-operative period only. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05; *vs.* control; ^{+}P<0.05 *vs* α-synuclein by one-way ANOVA with *post-hoc* Newman-Keuls.

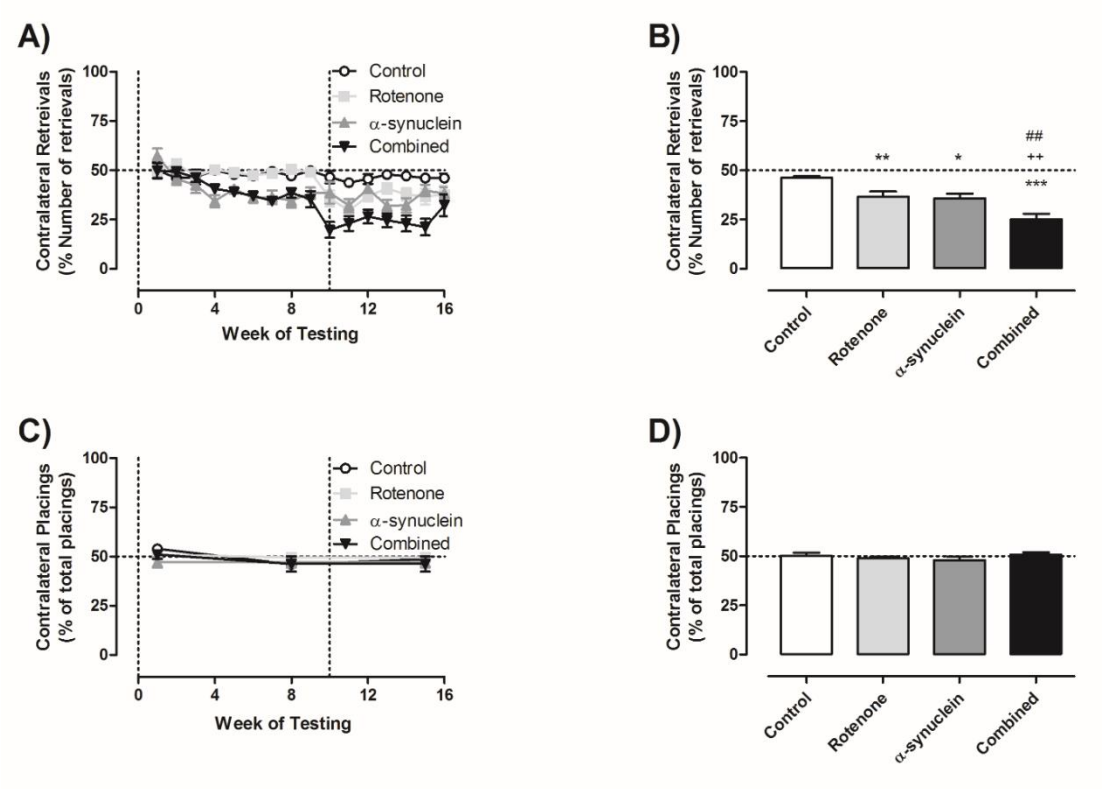
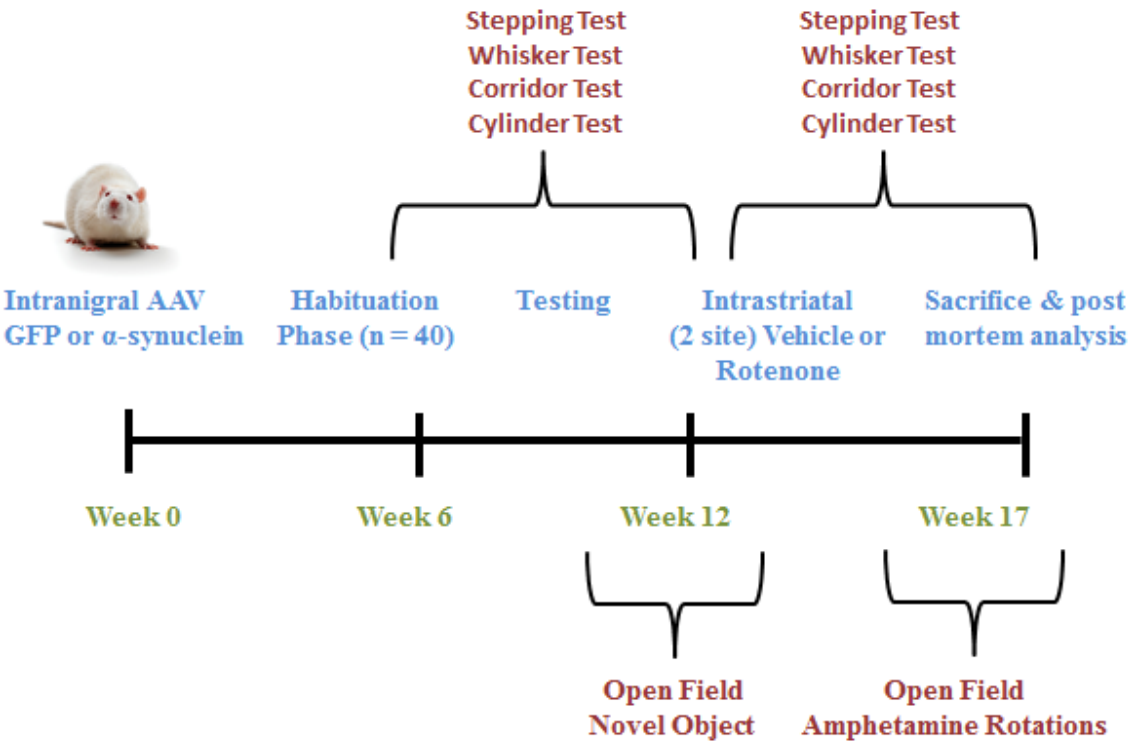


Fig. 5.8: Unilateral intranigral infusion of $AAV_{2/6}$-α-synuclein and/or intrastriatal rotenone induces a significant contralateral motor dysfunction in the Corridor test but not in the Cylinder test. In the Corridor test (A and B) of contralateral neglect, the ability of $AAV_{2/6}$-α-synuclein and rotenone treated animals to make contralateral retrievals was significantly impaired. Importantly, when the Parkinsonian challenges were administered sequentially to the same animals, this impairment was significantly greater than either insult alone. No significant difference emerged in the Cylinder (C and D) test. The XY plots on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data from the post-operative period. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 *vs.* control; ^{++}P<0.01 *vs.* α-synuclein; $^{\#\#}P$<0.01 *vs.* rotenone by one-way ANOVA with *post-hoc* Newman-Keuls.

As indicated above, for technical reasons the tissue from this study was unable to be processed for immunohistochemical analyses and therefore the neuropathology underlying these behavioural features was unable to be assessed.

5.4.3 Study 3: The effect of subclinical intranigral $AAV_{2/5}$-α-synuclein followed by subclinical intrastriatal rotenone infusion

Although the $AAV_{2/6}$ vector used in Study 2 led to a more pronounced phenotype than the $AAV_{2/5}$ vector used in Study 1, we were unable to source more $AAV_{2/6}$-α-synuclein for further studies (i.e. the $AAV_{2/6}$ used in Study 2 was a gift from Prof. Aideen Sullivan). Therefore, we reverted to in-house $AAV_{2/5}$ for the final study in this chapter which was completed in an attempt to generate tissue that could be used for *post-mortem* analysis. Below is a schematic representation of the timeline of habituation, surgery, testing and sacrifice.



5.4.3.1 Unilateral sequential administration of intranigral $AAV_{2/5}$-α-synuclein and intrastriatal rotenone is not detrimental to the rats general health

Intranigral infusion of $AAV_{2/5}$-α-synuclein and/or rotenone did not induce mortality in any of the rats. Moreover, the single or combined treatments did not have any effect on the body weight of the rats and they continued to gain weight over the course of the study (Fig 5.9: Group, $F_{(3,35)} = 0.23$, $P>0.05$).

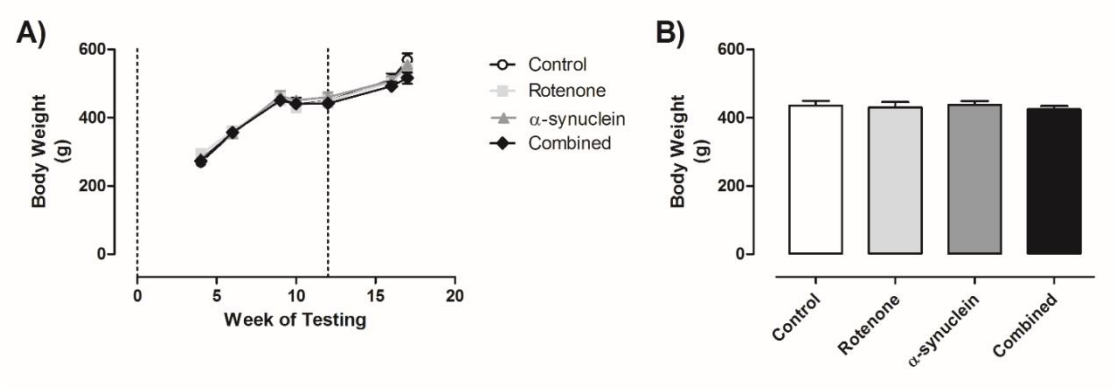


Fig. 5.9: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone did not affect the ability of the rats to gain weight over the course of the study. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone did not adversely affect the rats' general health over the time course of the study and they continued to gain weight. The XY plot on the left depict the data collected over the course of the study whereas the bar charts on the right represent collapsed data from the post-operative period only. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. $P>0.05$ by one-way ANOVA and *post-hoc* Newman-Keuls.

5.4.3.2 Unilateral sequential administration of intranigral $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone does not impair ipsilateral motor function

In line with our previous studies, the unilateral infusion of neither $AAV_{2/5}$-α-synuclein nor intrastriatal rotenone, alone or in combination, induced any significant impairment in the Stepping, Whisker, Corridor or Cylinder tests (Fig 5.10 and Fig 5.11). This was also further supported by performing the open field test where no difference was observed in the animals' general ambulatory behaviour (Fig 5.12) indicating that the motor dysfunction that we observed was specific for the lateralised motor tasks incorporated into this study. Due to their significant contralateral impairment (Fig. 5.14B), animals injected with the combined $AAV_{2/5}$-α-synuclein and rotenone developed a significant bias towards their ipsilateral side in the Corridor Test, and they preferentially retrieved CocoPops® from this side of their body over the course of testing (Corridor: Group, $F_{(3,35)} = 10.14$, $P<0.0001$; results of *post hoc* analyses shown in Fig. 5.11 B).

