Title: Development of a dual-hit model of Parkinson's disease by combining environmental and genetic risk factors

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Publication Date: 2012-06-06

Item record: http://hdl.handle.net/10379/4894
Development Of A Dual-Hit Model of Parkinson’s Disease By Combining Environmental And Genetic Risk Factors.

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March 2012

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Dedication

For my parents, Patsy and Ann Jo. I will be forever grateful.

“It may be that the gulfs will wash us down;
It may be that we shall touch the Happy Isles, and though
We are not now that strength which in old days
Moved earth and heaven, that which we are, we are---
One equal temper of heroic hearts,
Made weak by time and fate, but strong in will
To strive, to seek, to find, and not to yield.”

-Alfred Lord Tennyson
Abstract

Even though half a century has passed since the discovery of the efficacy of the dopamine precursor, levodopa, it remains the most effective therapy for the motor symptoms of Parkinson’s disease to this day, despite its serious side effects. One factor that is thought to have contributed to the paucity of novel therapies for this condition is the lack of relevant animal models. Since Parkinson’s disease is thought to arise from complex interactions between genes and the environment, modelling the condition using relevant genetic and environmental risk factors may yield a more relevant model. Thus, the aim of this body of work was to develop a novel model of Parkinson’s disease using the disease-associated pesticide, rotenone, and/or virally-mediated overexpression of the disease-associated protein, α-synuclein. This was addressed using three main approaches: 1) direct intracerebral infusion of rotenone alone, 2) systemic administration of rotenone combined with intranigral administration of AAV-α-synuclein, and 3) intracerebral infusion of rotenone with intranigral administration of AAV-α-synuclein. In all studies, the impact of the insults on motor function, nigrostriatal integrity and α-synuclein expression were assessed. The main findings were 1) that intracerebral infusion of rotenone induces motor impairments with nigrostriatal degeneration but without any α-synucleinopathy, 2) that systemic administration of rotenone with intranigral infusion of AAV-α-synuclein can induce the classical features of Parkinson’s disease but is associated with peripheral toxicity, and 3) that sequential intranigral infusion of AAV-α-synuclein and rotenone leads to progressive motor dysfunction with nigrostriatal degeneration and α-synuclein overexpression. Overall, this research has shown that the sequential intranigral “dual-hit” model provides a relevant and robust Parkinson’s disease model, which, with further validation, may be useful for gaining a greater understanding of the complex gene-environment interactions associated with the disease, and also for assessing novel pharmacological, neuroprotective or neuroreparative treatment approaches.
Acknowledgements

I would like to sincerely thank my supervisor, Dr. Eilís Dowd for all her help and support over the past few years. Your encouraging words were always very much appreciated.

I would also like to thank Prof. Deniz Kirik for the kind gift of plasmids provided and Dr. Aideen O’Doherty for the time, effort and advice that was always given when needed.

To my parents, Patsy and Ann Jo, the end is finally here! Thank you both for everything. I would be truly lost without you. The candles you had lighting for me all the way through really helped Mom, thanks!

The past few years would have been a lot tougher without the help from the gang in the CNS lab. To the remainder of the Dowd supergroup, best of luck and thank you! Thank you Teresa for all the advice and help during my time here. Sandra and Orla, it’s been awesome, you guys are amazing. Don’t forget that no matter how busy things get, there is always time for Westlife Friday and of course NRF if Ger is about. Sincere thanks to Danny and Ambrose for all your help, the place would fall down without you.

Finally, to my Daragh who has been there for me all the way through my PhD. Words really cannot express what you mean to me. Thank you.
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 exception:

Stereotaxic surgeries, behavioural data acquisition and histology for initial pilot study in section 3.3.1 was carried out by Dr. Sinéad Walsh

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Chapter 1: General introduction

Parkinson’s disease is the second most common neurodegenerative condition after Alzheimer’s disease which clinically manifests following the development of the pathology to an advanced stage (de Lau & Breteler, 2006). It is a progressive disorder inducing muscle tremor, stiffness and slowness of movement caused by the degeneration of the dopamine producing neurons in the basal ganglia which control movement. Pathologically, one of the main features of the disease is the formation of Lewy bodies composed primarily of accumulations of the protein α-synuclein (Braak et al., 2003). While the exact causes of Parkinson’s disease are not fully understood, it is believed that genetic factors, exposure to toxins, brain injury or infection, and adverse drug reactions may all be risk factors in the onset and progression of the disease (Priyadarshi et al., 2001; Warner & Schapira, 2003).