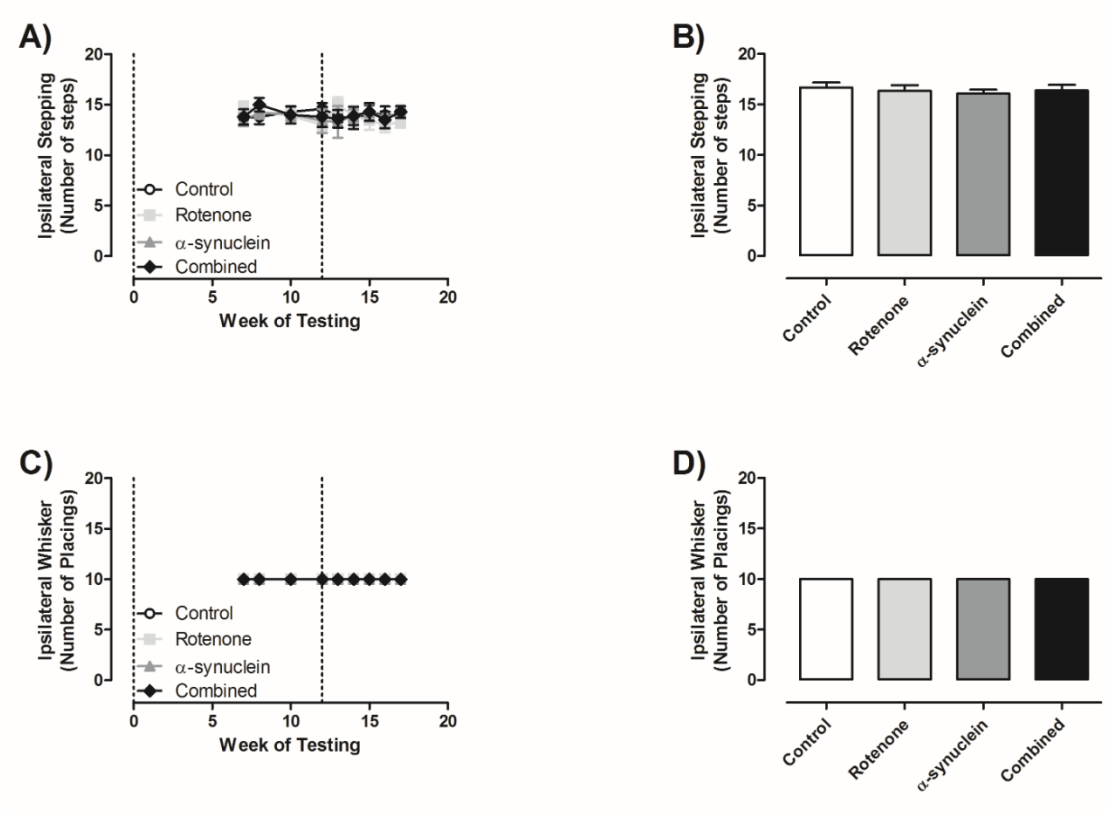


Fig. 5.10: Unilateral sequential intra-nigral administration of $AAV_{2/5}$-α-synuclein and intrastriatal rotenone does not impair ipsilateral motor function. Unilateral administration of intranigral $AAV_{2/5}$-α-synuclein and intrastriatal rotenone, alone or in combination with each other, did not cause any motor impairment on the side of the body ipsilateral to the lesion in either the Stepping (A and B) or the Whisker (C and D) tests. The XY plots on the left depict the data collected over the course of the study, whereas the bar charts on the right represent the collapsed data collected from the post-operative period only. Vertical lines indicate the day of viral vector administration and the subsequent environmental challenge. Data are shown as mean ± SEM. P>0.05, by one-way ANOVA and *post-hoc* Newman-Keuls.

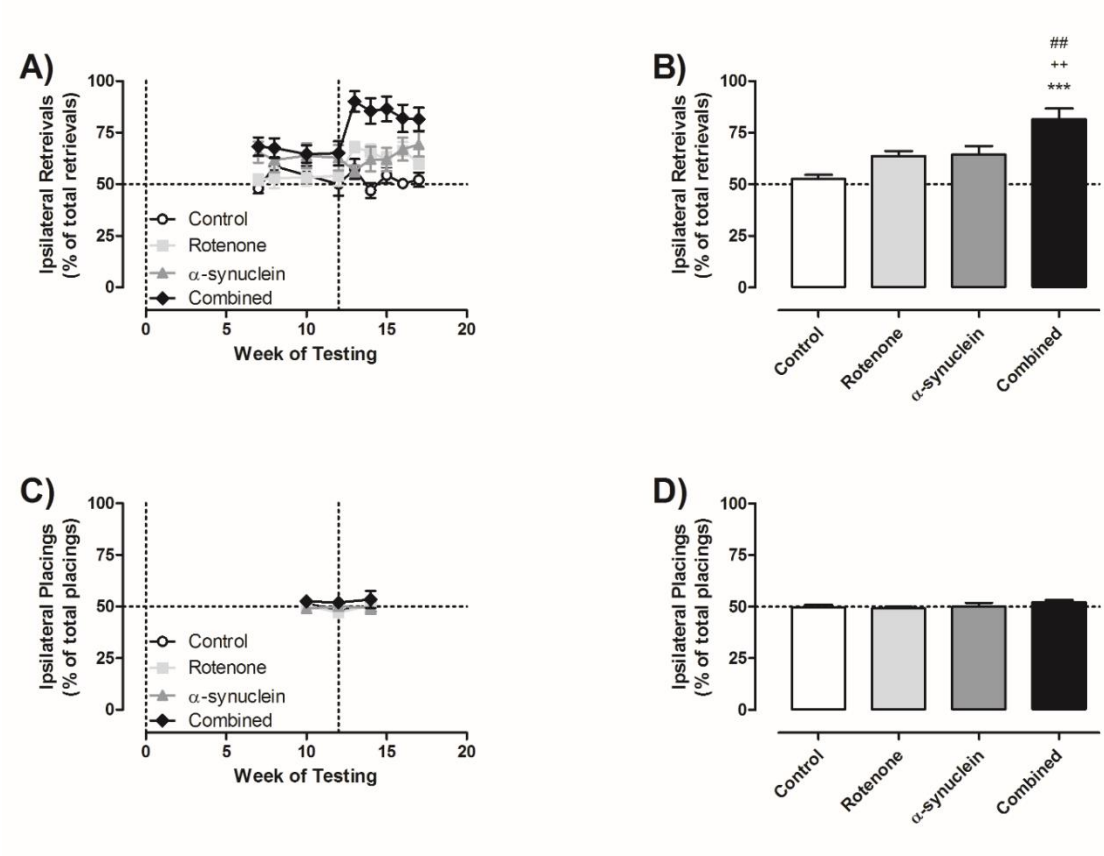


Fig. 5.11: **Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone does not impair ipsilateral motor function in the Corridor and Cylinder tests.** Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone did not cause any motor dysfunction on the ipsilateral side of the Corridor (A and B) or the Cylinder (C and D) tests. Due to their significant contralateral impairment, the combined group of $AAV_{2/5}$-α-synuclein and rotenone developed a significant bias towards their ipsilateral side in the Corridor Test. The XY plot on the left depict the data collected over the course of the study whereas the bar charts on the right represent collapsed data from the post-operative period. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001 *vs.* control; ^{++}P<0.01 *vs.* α-synuclein; $^{\#\#}P$<0.01 *vs.* rotenone by one-way ANOVA with *post-hoc* Newman-Keuls.

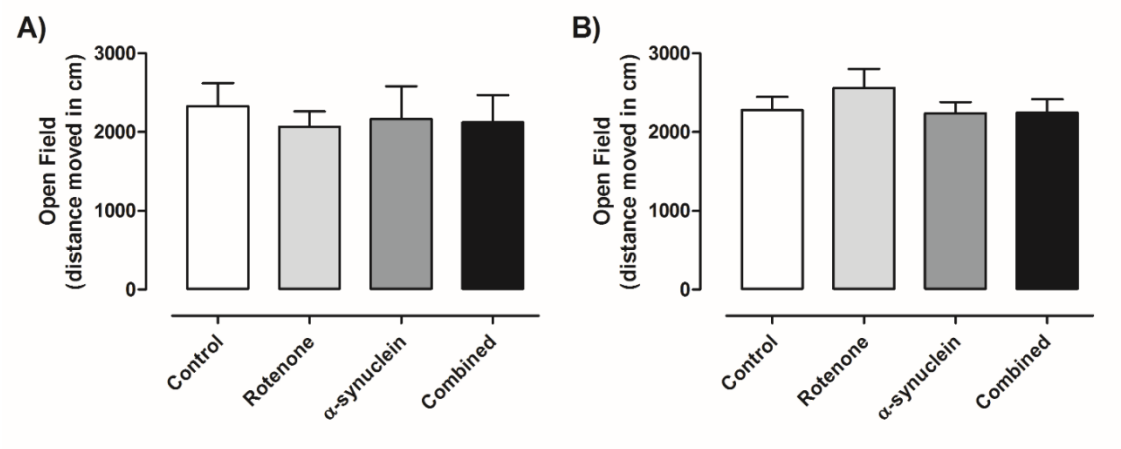


Fig. 5.12: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone does not adversely affect the rats' general locomotor activity. Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone did not cause any general motor dysfunction in the Open Field test at 12 (A) and 17 (B) weeks post viral surgery (immediately before and 5 weeks after rotenone infusion). Data are shown as mean ± SEM. $P>0.05$ by one-way ANOVA with *post-hoc* Newman-Keuls.

5.4.3.3 Unilateral sequential administration of intranigral $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone induces a profound contralateral motor dysfunction

On the contralateral side (Fig. 5.13 – Fig. 5.15), although a mild impairment in motor function emerged over the course of the study, administration of neither $AAV_{2/5}$-α-synuclein nor rotenone alone was sufficient to produce a statistically significant overall motor dysfunction in any of the tasks used. However, when the $AAV_{2/5}$-α-synuclein-injected animals were subsequently administered intrastriatal rotenone, this led to a profound impairment in the rats' ability to make adjusting steps in the Stepping Test (Fig. 5.13B: Group, $F_{(3,35)}=8.45$, $P<0.001$), in their capacity to make

vibrissae-elicited forepaw placings in the Whisker Test (Fig. 5.13D: Group, $F_{(3,35)}$=8.71, P<0.001), and in their ability to retrieve CocoPops® in the Corridor Test (Fig. 5.14B: Group, $F_{(3,35)}$=10.14, P<0.0001), and these rats also displayed pronounced and significant turning behaviour after systemic administration of amphetamine (Fig. 5.15B: Group, $F_{(3,35)}$=9.63, P<0.0001). Moreover, the impairment induced by this gene-environment lesion was significantly greater in all motor challenges than that induced by $AAV_{2/5}$-α-synuclein or rotenone alone (results of *post hoc* analyses shown in Fig. 5.13, Fig 5.14 and Fig. 5.15). This data confirms that striatal rotenone exposure of rats with a subclinical nigrostriatal burden of α-synuclein profoundly exacerbates the motor dysfunction caused by either the environmental or genetic insult alone.