Currently, there is no cure for Parkinson’s disease and all presently available pharmacological treatments are based on the concept of increasing or replacing central dopamine levels so as to minimise the associated symptoms (Lang & Obeso, 2004). Administration of levodopa, which is the dopamine precursor, has remained the most effective treatment for Parkinson’s disease for the past half century. While short term levodopa treatment is well tolerated, its longer term effectiveness is limited by both the progression of the disease, whereby it is purely symptomatic and does not halt neurodegeneration, and the emergence of motor related side effects such as dyskinesias (Cotzias et al., 1969; Marsden & Parkes, 1976; Obeso et al., 2004a).
One of the reasons that levodopa remains at the forefront of Parkinson’s disease treatment despite its serious limitations is because the lack of relevant animal models has hampered the development of novel treatments. These models should incorporate the progressive nigrostriatal degeneration, motor impairments and α-synuclein accumulation present in the human condition (Meredith et al., 2008; Willis & Kennedy, 2004). Within this paradigm, neurotoxin, transgenic, viral gene transfer and current pesticide models have succeeded in consolidating some but not all of the required features.

Both exposure to environmental risk factors and genetic defects are linked to Parkinson’s disease. The pesticide rotenone has recently been shown to be a direct causal agent of Parkinson’s disease whereby exposure has been linked to a 2.5 fold increase in Parkinsonian features via its action as a mitochondrial Complex I inhibitor (Tanner et al., 2011). Systemic administration of this compound to experimental animals has led to some modelling success (Nistico et al., 2011), however, its major practical limitations effectively preclude its use as a relevant and reliable Parkinson’s disease model (Ferrante et al., 1997). Alternative rotenone administration routes, such as acute and chronic direct intracerebral infusion, have shown promise but a more complete characterisation of its effects need first be investigated to determine its potential as a novel approach to modelling the disease. As many as 16 genetic mutations have been discovered to play a role in disease onset and it is believed that a substantial percentage of sporadic cases are due to a synergistic effect between genetic and environmental risk factors (Gao & Hong, 2011a).
This introductory chapter will provide an evidence-based overview of Parkinson’s disease causes and effects along with current treatments, models and their associated limitations. A detailed summary of the more established rotenone models will also be provided.

1.1 Parkinson’s disease

Parkinson’s disease is a progressive neurodegenerative disease which affects approximately 1% of the population over 65 years of age (Satake et al., 2009). The vast majority of cases develop in elderly patients with about 4% of all diagnoses occurring before the age of 50 (de Rijk et al., 1995; Van Den Eeden et al., 2003). Sporadic cases account for more than 90% of the entirety, while the remainder have a purely genetic basis (Belin & Westerlund, 2008).

There are four cardinal motor symptoms of Parkinson’s disease which include tremor, rigidity, bradykinesia or postural instability (Jankovic, 2008). Other motor features of the disease include gait disturbances such as shuffling, freezing, festination (an involuntary quickening of gait), scoliosis and dystonia (Giladi et al., 2001; Schaafsma et al., 2003). These tend to progressively deteriorate as the disease state advances. Other non-motor complications associated with Parkinson’s disease include speech & swallowing disturbances, sleep & perception disarrangement, autonomic, gastrointestinal & neuro-opthalmological dysfunction, depression, dystonia and dementia (Chaudhuri et al., 2006). Standard Parkinsonian therapy fails to address these problems, though some can be controlled by specific individual treatments (Rascol et al., 2011).

The main neuropathological feature of Parkinson’s disease is the degeneration of the nigrostriatal dopaminergic pathway. By the time the disease is clinically diagnosed there
is a loss of dopaminergic neurons of approximately 50-80% (Fearnley & Lees, 1991). Formation of Lewy bodies in the midbrain is a feature of the disease which can be assessed \textit{post-mortem} and is used as a definitive determination of the disease state (Schmidt \textit{et al.}, 1991).

A criterion has been created in order to standardise the diagnostic process, where, for Parkinson’s disease to be diagnosed, the clinician requires bradykinesia in conjunction with either rigidity, resting tremor or postural instability to be evident. Other potential causes for these symptoms need also be ruled out. Finally, three or more of the following features are required during onset or evolution: unilateral onset, tremor at rest, progression over time, asymmetry of motor symptoms, response to levodopa for at least five years and the appearance of dyskinesias induced by the intake of excessive levodopa (Jankovic, 2008).

\textbf{1.1.1 Historical milestones in Parkinson’s disease research}

While much of our knowledge of Parkinson’s disease has come from research carried out in the past number of decades, its existence has been noted throughout history. One of the earliest references to the disease comes from the Indian medical doctrine called Ayurveda. The symptoms of Parkinson’s disease, then called Kampavata, were described as far back as 5,000 B.C. (Lang & Lozano, 1998). To treat Kampavata, a tropical legume called \textit{Mucuna pruriens} was used. The seeds of \textit{Mucuna pruriens} are a natural source of therapeutic quantities of levodopa, which is the basis for most current day treatments (Zhang \textit{et al.}, 2006). 30 g of \textit{Mucuna pruriens} seeds (containing 1000 mg of levodopa) has been shown to have a therapeutic effect similar to standard levodopa/carbidopa treatment (Katzenschlager \textit{et al.}, 2004).
Parkinson’s disease was first formally described by the English physician James Parkinson in his work “An Essay on the Shaking Palsy”, published in 1817. In it he systematically described the medical history of six individuals who had symptoms of the disease which eventually bore his name (Parkinson, 1817 (republished 2002)). He used the essay to document the symptoms of the disease which he described as “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 senses and intellect being uninjured.” While Parkinson tried to encourage the medical community to study the disease at the time, little further work was carried out until 1861 when French neurologist Jean Martin Charcot and his colleagues distinguished the disease from other neurological conditions and termed it “Parkinson’s disease” (Lees, 2007).

The latter half of the 19th and the beginning of the 20th centuries saw an expansion of research into Parkinsonian symptoms and pathology with definitions of rigidity and bradykinesia being associated with the disease, and in 1912, Friedrich Lewy, a German neurologist, described the cardinal neuropathological lesion that would later bear his name: the Lewy body (Rodrigues e Silva et al., 2010). The underlying biochemical changes in the Parkinsonian brain were identified in the 1950s, due largely to the work of Swedish scientist Arvid Carlsson (Carlsson, 1959; Carlsson et al., 1957). He demonstrated that dopamine was a neurotransmitter in the brain and not just a precursor for noradrenaline, as had been previously believed. He developed a method for measuring the amount of dopamine in brain tissues and found its levels in the basal ganglia, the area important for movement, were particularly high. He then showed that administering reserpine to animals caused a decrease in dopamine levels and a loss of movement
control. These effects were similar to the symptoms of Parkinson’s disease. The work carried out by Carlsson and colleagues commenced a new era in Parkinson’s research which saw investigation of the disease at the biochemical and neuronal level.

1.1.2 Pathophysiology of Parkinson’s disease

As outlined earlier, the two main pathophysiological features of Parkinson’s disease are the formation of Lewy bodies and the progressive destruction of the nigrostriatal pathway in the basal ganglia.