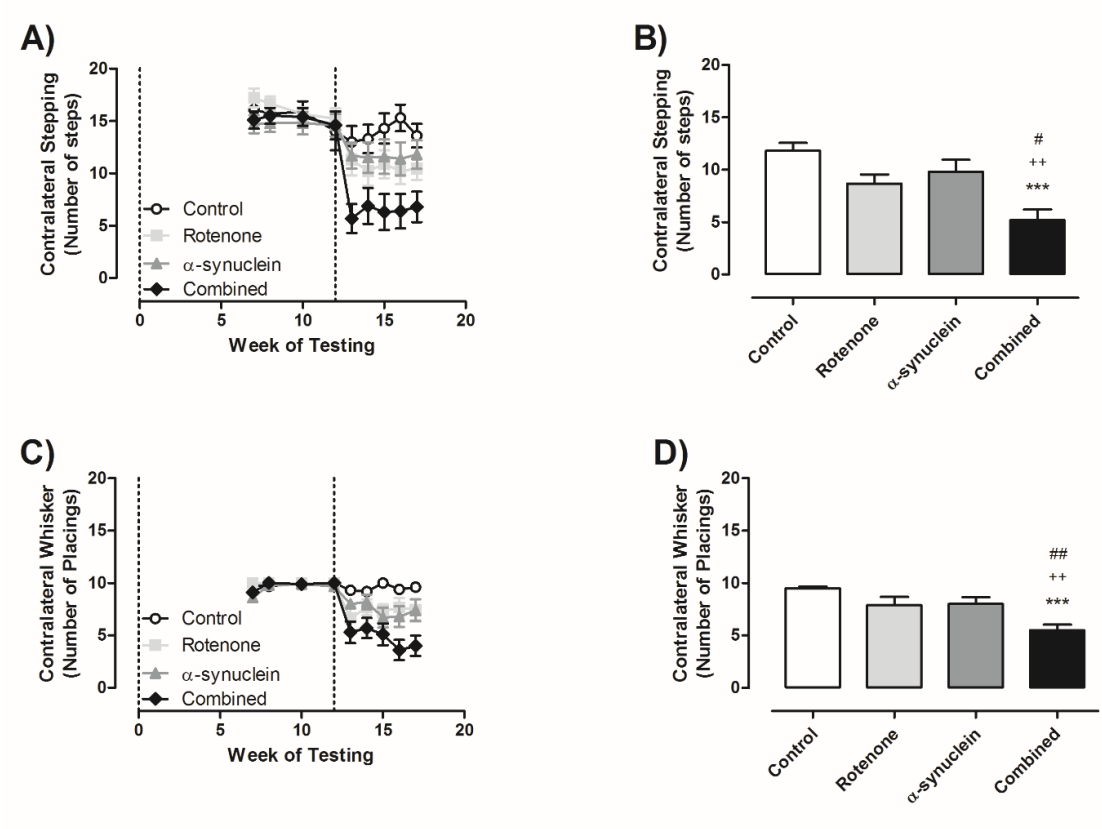


Fig. 5.13: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and intrastriatal rotenone results induces contralateral motor dysfunction in the Stepping and Whisker Tests. Unilateral administration of neither intranigral $AAV_{2/5}$-α-synuclein nor intrastriatal rotenone alone was sufficient to induce a significant contralateral motor dysfunction in any of the tests used. In contrast, when the Parkinsonian challenges were administered sequentially to the same animals, the rats developed a profound contralateral impairment in the Stepping (A and B) and Whisker (C and D) test. The XY plots on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data from the post-operative period only. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001 *vs.* control; ^{++}P<0.01 *vs.* α-synuclein; $^{\#\#}P$<0.01, $^{\#}P$<0.05 *vs.* rotenone by one way ANOVA with *post hoc* Newman-Keuls.

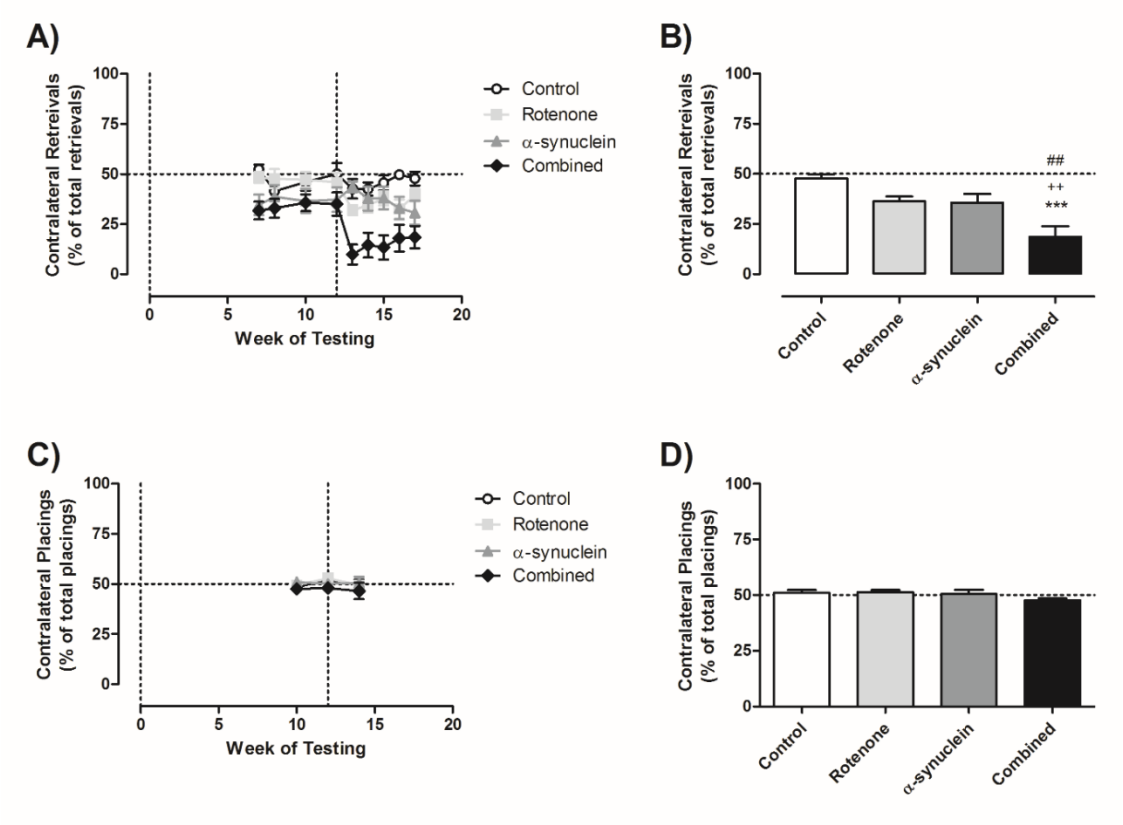


Fig. 5.14: Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and intrastriatal rotenone results induces contralateral motor dysfunction in the Corridor test but not in the Cylinder test. Unilateral administration of neither intra-nigral $AAV_{2/5}$-α-synuclein nor intra-striatal rotenone alone was sufficient to induce a significant contralateral motor dysfunction in any of the tests used. In contrast, when the Parkinsonian challenges were administered sequentially to the same animals, the rats developed a profound contralateral impairment in the Corridor test (A and B) but not in the Cylinder (C and D) test. The XY plots on the left depict the data collected over the course of the study, whereas the bar charts on the right represent collapsed data from the post-operative period only. Vertical dotted line indicates the day(s) of surgery. Data are shown as mean ± SEM. ***P<0.001 *vs*. control; ^{++}P<0.01 *vs*. α-synuclein; $^{\#\#}P$<0.01 *vs.* rotenone by one way ANOVA with *post hoc* Newman-Keuls.

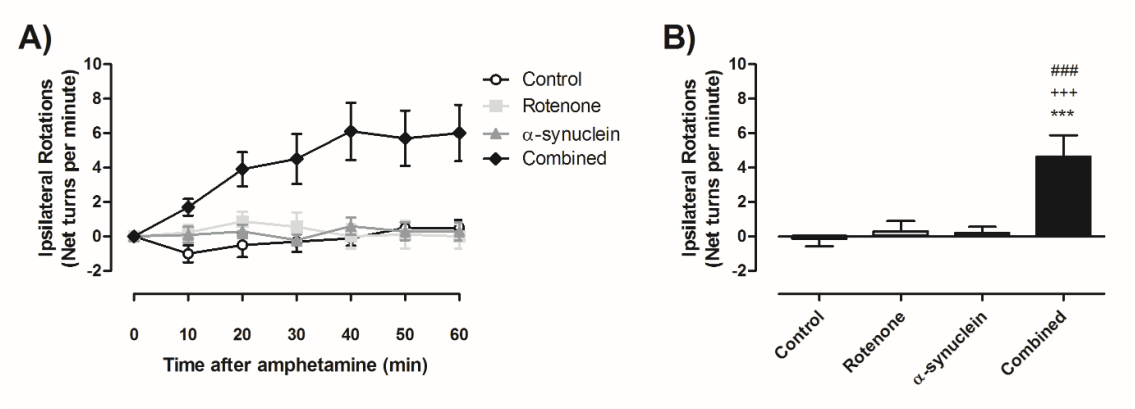


Fig 5.15: Sequential unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and intrastriatal rotenone is associated with amphetamine-induced rotational behaviour. Unilateral administration of neither intra-nigral $AAV_{2/5}$-α-synuclein nor intra-striatal rotenone alone was sufficient to induce significant ipsilateral turning. In contrast, when the challenges were administered sequentially, the rats developed profound ipsilateral turning behaviour. Data are shown are mean ± SEM. ***P<0.001 *vs*. control; ^{+++}P<0.001 *vs*. α-synuclein; $^{###}P$<0.001 *vs*. rotenone by one-way ANOVA and *post-hoc* Newman-Keuls.

5.4.3.4 Unilateral intranigral infusion of $AAV_{2/5}$-α-synuclein and/or intrastriatal rotenone induces significant nigrostriatal neurodegeneration

Central to the Parkinsonian neuropathology is degeneration of the dopaminergic neurons of the nigrostriatal pathway. Thus, in our *post mortem* analyses, we sought to determine the impact of the single or combined insults on the integrity of the nigrostriatal pathway using tyrosine hydroxylase immunohistochemistry (Fig. 5.16). At the levels of the terminals in the striatum, administration of either rotenone or $AAV_{2/5}$-α-synuclein resulted in significant loss of nigrostriatal terminals (Group, $F_{(3,35)}$=15.56; P<0.0001), but there was no additive effect of sequential exposure to both insults (results of *post hoc* analyses shown in Fig. 5.16B). At the levels of the cell bodies in the substantia nigra, the administration of either rotenone or $AAV_{2/5}$-α-synuclein resulted in significant loss of cell bodies (Group, $F_{(3,31)}$=18.24; P<0.0001; P<0.0001), and there was a pronounced additive effect of combining both insults (results of *post hoc* analyses shown in Fig. 5.16C).