1.1.2.1 Lewy body formation

Lewy bodies are one of the main pathological hallmarks of Parkinson’s disease. They are eosinophilic, cytoplasmic inclusions which consist of a dense core surrounded by a halo of radiating fibrils. The primary structural component of these fibrils is the protein α-synuclein and they may also be associated with other proteins such as ubiquitin, neurofilament protein and tau proteins (Gibb & Lees, 1988; Spillantini et al., 1998b). Upon autopsy, brains of individuals with Parkinson’s disease are additionally characterised by α-synuclein-positive accumulations within neuronal processes, termed Lewy neurites (Galvin et al., 1999). While it is known that Lewy pathology is found throughout the nervous system, a proposed stereotyped caudal-to-rostral spread of pathology as the disease progresses remains a popular hypothesis (Braak et al., 2003). The earliest Lewy pathology affects the enteric and peripheral autonomic nervous system as well as the olfactory bulb, and it subsequently spreads caudo-rostrally from the lower brainstem (stage 1) to diffuse involvement of the neocortical ribbon (stage 6) (Braak et al., 2004) (Fig. 1.1). α-Synuclein pathology in the Braak paradigm is not apparent in the substantia nigra until stage 3 which fits with the hypothesis that a substantial precursory
syndrome precedes the development of Parkinson’s disease motor symptoms. Also, the anatomical pattern of the earlier Braak stages fits well with disease manifestations, including hyposmia (olfactory bulb), constipation (enteric nervous system), and sleep disorder (brainstem reticular formation) which emerge before motor symptoms associated with the disease (Wolters et al., 2006). As the disease progresses, widespread cortical involvement with Lewy bodies correlates with the frequent occurrence of cognitive impairment in advancing Parkinson’s disease (Mattila et al., 2000).

Fig. 1.1 Spread of idiopathic Parkinson’s disease pathology alongside the development of clinical symptoms. As proposed by Braak and co-authors, Lewy body pathology may arise in the periphery/enteric nervous system, possibly in the gastrointestinal tract, and transfer to the brain stem via the glossopharyngeal and vagus nerves. Finally, it spreads to the cortex at a later stage of disease progression (red arrows). Alternatively, the pathology may initiate at the olfactory bulb and the anterior olfactory nucleus and from there spread to the midbrain and the cortex (orange arrows) (Hansen & Li, 2012; Vernon et al., 2010).
1.1.2.2 Nigrostriatal degeneration

1.1.2.2.1 The basal ganglia

The basal ganglia are a collection of nuclei found on both sides of the thalamus, outside and above the limbic system, but below the cingulate gyrus and within the temporal lobes (Parent & Hazrati, 1995). The largest group of these nuclei are the corpus striatum, made up of the caudate nucleus, the putamen, the globus pallidus pars interna, the globus pallidus pars externa and the nucleus accumbens. The inhibitory neurotransmitter gamma-aminobutyric acid (GABA) plays a major role in the functioning basal ganglia where GABAergic medium spiny neurons make up about 95% of all neurons in the striatum (Kemp & Powell, 1971). There are two major basal ganglia pathways: a ‘direct’ pathway from the striatum to the basal ganglia modulatory output structures of the globus pallidus pars interna and substantia nigra, and an ‘indirect’ pathway projecting to the globus pallidus pars externa and sub-thalamic nucleus before progressing to the output structures that modulates the direct one (DeLong & Wichmann, 2007). Under normal physiological conditions (Fig. 1.2A), the two pathways exert opposing effects on movement whereby activation of the direct striatonigral pathway disinhibits thalamocortical neurons and facilitates motor activity and activation of the indirect striatopallidal pathway enhances inhibition of thalamocortical neurons and reduces motor activity (Fisone et al., 2007). The GABAergic neurons of the direct and indirect pathways express high levels of the post-synaptic dopamine D₁ and D₂ receptors, respectively. Activation of the dopamine D₁ receptors, via release of dopamine in the striatum, stimulates the neurons of the direct pathway by direct disinhibition of the thalamocortical neurons while activation of the dopamine D₂ receptors inhibits the neurons of the indirect
pathway by indirect disinhibition of the thalamocortical neurons, with both actions working to facilitate movement (Surmeier et al., 2007).