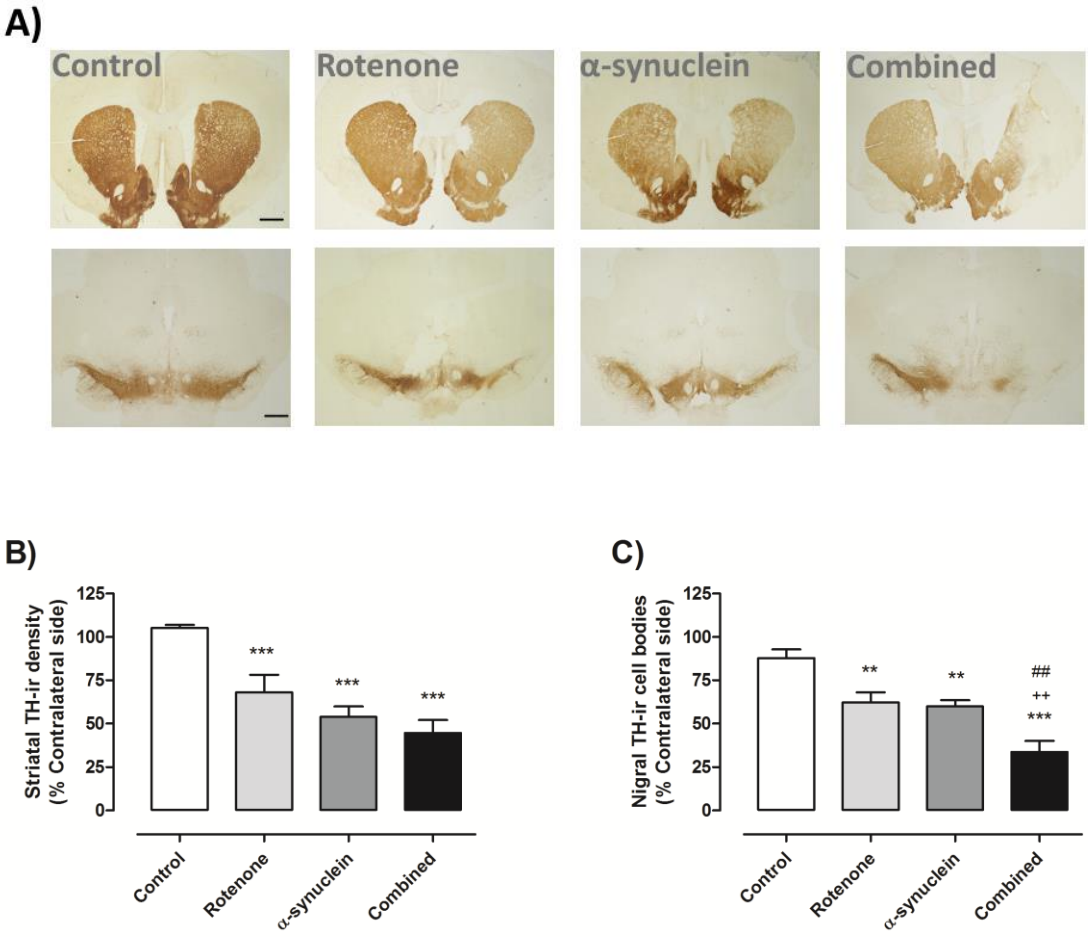


Fig. 5.16: Sequential unilateral administration of intra-nigral $AAV_{2/5}$-α-synuclein and/or intra-striatal rotenone induces significant nigrostriatal neurodegeneration. Administration of rotenone and/or $AAV_{2/5}$-α-synuclein resulted in significant nigrostriatal neurodegeneration which was evident at the level of the terminals in the striatum (B) and the cell bodies in the substantia nigra (C) with representative photomicrographs in (A). Note that the impact of the combined genetic and environmental insults was significantly greater than either insult alone at the level of the cell bodies in the substantial nigra. Data are shown ± SEM. ***P<0.001, **P<0.01 *vs*. control; $^{\#\#}P$<0.01 *vs*. rotenone; ^{++}P<0.01 *vs*. α-synuclein by one way ANOVA with *post hoc* Newman-Keuls. TH-ir – tyrosine hydroxylase immunoreactivity. Scale bar in striatal represents 3 mm and in nigral images represents 1 mm.

5.4.3.5 Sequential unilateral administration of intrastriatal rotenone does not affect $AAV_{2/5}$-α-synuclein induced α-synuclein expression

As expected, intranigral infusion of $AAV_{2/5}$-α-synuclein led to a significant expression of α-synuclein at the levels of the terminals of the striatum (Fig. 5.17: Striatum: Group, $F_{(3,35)} = 37.94$, $P<0.0001$) and the cell bodies of the substantia nigra (Cell bodies: Group, $F_{(3,33)} = 21.36$, $P<0.0001$). Subsequent administration of rotenone did not exacerbate the level of α-synuclein expression. This pathological expression of α-synuclein also induced the formation of dystrophic neurites in the nigrostriatal terminals of the striatum (Group, $F_{(3,32)} = 13.17$, $P<0.0001$), a well-established feature of the AAV-α-synuclein model (Fig 5.18). Subsequent administration of rotenone did not exacerbate the formation of these swollen varicosities in striatal terminals.

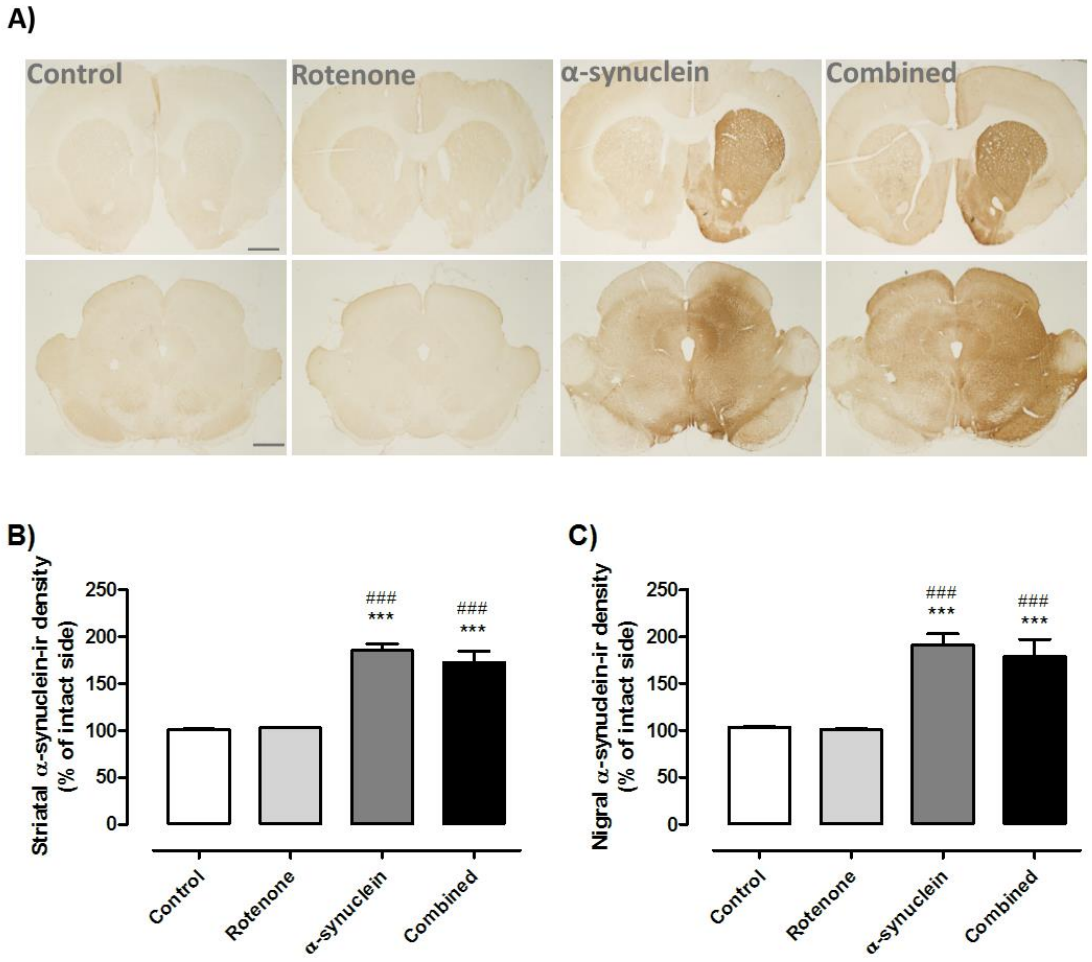


Fig 5.17: Sequential administration of $AAV_{2/5}$-α-synuclein and rotenone infusion did not affect $AAV_{2/5}$-α-synuclein induced α-synuclein expression. Unilateral administration of $AAV_{2/5}$-α-synuclein into the rat substantia nigra resulted in significant expression of α-synuclein in the nigrostriatal pathway ay the level of the terminals of the striatum (B) and the cell bodies of the substantia nigra (C). There was no significant difference in the level of α-synuclein upon subsequent administration of intrastriatal rotenone. Data is shown as mean ± SEM. ***P<0.001 *vs.* control; $^{###}P$<0.001 *vs.* rotenone by one-way ANOVA with *post-hoc* Newman-Keuls. α-synuclein-ir – α-synuclein immunoreactivity. Scale bar in striatal images represents 2 mm and in nigral images represents 1 mm.

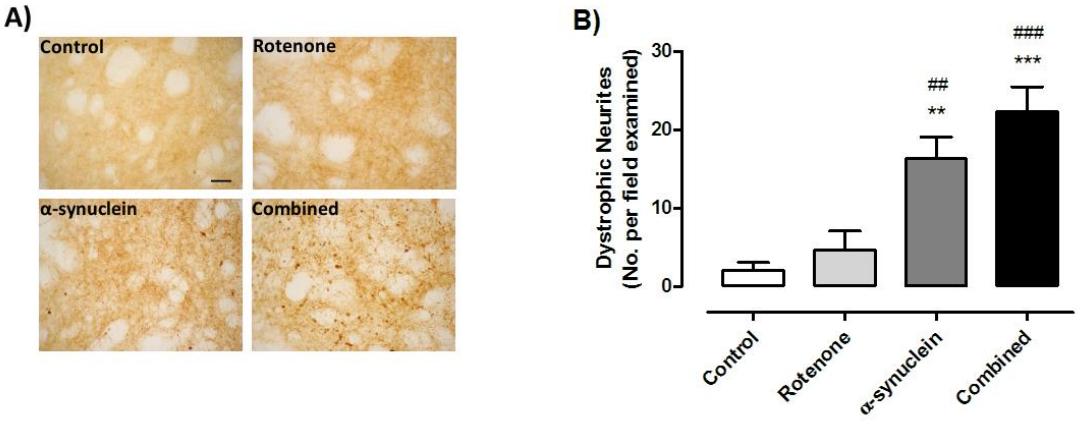


Fig 5.18: Sequential administration of $AAV_{2/5}$-α-synuclein and rotenone infusion does not exacerbate α-synuclein induced neuronal dystrophy. Immunohistochemical analysis for tyrosine hydroxylase revealed significant neuronal dystrophy in the ipsilateral striatum in animals infused with α-synuclein (A). Subsequent rotenone administration did not exacerbate this level of dystrophic neurite formation (B). Data is shown as mean ± SEM. ***P<0.001, **P<0.01 *vs.* control; $^{###}P$<0.001, $^{##}P$<0.01 *vs.* rotenone by one-way ANOVA with *post-hoc* Newman-Keuls. Scale bar in striatal images represents 0.2 mm.

5.5 Discussion

As indicated above, this chapter detailed the results of three separate studies which combined intra-nigral infusion of AAV-α-synuclein with intra-striatal infusion of rotenone. However each study differed with respect to the viral serotype, viral titre and/or rotenone injection regime which led to differences in the "clinical" manifestation (i.e. behavioral manifestation) of a motor disorder in this model. Studies 1 and 2 were performed in order to assess the behavioural impact of exposing rats with a clinical load of α-synuclein to a clinical dose of rotenone, but differed with respect to the viral serotypes used (Study 1 used $AAV_{2/5}$ while Study 2 used $AAV_{2/6}$). Study 3 was performed in order to investigate the impact of exposing rats with a subclinical load of α-synuclein (using $AAV_{2/5}$ vectors) to a subclinical dose of rotenone.

Unfortunately, for technical reasons, the tissue from Studies 1 & 2 was unable to be processed for immunohistochemical analyses and therefore it is difficult to draw any conclusions about α-synuclein-rotenone interactions in these studies except at a behavioural level. In both of these studies, there was some indication of an additive interaction between α-synuclein and rotenone in that the contralateral motor impairment (Stepping Test performance in Study 1 and Corridor Test performance in Study 2) induced by either toxin alone was enhanced when both were administered sequentially. Given the positive indications from these studies, we decided to proceed to a 3rd study from which we could harvest tissue for immunohistochemical analyses. Indeed, we found in Study 3, a robust interaction between intranigral α-synuclein and intrastriatal rotenone infusion at a behavioural level which was

underpinned by an additive effect of the two challenges at the level of the nigrostriatal cell bodies. Thus, Study 3 highlights that subclinical, or presymptomatic, α-synuclein-induced Parkinsonism can be precipitated into an overt motor syndrome by subsequent exposure to pesticides. This sheds further light on α-synuclein-rotenone interactions in the etiology of Parkinson's disease, and also provides a novel gene-environment model for testing potential therapies for this condition.

As previously mentioned, the role that the Parkinson's disease associated gene, *α-synuclein,* plays in both idiopathic and familial forms of the disease has been very well established. Viral vector mediated overexpression of this protein in animal models has now become one of the most etiologically relevant models of the disease. In Study 3, as expected, $AAV_{2/5}$-α-synuclein led to pronounced and widespread α-synuclein overexpression in the midbrain and striatum. This led to nigrostriatal dystrophy and partial loss of nigral cell bodies and terminals. Despite this α-synuclein pathology and consequent nigrostriatal degeneration, animals only developed a mild behavioural impairment which was not sufficient to produce a statistically significant overall impairment. This is not unlike the human condition where α-synuclein pathology and even Lewy body formation have been found in presymptomatic individuals without any evidence of a movement disorder (Mikolaenko et al., 2005, Markesbery et al., 2009), and this allowed us to assess the impact of subsequent rotenone exposure in this subclinical model.

Rotenone is an organic pesticide which has been causally linked to the development of Parkinson's disease and it is extracted from the roots of tropical plants belonging to the genera *Lonchocarpus* and *Derris* (Tanner et al., 2011). Rotenone is a highly lipid soluble compound and freely crosses cellular membranes where it causes mitochondrial dysfunction by inhibiting complex I of the electron transport chain (Degli Esposti, 1998). This leads to the production of reactive oxygen species, activation of apoptotic pathways and neuronal degeneration (Li et al., 2003). In Study 3, as expected, rotenone led to loss of nigrostriatal cell bodies and a reduction in terminal innervation density in the striatum. Despite this nigrostriatal neurodegeneration, animals only developed a mild behavioural impairment which was not sufficient to produce a statistically significant overall impairment in motor function in any of the motor rests used. Similarly to the α-synuclein-induced lesion, the lack of an overt rotenone-induced motor deficit allowed for assessment of the impact of subclinical rotenone exposure on rats with subclinical α-synuclein-induced Parkinsonism.

Surprisingly, relatively few researchers have focused on developing relevant gene-environment interaction animal models of Parkinson's disease even though this has been highlighted as being of crucial importance to the field (Horowitz and Greenamyre, 2010). In this regard, in the current studies, we investigated the effect of exposing nigrostriatal terminals with a presymptomatic burden of α-synuclein to rotenone. As outlined above, in the absence of tissue analysis, it is difficult to draw any conclusions about Studies 1 & 2. However, in Study 3, although rotenone and α-synuclein alone were subclinical and did not develop an overt motor disability, when

these toxins were sequentially administered, the rats developed pronounced impairment in their ability to make adjusting steps in the Stepping Test, in their capacity to make vibrissae-elicited forepaw placings in the Whisker Test, and in their ability to retrieve CocoPops® in the Corridor Test. Moreover, when the brains of the rats were assessed *post mortem*, we found that the subsequent exposure to rotenone exacerbated the α-synuclein-induced degeneration of the nigrostriatal neurons. This indicates that rats with a compromised nigrostriatal system due to pathological α-synuclein expression are highly sensitive to rotenone. This points to a pronounced and synergistic interaction between these two Parkinsonian insults which was more pronounced than we have observed previously by administering sequential intranigral $AAV_{2/5}$-α-synuclein and rotenone (Mulcahy et al., 2012) or by administering intranigral $AAV_{2/5}$-α-synuclein and systemic rotenone (Mulcahy et al., 2013).

Rotenone is well known to produce neuronal death through mitochondrial inhibition and subsequent production of reactive oxygen species (Degli Esposti, 1998). In contrast, the mechanism of α-synuclein-induced neurodegeneration has still not been fully elucidated but it is thought that it may result from the detrimental effects of insoluble α-synuclein fibrils on membrane structure and permeability. Although the interaction between these two Parkinson's disease risk factors has been largely neglected *in vivo*, several *in vitro* studies point to potential mechanisms which may underlie their synergistic effects in the current study. For example, previous studies have shown that rotenone enhances α-synuclein production and/or oligomerisation in cellular systems (Dokleja et al., 2014, Chorfa et al., 2014, Yuan et al., 2015, Sala et

al., 2013, Ma et al., 2013) and that it can also reduce autophagy which is required for destruction of aggregated forms of α-synuclein (Yuan et al., 2015).

Thus, the 3rd study described in this chapter has revealed that rats with a subclinical nigrostriatal burden of α-synuclein that were subsequently exposed to rotenone, develop a profound and progressive motor decline which is underpinned by dystrophy and degeneration of the nigrostriatal pathway. Moreover, the level of motor impairment and nigrostriatal neuropathology induced by dual exposure to these Parkinsonian risk factors was significantly greater than that induced by either alone which point to a synergistic interaction between these genetic and environmental factors. This indicates that presymptomatic α-synuclein-induced Parkinsonism can be precipitated into an overt motor syndrome by subsequent exposure to pesticides. Further studies using this approach could shed further light on α-synuclein-rotenone interactions in the etiology of Parkinson's disease, and could also provide a novel and relevant model for testing potential neuroprotective and disease-modifying therapies for Parkinson's disease.

Chapter 6: General Discussion

The work presented in this thesis sought to develop and characterise animal models of Parkinson's disease. We were particularly focused on developing a novel model of the disease by using the Parkinson's disease associated protein, α-synuclein, and subsequently exposing α-synuclein-overexpressing animals to environmental risk factors. The main findings from this body of work are: 1) that a differential pattern of motor dysfunction is induced by unilateral intrastriatal infusion of the bacterial inflammagen, LPS, and the agritoxin, rotenone, when compared to the 'gold standard' model of the disease, the 6-OHDA model, despite similar levels of nigrostriatal neurodegeneration (Naughton et al., 2016a); 2) that the sequential exposure of α-synuclein and LPS does not precipitate a profound motor dysfunction which is in agreement with the findings that dual exposure to these genetic and neuroinflammatory challenges does not drive further nigrostriatal neurodegeneration; and 3) that subclinical α-synuclein pathology can be precipitated into overt motor dysfunction by exposing these animals to a subclinical dose of rotenone, and this motor dysfunction is preceded by significantly enhanced degeneration the nigrostriatal pathway (Naughton et al., 2016b, submitted). Thus, we have shown that exposing striatal terminals overexpressing α-synuclein to rotenone can reliably model the main pathological features and motor dysfunction associated with the clinical condition.

Despite decades of research and several seminal discoveries in pharmacotherapy, levodopa has remained the mainstay of treatment for the motor symptoms of

Parkinson's disease. However, this, and related dopaminergic pharmacological interventions, do not target the underlying neuropathology which drives disease progression. One of the reasons proposed for the paucity for effective therapeutic interventions is the lack of etiologically relevant animal models of the disease. Due to the ever increasing longevity of our population, the incident rates of Parkinson's disease are also increasing with an anticipated rise in patients presenting at clinics from 4.1 million to 9.3 million people by 2030 (Dorsey et al., 2007). The lack of a viable treatment option for patients has resulted in a considerable drive to identify novel strategies to address the underlying degeneration in an attempt to halt disease progression. Thus, there is an urgent need to develop better animal models of Parkinson's disease in order to allow for improved prediction of the potential clinical efficacy of emerging therapies. With this in mind, the experiments described in Chapters 3-5 attempted to characterise animal models of Parkinson's disease, namely the newer emerging LPS and rotenone models (Chapter 3), and then to combine these environmental toxins with the Parkinson's disease associated protein, α-synuclein, in an attempt to incorporate the genetic links to the disease (Chapters 4 and 5). Over the course of the studies described, we attempted to generate a novel gene-environment interaction model of Parkinson's disease using AAV-α-synuclein in combination with LPS or rotenone. These approaches and a summary of their associated features are presented in Table 6.1. This demonstrates that the final approach taken in this thesis, namely, sequential intra-nigral infusion of AAV-α-synuclein and intra-striatal rotenone was capable of modelling the classic triad of features associated with human Parkinson's disease, namely, motor dysfunction, nigrostriatal neurodegeneration and α-synucleinopathy.