In the Parkinsonian brain (Fig. 1.2B), decreased levels of dopamine leads to decreased activity of striatal direct pathway neurons and also increased activity of striatal indirect pathway neurons. The excessive stimulation of the indirect pathway results in increased inhibition of globus pallidus *pars externa* causing disinhibition of the subthalamic nucleus and subsequent increased excitation of globus pallidus *pars interna* and substantia nigra *pars reticulata*. The overall effect of dopamine loss is an increase in the inhibitory output from globus pallidus *pars interna* and substantia nigra *pars reticulata* and decreased activity in thalamocortical neurons (DeLong et al., 2007). The decreased dopamine levels which cause this dysregulation in the basal ganglia is due to a severe loss of neurons of the dopaminergic nigrostriatal pathway.
Fig. 1.2 Schematic diagram of the basal ganglia under normal physiological conditions (A) and in Parkinson’s disease brains (B). Dopamine released from the nigrostriatal neurons excites D$_1$ receptors of the direct pathway resulting in direct disinhibition of the thalamocortical neurons and facilitation of motor activity. Dopamine inhibits D$_2$ receptors of the indirect pathway resulting in indirect disinhibition of the thalamocortical neurons and facilitation of motor activity. The net effect of nigrostriatal dopamine in Parkinson’s disease is loss of the facilitatory effects of the neurotransmitter with consequent effects on motor activity. Glu: glutamate; DA: dopamine; Gpe: globus pallidus pars externa; Gpi: globus pallidus pars interna; MSNs: medium spiny neurons; SNpr: substantia nigra pars reticulata; SNpc: substantia nigra pars compacta; STN: subthalamic nucleus. Image taken from (Di Filippo et al., 2008).

1.1.2.2.2 Loss of dopaminergic nigrostriatal neurons

The main pathological hallmark associated with Parkinson’s disease is the progressive degeneration of the nigrostriatal dopaminergic pathway in the basal ganglia, as just described (Bernheimer et al., 1973). The loss of neurons is clearly apparent in the post-mortem brain. This degeneration within the basal ganglia can often progress without the onset of motor impairments for many years and it may not be until there is an approximate loss of half the neuronal population of the substantia nigra pars compacta
that the clinical manifestations associated with the disease appear (Riederer & Wuketich, 1976).

The loss of neurons in Parkinson’s disease appears to be heterogeneous, where the ventrolateral part of the substantia nigra is almost completely destroyed, whereas there is only partial damage to the dorsal portion (Damier et al, 1999). The projections from this area of the substantia nigra extend predominantly to the striatum and such extensive damage is responsible for the loss of striatal dopamine as seen in the disease (Fearnley et al., 1991). By the time the disease has progressed to the stage where symptoms have manifest clinically, there is a loss of approximately 40-50% of the substantia nigra neurons with a resultant 80% loss in striatal dopamine levels (Bernheimer et al., 1973; Terzioglu & Galter, 2008). The reason for the lag between onset of the disease and the appearance of clinical symptoms is due to a mechanism of internal compensation where the remaining dopamine neurons can become over-active to produce enough dopamine to retain homeostasis within the local environment (Hornykiewicz & Kish, 1987; Zigmond et al., 1990). Recent advances in understanding changes in neuronal function, as degeneration of dopaminergic cells progresses, have shown the existence of a dissociation between three stages of events associated with Parkinson’s disease that until recently were considered to occur simultaneously. An emerging understanding suggests a sequential activation of compensatory mechanisms, as quantified by electrochemical measurement of dopamine release (Perez et al., 2008), which leads to the belief that the presymptomatic period in Parkinson’s disease comprises stages of increased compensatory intensity (Obeso et al., 2004b).

- In the first stage, dopamine homeostatic compensatory mechanisms are able to ‘mask’ the disease.
• The second stage begins with the breakdown of striatal dopamine homeostasis and ends with changes in the activity of the basal ganglia output structures, during which more powerful compensation takes place within the basal ganglia.

• The final stage begins with increased globus pallidus pars interna activity and finishes with the emergence of Parkinsonian motor abnormalities, during which the compensation of maximum intensity takes place in structures outside the basal ganglia (Bezard & Gross, 1998a; Bezard et al., 2003).

Also, post-synaptically, striatal D₁ and D₂ receptor up-regulation may contribute to compensation of early dopaminergic cell loss. This receptor activation hyperpolarises medium spiny neurons projecting from the striatum to the globus pallidus and inhibits GABA release (Shin et al., 2003). In later disease stages, however, these same modifications of receptor sensitivity, especially D₁, may contribute to the emergence of motor fluctuations (Lebel et al., 2007).