Approach attempted	Motor dysfunction	Nigrostriatal degeneration	α-Synuclein expression	Chapter/Reference
6-OHDA	Yes	Yes	No	Chapter 3 (Naughton et al., 2016a),
LPS	Yes	Yes	No	Chapter 3 (Naughton et al., 2016a),
Rotenone	Yes	Yes	No	Chapter 3 (Naughton et al., 2016a)
α-Synuclein & LPS	No additive effect	No additive effect	Yes	Chapter 4
α-Synuclein & Rotenone	Pronounced additive effect	Additive effect	Yes	Study 3, Chapter 5 (Naughton et al., 2016b, submitted)

Table 6.1: A general overview of the approaches to modelling Parkinson's disease used in this thesis and their associated key features. In all studies, the neurotoxins were administered to the nigrostriatal terminals at the level of the striatum, while α-synuclein was delivered to the cell bodies in the substantia nigra using AAV vectors.

The first approach we used (Chapter 3) was to comparatively assess the neurotoxic, inflammatory and environmental models of Parkinson's disease. This was achieved by stereotaxically infusing 6-OHDA, LPS and rotenone at four sites along the rostro-caudal axis of the striatum using coordinates established for the Parkinsonian neurotoxin 6-OHDA (Kirik et al., 1998). In this study, we demonstrated the distinct behavioural patterns induced by administration of each of these neurotoxins despite similar levels of neurodegeneration across all groups (Naughton et al., 2016a). The four site intrastriatal rotenone and LPS models of the disease compare favourably with the more established 6-OHDA model in so far as they each cause similar levels

of nigrostriatal neurodegeneration and motor dysfunction albeit without any α-synuclein expression. Moreover, the rotenone model confers a distinct advantage over the 6-OHDA model due to the fact that rotenone has been causally linked to Parkinson's disease. Similarly, neuroinflammation which is mimicked by LPS, has also been directly associated to disease pathogenesis (Niehaus and Lange, 2003). However, these unitary models are not without their limitations; specifically, 1) their acute degeneration process, 2) the fact that each of these models is induced by a single insult, 3) there is no evidence of a progressive motor dysfunction, and finally, 4) that there is no associated α-synuclein pathology. To overcome these limitations, we incorporated the use of AAV vectors overexpression normal wildtype α-synuclein in all of the remaining studies.

The most important question addressed in this body of research was whether combining relevant genetic and environmental Parkinson's disease risk factors which are known to be associated with the human condition would produce a more robust and reliable animal model. There is a wealth of evidence which supports this multi-hit approach as Parkinson's disease is now widely accepted to arise as a result of complex interactions between genetics and exposure to exogenous factors. Moreover, it has been previously established that the nigrostriatal pathway is readily able to compensate for the effects of exposure to a unitary environmental or genetic factor acting alone (Agid et al., 1973, Anglade et al., 1995, Zigmond et al., 1990c). Therefore, exposing the circuitry of the nigrostriatal pathway to multiple factors may result in sustained or continuing degeneration which is more reflective of the progressive nature of the disease. Numerous studies to date have shown that viral

vector mediated overexpression of the protein, α-synuclein, when delivered directly to the substantia nigra, results in robust α-synuclein pathology in the nigrostriatal system causing degeneration and dystrophy of nigrostriatal dopaminergic neurons (Kirik et al., 2002, Kirik et al., 2003, Chung et al., 2009, Ulusoy et al., 2010, Decressac et al., 2012b). However, whilst this model is the most etiologically relevant model of the disease, it is associated with a high degree of variability with respect to the neurodegeneration and the behavioural profile it induces. Typically, only 25-30% of animals infused with AAV-α-synuclein develop motor deficits underpinned by significant neurodegeneration (Kirik et al., 2002). Indeed this may somewhat reflect the clinical situation there α-synuclein pathology, and even Lewy body formation, is not in itself sufficient to induce an overtly manifest clinical syndrome with both having being identified in the normal aged human brain (Mikolaenko et al., 2005, Markesbery et al., 2009). However, in the Markesbery *et al*. (2009) study, the "normal" patients with α-synuclein pathology were suggested to represent a population with preclinical or presymptomatic Parkinsonism. Given that Parkinson's disease is thought to emerge after interaction between genetic and environmental risk factors, it follows that sufficient exposure of such presymptomatic individuals to environmental risk factors (or conversely, insufficient exposure to environmental protective factors) could result in clinically manifest Parkinson's disease. Therefore, from a preclinical perspective, we hypothesised that exposing rats with AAV-α-synuclein-induced pathology to a second Parkinson's disease-associated insult could produce a more robust and reliable model.

One feature that has become well established as a driving factor in the pathogenesis of Parkinson's disease is the contribution of neuroinflammation to the degenerative process. Indeed, it is believed that ongoing active neurodegeneration (Tansey and Goldberg, 2010, Tzeng et al., 2005) harnesses a persistent neuroinflammatory response establishing a chronic and self-sustaining cycle of neurodegeneration and neuroinflammation (Banati et al., 1998, Bronstein et al., 1995, McGeer and McGeer, 1998). Therefore, with this in mind, our next approach was to expose striatal terminals, with a high α-synuclein burden to the bacterial inflammagen, LPS. Interestingly, despite the fact that there was a significant contralateral motor dysfunction when LPS and α-synuclein were administered in their own right, there was no additive effect of combining these Parkinsonian challenges. This was also reflected in the *post-mortem* analysis where sequential exposure to α-synuclein and LPS did not drive further nigrostriatal neurodegeneration. Strikingly, we did observe an intriguing result whereby LPS significantly increased the expression of the α-synuclein protein in both the levels of the terminals in the striatum and the cell bodies of the substantia nigra. Although reasons for this are not known it is becoming increasingly apparent that a complex relationship exists between α-synuclein expression and microglia, and the distinct profiles which they harness in response to α-synuclein overexpression (Roodveldt et al., 2013, Sanchez-Guajardo et al., 2010). Bearing the limitations observed in this dual exposure model in mind, we opted to focus subsequent studies on investigating the impact of exposing rats already exhibiting α-synuclein pathology to the agritoxin, rotenone.

Although decades of research have been conducted into investigating the environmental etiology of Parkinson's disease, it is only very recently that rotenone, a potent complex I inhibitor, was confirmed to be causally linked with the disease (Tanner et al., 2011). Prior to this discovery, the increased risk associated with exposure to this agritoxin was predicted through numerous preclinical studies demonstrating that exposure to rotenone induced a relatively selective degeneration of the nigrostriatal pathway in animal models (Greenamyre et al., 2003, Betarbet et al., 2000, Fleming et al., 2004b, Ferrante et al., 1997). However, these studies yielded models which were not without their limitations, with the primary concern being the toxicity of systemic rotenone and the lack of α-synuclein pathology. Therefore, in an attempt to address these limitations, and building on previous studies conducted within our laboratory (Mulcahy et al., 2012, Mulcahy et al., 2013, Naughton et al., 2016b, submitted), in Chapter 5, we sought to determine the effect of exposing rats with a high nigrostriatal α-synuclein burden to direct-intracerebral delivery of rotenone. The most important findings from this chapter emerged from Study 3 (for which we were able to complete immunohistochemical analyses). This study showed that, intriguingly, despite the fact that subclinical doses of α-synuclein and rotenone were not sufficient to induce an overall motor dysfunction in their own right, when delivered in combination with each other, they precipitated a profound impairment in the rats' ability to perform motor tasks which was underpinned by a significantly enhanced nigrostriatal neurodegeneration. Thus, this sequential intranigral AAV-α-synuclein and intrastriatal rotenone model was the only model assessed in this thesis that displayed the triad of classic features (that is, motor dysfunction, nigrostriatal neurodegeneration and α-synucleinopathy) associated with

Parkinson's disease (Naughton et al., 2016b, submitted). The behavioural and neuropathological effects we observed by exposing striatal terminals, with a high burden of α-synuclein, to a rotenone challenge were more pronounced than we have previously observed with; 1) sequential intranigral AAV-α-synuclein and rotenone and 2) intranigral AAV-α-synuclein and systemic rotenone (Mulcahy et al., 2012, Mulcahy et al., 2013).

It is clear from the literature that relatively few researchers have focused on developing gene-environment interaction models even though this has been highlighted as being of crucial importance to the field (Horowitz and Greenamyre, 2010). Further development of gene-environment interaction models of Parkinson's disease, such as the one developed in the present thesis, would undoubtedly be useful to preclinical researchers investigating the etiology and pathogenesis of Parkinson's disease. Furthermore, as Parkinson's disease therapeutics has been greatly hampered by the lack of relevant animal models of the disease, such multi-hit models may provide a better platform to investigate any pioneering therapeutics which could potentially modify the disease pathology.

The future of Parkinson's disease modelling

The studies incorporated into this body of work have developed a novel rotenone and α-synuclein model of Parkinson's disease whereby the combination of subclinical doses of both of these toxins precipitated an overt and profound motor dysfunction underpinned by significant neurodegeneration. However, it has become increasingly apparent that Parkinson's disease is a multifactorial disease and not exclusive to

nigrostriatal neurodegeneration and motor dysfunction. Therefore, it is important that the pathological role of non-dopaminergic systems and the human burden of non-motor features should not be overlooked. In this context, for truly relevant models of the disease, these factors need to be considered, and in doing so, they may provide us with a greater understanding of the multi-system effects which all interact and contribute to the progression of this disabling condition.

Concluding remarks

There are few areas of Parkinson's disease research that elicit as much controversy as animal models for the disease. Animal models of the disease are not only required in order to gain further insight into disease etiopathogenesis, but also to identify potential therapeutic targets and to screen and/or test neuroprotective treatments. Prior to clinical trials, it is crucial to have relevant animal models of the clinical condition which exhibit easily measurable responses that reflect the desired outcome for patients. Opinions are diverging on what constitutes an ideal animal model of Parkinson's disease, and particularly, what aspects of the disease should a model reproduce in order to be considered a valuable model. Currently, the answer to that question is that different models are useful for different purposes (Litvan et al., 2007). Although, it is easy to concede that the paucity of animal models is the reason for the paucity of novel neuroprotective therapies for Parkinson's disease, etiology is also a compounding factor. As the etiology of the disease (sporadic vs. familial; environmental vs. genetic) differs significantly from patient to patient, adapting different etiological triggers into preclinical trials may help to improve the predictive validity of such trials.

The undeniable link between genetics and exposure to environmental toxins should ideally drive the field of research towards the generation of more etiologically relevant gene-environment interaction models as they hold great potential in reproducing features of the clinical condition. Moreover, consistent and practical approaches in the study of gene-environment interactions may provide a better estimate of the relevant risk to genetically-susceptible individuals upon their exposure to environmental factors. To date, current research investigating potential novel pharmacologically effective treatments for the disease may have been largely hampered by the lack of incorporation of a gene-environment interaction paradigm in preclinical studies. However, until the approach of generating and refining combined animal models of Parkinson's disease is unified, recognising the true potential of any disease-modifying therapies remains limited. Nevertheless, if these preclinical challenges can be overcome, these combined models may provide the platform for screening any novel potentially disease-modifying pharmacotherapies in the treatment of Parkinson's disease.

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Appendix 1.1: SOP for the Production, Purification and Titration for AAV

Growing and amplification of colonies

1. From frozen glycerol stocks, scrape the frozen surface with a sterile inoculating loop, and then immediately streak the bacteria that adhere to the loop onto the surface of an LB agar plate containing the appropriate antibiotics. Return the glycerol stocks to -80°C and incubate the plate overnight at 37°C upside down.

2. Pick 4-8 colonies and place into 4-8mls of LB medium in sterile tubes. Label each tube with appropriate colony number and grow overnight at 37°C with continuous shaking.

3. Next door pour 1.5mls of culture into an eppendorf and spin @ 12,000 rpm at 4°C for 1 minute. Store the remainder at 4°C.

4. Remove the entire medium, leaving the pellet as dry as possible

Appendix 1.2: Determining Plasmid Concentration by Spectrophotometry

1. Using the nanodrop systems, equilibrate and zero machine by 1µl of water
2. Place 1µl of AAV plasmid on nanodrop and read at 260: 280nm. Take the 260 reading and record the calculated reading.
3. Place 1µl of pTRUF plasmid on nanodrop and read at 260: 280nm. Take the 260 reading and record the calculated reading
4. Example: Nanodrop reading gives 133.5ng/ µl
5. On normal spec: OD @ 260 X Dilution factor X 50 = µg/ml

Appendix 1.3: General instructions for AAV work

Splitting of cells (factories)

- Add 250 ml PBS to an aspirator bottle.
- Remove old medium from cell factory.
- Rinse with 250 ml PBS and drain immediately.
- Add 250 ml Trypsin/EDTA and incubate for 1.5 min.
- Shake CF with flat motion and place cells in a bottle with 350 ml medium.
- Rinse CF with 350 ml medium and combine cells into above bottle.
- Add medium to a total volume cell of 1000 ml.
- CF old (transfection): add 400 ml cells + 600 ml medium
- CF (new): add 150 ml cells + 1170 ml medium

Thawing: thaw cells in 37°C. Stop as soon as it is completely thawed. Add to flask with 25-30 ml medium. 293FL (Florida): rack 5, box 2

Flasks: 25-30 ml medium. Splitting ratio 1:3. Wash cells with 10 ml PBS and split with 3 ml trypsin. Add 7-8 ml medium and aliquot to new flasks. About 10 flasks to 1 factory.

Transfection of a cell factory

Cells should be 70-80% confluent at the time of transfection.

MilliQ-H2O should be at room temperature.

Equilibrate the 2xHBS solution and the medium to 37°C.

1l complete 293-cell medium (for 1 cell factory).

Do not freeze CaCl2 -> prepare freshly [2.5M] (3.68g CaCl2 dissolved in autoclaved millipore water to a total volume of 10 ml, sterile filtered)

DNA: Either endofree purified or CsCl purified (use buthanol instead of phenol)
Use a total of 2.5 mg of DNA with equimolar amounts of helper and vector DNA.

Add the following components in a 250ml conical in order:

1. plasmids
2. Autoclaved MilliQ-H2O or endotoxin free water for cell biology (calculates the volumes of the other components and adds water to a final volume of 52ml)
3. CaCl2 (5,2ml)

Mix thoroughly with 52 ml 2xHBS by swirling and inverting.

Wait 45 sec, solution should get a "milky" white colour.
Stop reaction by adding it to 1l medium.

Pour off medium and add transfection mix (do not wash the cells).

Harvesting

Harvest the cells 3 days (~ 72h) after transfection.

Pour off medium.

Rinse with 500 ml PBS /5 mM EDTA to detach cells (shake with flat motion) and pour it into a 500ml conical.

Rinse with 500 ml plain PBS and pour it into a 500ml conical.

Centrifuge 1000*g, 15 min, 4°C. Pour off supernatant.

Dissolve pellets in 60 ml Lysis buffer (50mM Tris pH 8.4, 150mM NaCl).

Pool in one of the 500 ml conicals. Freeze 15 min in dry ice/ethanol bath.

Thaw 35 min, 37°C, and mix vigorously (by shaking). DO NOT VORTEX.

Freeze 15 min in dry ice/ethanol bath. Store frozen in -20°C freezer.

Benzonase

Thaw lysed cells (in 37°C) and add 1250 units Benzonase + 60 µl 1M MgCl2

Incubate 37°C, 30 min.

Transfer lysate to 2x50 ml conicals.

Centrifuge 1800*g for 20 min.

Iodixanol (IOD) gradient

Add 15 ml lysate supernatant from above centrifugation to each of 4 centrifugation tubes using syringes with needles.

Set up the pump by connecting glass capillaries to the tubings on each side of the pump.

Add 30 ml of 15% IOD to a 50 ml tube and place the capillaries into the tube.

Start pumping the IOD in order to completely fill the system with IOD and avoid bubbles.
Place capillary tubes into each centrifuge tube so that they reach the bottom.

Adjust the 15% IOD volume to 27.5 ml in the 50 ml tube (2.5 ml volume is already in the pump tubing).

Run 15% IOD (pump set to 37 rpm) until near the bottom of 50 ml conical.

Add 20 ml of 25% IOD to the 50 ml conical.

Run 25% IOD until near the bottom of 50 ml conical.

Add 30 ml of 40% IOD to the 50 ml conical.

Run 40% IOD until near the bottom of 50 ml conical.

Add 20 ml of 60% IOD to the 50 ml conical.

Run 60% IOD until near the bottom of 50 ml conical.

Remove the capillaries from the centrifuge tubes and run sterile dH2O through the pump (IOD will crystallize if not removed from the pump tubing!)

Use a 2 ml or 5ml syringe fitted with a needle (pink) to top off the centrifuge tubes with Lysis buffer.

Seal tubes with a heat sealer and gently squeeze centrifuge tubes to check for heat seal failure.

Place tubes into 70Ti Beckman rotor, add red caps on top of each centrifuge tube and properly seal the rotor.

Centrifuge at 69 000 rpm (~350.000g), 1.5 h, 18°C.
Use slow acceleration and deceleration. Place centrifuge tube in holder.

Clean with alcohol on top and bottom.

Use needle (pink) and tissue to prick hole in top.

Use 10 ml syringe with needle (pink) to poke the centrifuge tube in the middle of the 60% phase.

Pull ≈7ml from the centrifuge tube and repeat for all 4 tubes. Put in 250 ml conical.

Add 32 ml FPLC start buffer and store in fridge.

Concentration

Materials and Solutions:

- Concentrator (Millipore Amicon Ultra 100kDa MWCO)

- DPBS

1. Rinse concentrator with 10 ml PBS+Ca+Mg and centrifuge at 1500 g, 2 min at 18°C.

2. Add eluted virus suspension to the concentrator.

3. Centrifuge at 1500 g, 18°C, for 2-3 min until the retained volume is about 250 µl.

5. Add 10 ml of PBS+Ca+Mg on top of the virus suspension.

6. Centrifuge at 1500 g, 18°C, in intervals of 1-3 min until the retained volume is about 250 µl.

7. Repeat steps 4+5.

8. Pipet the concentrated virus solution into a glass. Use 100 µl of PBS+Ca+Mg to wash the concentrator and add to the vial. Mix and store in –80°C freezer as 50 µl aliquots in glass vials.

DNA Miniprep (Following Sambrook et al Book)

1. Resuspend the pellet in 200µl of ice cold solution I by vortexing. Ensure the pellet is completely dispersed in sol I.

2. Then add 400µl of freshly prepared Solution II. Close the tube and invert the tubes rapidly 5 times. Store the tube on ice.

3. Add 300µl of ice-cold solution III. Close the tubes and vortex it gently in an inverted position for 10 seconds to disperse sol III through the viscous bacterial lysate. Store on ice for 5mins.

4. Centrifuge at 12000g for 5mins at 4°C in a microfuge. Transfer the supernatant to a fresh tube.

5. Add an equal volume of phenol:chloroform and mix by vortexing. Centrifuge at 12,000g for 2mins at 4°C in a microfuge and transfer the supernatant to a fresh tube.
6. Precipitate the double-stranded DNA with 2 volumes of ethanol at room temperature. Mix by vortexing and allow the mixture to stand for 2 mins at RT.

7. Centrifuge at 12,000g for 5mins at 4°C in a microfuge.

8. Remove the supernatant by gentle aspiration. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Remove any drops of fluid adhering to the walls of the tube.

9. Rinse the pellet of double stranded DNA with 1ml of 70% ethanol at 4°C and remove the supernatant as described previously. Allow the pellet of nucleic acid to dry in te air for 10mins.
10. Re-dissolve the nucleic acids in 50µl of TE (pH8.0) and RNAse at 20ng/ml. Vortex briefly, and store at -20°C.

Further amplification of appropriate colonies

1. Once the miniprep digest confirms which colonies are correct place the remainder of the initial 5ml culture (left at 4°C) into a large 1L LB (+ antibiotics)*** conical flask, sealed with cotton wool.

2. Leave overnight at 37°C with continuous shaking.

1.5 Maxiprep (Using a Jetstar 2.0 Giga or Qiagen)

[SIDE NOTE: Prior to spinning take 1ml aliquot of the culture for glycerol stocks- 850µl of culture with 150µl of sterile glycerol and label and store at -70°C.]

1. Place the culture into 500ml centrifuge tubes and spin down at 4000g. Remove all traces of medium carefully.

2. **Cell Resuspension**: Add 125ml of buffer E1 to the pellet and resuspend the cells until the suspension is homogeneous. No cell clumps must be visible.

3. **Cell lysis:** Lyse the bacterial cells by adding 125ml of buffer E2. Mix gently but thoroughly until a homogeneous lysate is obtained. This will be very viscous. **Do not vortex** and incubate at room temperature for 5mins.

4. **Neutralization:** Neutralise the lysis mix from above with 125ml of buffer E3. Mix gently but thoroughly until a homogeneous mixture is obtained. **DO NOT VORTEX**. The liquid must be completely thin-bodies with no viscous solution left.

5. Centrifuge the mixture for 30min at 12,000g at room temperature. The supernatant should be clear after centrifugation and collect the supernatant in an **autoclaved clean bottle.**

6. **Equilibration:** Screw the GIGA cartridge onto a 1 litre laboratory bottle with 45mm neck (Duran) and fill 200ml of equilibration buffer E4. Apply the vacuum to the cartridge through the side-arm with tubing-connector and suck through the complete amount of liquid. Keep the vacuum on until all liquid has drained from the resin. Discard the flow through.

7. **Loading the lysate:** Fill the cleared lysate from above into the cartridge with the equilibrated Jetstar 2.0 resin (may need to place gauze over the cartridge and pour the lysate through this to collect extra debris). Apply the vacuum to the cartridge through the sidearm with tubing-connector. Keep te vacuum on until all of the lysate has passed through the resin.

8. **Wash 1:** Fill 300ml of buffer E5 into the cartridge and apply vacuum to the cartridge through the side-arm with tubing connector. Keep the vacuum on until all liquid has drained from the resin.

9. **Wash 2:** Repeat step 7 once with 300ml of buffer E5.

10. Plasmid Elution: take off the filter cartridge from the 1 litre bottle and screw it onto a clean, **sterile 200ml Duran autoclaved bottle** with 45mm neck.

11. Apply 100ml of elution buffer E6 into the cartridge. Apply a soft vacuum to the cartridge through the side-arm with tubing-connector until approx. 30-40ml of buffer E6 have elutes from the cartridge. Release the vacuum from the cartridge, so that no further liquid is pulled through the resin. **Let stand for 1min without agitation**. Then switch on the vacuum again

and draw the remaining liquid from the resin into the receiver bottle. Keep the vacuum on until all liquid has drained from the resin.

12. **Plasmid Precipitation:** Precipitate the DNA with 0.7 volumes of isopropanol. Transfer everything to a tube you can spin down in. Centrifuge at 4°C and at 15000g for 40mins.

13. Wash the precipitated DNA with 10ml of 70-80% ethanol per tube and re-centrifuge for 5 min. Air dry the pellet for 10min and re-dissolve it in a suitable volume of buffer. 10 or 20mls of TE or dH2O (sterile) is suitable. Leave at 4°C overnight to allow the DNA dissolve throughout the solution. Then freeze at - 20°C.

1.8: final plasmid volume required for 20 Plate Transfection:

From example above for 500µg (10 plate) transfection:
pTRUF = 604.3µl
AAV5 = 2697.94 µl

But for a 1000µg (20 plate) transfection:
pTruf = 1208.6µl
AAV5 = 5395.88 µl

1.9 Cell transfection

1. Thaw plasmids first thing in the am and allow warming up:
   Prewarm to 37°C:
   2.5M CaCl2
   2X HBS
   Complete DMEM media with 5% FCS and Pen-Strep and L-Glut.

2. In a tube labelled 1, for a 20 plate transfection add:
   2.5ml $CaCl_2$
   1.2086ml of pTRUF
   5.39588ml of AAV5
   Make the remainder up with water up to 25mls

3. In a tube labelled 2 add: 25ml of 2X HBS pH7.05 (NB).

4. Place tube 1 on holder and gently bubble it slowly using pipette and pipette aid. Then drop-wise add the contents of Tube 2 into tube 1. Once all has been added in allow the mixed solution to settle for 10mins.

5. In a tissue culture flask add 400ml of pre-heated DMEM media and then add the 50ml mixture.

6. Aspirate off media of the 20 plates and place 22mls of the solution onto each plate. Be careful not to wash of the cells from the plate-do it very slowly.

7. Incubate for 48hrs at 37°C at 5% CO2.

8. 48hrs after transfection aspirate all but 3-5mls from each plate, and then scrape the cells off using a cell scraper and transfer the media/cells into two 50mls falcon tubes. Wash the plates with a total of 10-15 ml complete DMEM or PBS (start with plate no 1 and transfer as you go along).

9. Spin at 2500rpm for 10mins at RT and aspirate supernatant.

10. Cell pellets are then taken up and lysed in a total of 15ml Lysis buffer per 10 plates.

11. Freeze/thaw three times in dry ice/ethanol bath and 37°C water bath.

1.10 AAV purification by iodixanol density gradient and heparin /Q Sepharose affinity chromatography.

1. Benzonase is then added to the lysate at a final concentration of 50U/ml. and is incubated at 37°C for 30mins.

2. The crude lysate is clarified by centrifugation at 4000g for 20min and the vector containing supernatant is stored until the iodixanol gradients are prepared

3. For the iodixanol gradients use quick seal tubes by underlaying and displacing the less dense cell lysate.

4. The iodixanol solutions are layered very carefully in the following order using a Pasteur pipette:
   - Your virus solution (15ml) - for 10 plates
   - 6ml of 15% iodixanol/1M NaCl in PBS-MK buffer
   - 4ml of 25% iodixanol in PBS-MK buffer containing Phenol Red
   - 3ml of 40% iodixanol in PBS-MK buffer
   - 2ml of 60% iodixanol (neat) containing Phenol Red

5. The tube is then filled up to the bottom of the neck of the tube with lysis buffer using either a pasteur pipette or a syringe with a small gauge needle.

6. The tubes are then sealed using rubber caps and then squeezed tightly to determine that there was no leakage.

7. Centrifuge in a Type 70Ti rotor (Beckman) at 69,000RPM for 1 Hour at 18°C.

8. After centrifugation the tubes are clamped in a retort stand and an 18-19 inch gauge needle was inserted into the interface between the 60 and 40% iodixanol steps (clear step) and then removed. A new needle was then inserted (bevel side up) attached to a 5ml syringe. Remove the cap and the 4mls of this fraction removed.

Equilibration of the Heparin column:

Heparin columns are good for AAV2 and AAV3. (Heparin: cat no: 17-0406-01) Q Sepharose are good for everything. (Q Speharose: cat no: 17-1153-01) Both from Amersham.

Heparin-1ml column for 10 plates:

1. Apply 20 ml of PBS-MK to the column.
2. Apply 3-4mls of virus from previous step.
3. Allow to drip through under gravity and collect eluent.
4. Apply this again.
5. Wash with 20mls PBS-MK.
6. Elute with 7mls PBS-MK-NaCl.

Q-Sepharose (1ml column for 10 plates):

1. Add 5ml of Buffer A (5ml/min)
2. Add 5ml of Buffer B.
3. Add 5ml of Buffer A.
4. Add 3-4mls of the virus collected from previous step, diluted with 1:1 of Buffer A.
5. Wash with 10mls of Buffer A.
6. Elute with 7mls of Buffer B.

Buffer A: 20mM Tris, 15mM NaCl pH8.5)
Buffer B: 20mM Tris, 500mM NaCl pH8.5)

1.11: Concentration and Desalting of rAAV preparations

1. AAV is concentrated and desalted by centrifuging through a BIOMAX 100 Ultrafree 15 centrifugal filter device (Millipore UFV2BHK 10 or 40).

2. Place filter devices in a 50ml falcon tube and 10ml of PBS-MK buffer added to the top. Centrifugation was at 2000g for 15mins. Approx 50µl of liquid should remain in the device.

3. Add the eluted sample from earlier to the top of the column and spin for 15- 20mins. The sample should concentrate about 10 fold. There should be no more then 300-500µl left. If so respin.

4. Add a further 5ml of PBS-MK buffer and spin again. Repeat 2 times more. Increase spin time for each wash by approx. 10mins as the spin will take successively longer to reduce the sample. Final volume should be under 700µl (generally 200-500µl).

5. Pipette up and down the solution several times to resuspend the AAV particles. Store in small aliquots to avoid freeze-thawing of the virus.

Appendix 1.4: Rotenone preparation

Rotenone preparation for intracerebral administration

For Intranigral or intrastriatal infusion, rotenone was dissolved in a 1:1:18 combination of DMSO, Cremophor® and 0.89% saline, respectively. Rotenone was initially dissolved in DMSO before the addition of Cremophor® and saline. Solutions were prepared freshly and stored in the dark until administration was completed. The following example demonstrates the stock calculations involved prior to a single intranigral 0.9 µg infusion or a four site intrastriatal 3.6 µg (4 x 0.9 µg) infusion:
This was infused at 1 µl min-1 over 3 minutes at a rate of 0.3 µg min^{-1} Thus, required drug concentration is: 0.3 µg µl -1 (i.e. 0.3 mg ml^{-1})
If 5 mg of rotenone was weighted out then it would have been dissolved in 16.67 ml of vehicle (i.e. weight/concentration or 5 mg / 0.3 mg ml-1 = 16.67 ml)). Therefore, it would have been dissolved in:

DMSO: Cremophor®: Saline
1: 1: 18
0.83ml: 0.83 ml: 15.0 ml

Appendix 1.5: Perfusion protocol

Buffers for Perfusion-Fixation

0.2M Phosphate buffer

Stock A
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$ MW=137.99)
1M=137.99g in 1L dH_2O
0.1M=13.799g in 1L dH_2O
0.2M=27.598g in 1L dH_2O

Stock B
Disodium hydrogen phosphate dehydrate ($Na_2HPO_4.2H_2O$ MW=177.99)
1M=177.99g in 1L dH_2O
0.1M=17.799g in 1L dH_2O
0.2M=35.598g in 1L dH_2O

→For 100ml PB
9.5ml stock A + 40.5ml stock B + 50ml dH_2O =100ml PB

→For 1000ml PB
95ml stock A + 405ml stock B + 500ml dH_2O =1000ml PB

→For 2000ml PB
190ml stock A + 810ml stock B + 1000ml dH_2O =1000ml PB
Stock A: 27.598g/L x 0.19L = 5.24362g in 190ml dH_2O
Stock B: 35.598g/L x 0.81L = 28.83438g in 810ml dH_2O
Alternatively add both to 1L of dH_2O 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_2O 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_2O
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 dH_2O (1 tablet per 200ml water)
2. Add 250g of sucrose
3. Stir until dissolved, apply heat if necessary
4. Make up to 1L with dH_2O

0.1% w/v TBS-Azide

1. Weigh out 12g Trizma, 9g NaCl, and 1g sodium azide
2. Add to 1L dH_2O and dissolve
3. PH to 7.4

Appendix 1.6: Solutions for Immunohistochemistry

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

Appendix 1.7: 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% H_2O_2 5 ml
   -Distilled H_2O 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^o 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^o 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^o 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^{o}C.*

12. Incubate in H_2O_2/DAB solution until colour develops.
    - -TNS 40 ml
    - -DAB 20 mg (frozen in 2 ml aliquots).
    - -30% H_2O_2 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

16. Clear in Xylene in the fume hood and coverslip using DPX mountant.
    - -1st Xylene for 5 min
    - -2nd Xylene for 5 min

Appendix 1.8: Slide Subbing

Materials

Gelatin (10g/L)

Chromic Potassium Sulphate (500mg/L)

Distilled H_20

Slides

Method

1. Heat H_20 to +40◦ C and add gelatin slowly allowing it to dissolve before adding more
2. Add chromic potassium sulphate
3. Subbing Medium is then cooled to ~+3◦0 C
4. Slides placed in slide holders and dipped into subbing medium for ~ 1 min
5. Remove slides and allow to dry for ~1 week