1.1.2.3 Mitochondrial dysfunction and oxidative stress

Mitochondria are found in virtually all eukaryotic cells and function to generate cellular energy in the form of adenosine triphosphate by oxidative phosphorylation (Frey & Mannella, 2000). They are also involved in regulation of cell death via apoptosis, calcium homeostasis, haem biosynthesis, and the formation & export of iron-sulphur (Szabadkai & Duchen, 2008). The electron transport chain is composed of five complexes including an adenosine triphosphate-synthase located in the inner mitochondrial membrane (Fig. 1.3). The function of the chain is to generate cellular energy in the form of adenosine triphosphate. This is accomplished by the transport of electrons between complexes causing proton movement from the matrix to the intermembrane space generating a
proton concentration gradient used by adenosine triphosphate-synthase to produce adenosine triphosphate (Chan, 2006).

Fig. 1.3 Schematic diagram of the Electron Transport Chain. The chain consists of these 4 complexes plus a 5th unlinked complex, ATP synthase. Three of the 4 complexes act as pumps, moving H+ ions across from the matrix into the intermembrane space. This builds up a concentration gradient on one side that wants to equilibrate with the matrix. So this is creating an electrochemical force that can be utilised as energy by ATP synthase. The electron transport chain accepts electrons from NADH that were produced from glycolysis and the TCA cycle, it passes these high energy electrons through a series of enzyme complexes that eventually oxidise it to form water in the presence of oxygen. At the same time, the energy released from oxidation of these molecules is used to pump H+ ions across a membrane which creates an electrochemical gradient or proton-motive force that is subsequently harnessed by ATP synthase to produce a more stable energy transport molecule that a wide range of reactions can harness. NADH: Ubiquinone oxidoreductase, Q: ubiquinone, ADP: adenosine diphosphate, ATP adenosine triphosphate, cyt c: cytochrome c, TCA: Tricarboxylic acid cycle. Taken from (Nelson, 2004).

Neurons have a considerable energy need and contain considerable numbers of mitochondria which are extremely sensitive to mitochondrial dysfunction. Complex I is composed of 43 subunits, 7 of which are encoded by the mitochondrial genome and the remainder by the nuclear genome. These mitochondrial encoded subunits are all
hydrophobic are essential for full Complex I activity. Several neurological disorders, such as epilepsy, autism spectrum disorders, amyotrophic lateral sclerosis, Alzheimers disease and Parkinson’s disease, are associated with mitochondrial dysfunction and demonstrate enhanced production of free-radical species (Lin & Beal, 2006). Mutations in genes associated with individual subunits result in differing phenotypes associated with individual mitochondrial diseases. Mutation in the mitochondrial ND4 gene is linked with degeneration of specific neuronal cells, those of the substantia nigra, and is associated with the onset of Parkinson’s disease (Greenamyre et al., 2001). The first line of evidence for a link between mitochondrial dysfunction and Parkinson’s disease came from the description of Complex I deficiency in the post mortem substantia nigra of Parkinson’s disease patients and has been suggested to be one of the fundamental physiological abnormalities of the disease. This defect may be due to oxidative damage to Complex I, since this is a feature of sporadic Parkinson’s disease brain mitochondria (Keeney et al., 2006). Studies of families who suffer from inherited forms of Parkinson’s disease have identified a number of genes encoding mitochondrial proteins or proteins implicated in mitochondrial dysfunction such as α-synuclein, Parkin, PINK1 and DJ-1 (Dodson & Guo, 2007; Wood-Kaczmar et al., 2006).

Complex I inhibition causes an increase in the release of electrons from the transport chain into the mitochondrial matrix which then react with oxygen to form reactive oxygen species such as \( \text{O}_2^- \), hydroxyl radicals and nitric oxide (Liochev & Fridovich, 1999). Amongst the most common mechanisms of protein damage caused by reactive oxygen species is oxidation to form carbonyl groups on proteins and this leads to cytotoxic protein aggregation, activation of cell death pathways, and impairment of neuroprotective pathways. An increase in these carbonyl groups has been reported in the substantia nigra,
the basal ganglia and prefrontal cortex of Parkinson’s disease patients (Floor & Wetzel, 1998).

1.1.2.4 Neuroinflammation

For the past number of decades, gliosis, which is the proliferation of astrocytes in damaged areas of the central nervous system, has been a well-recognised neuropathological feature of Parkinson’s disease, thought of as secondary and insignificant, as far as the pathogenesis of the disease is concerned. More recently, however, human epidemiological studies have suggested that inflammation increases the risk of developing Parkinson’s disease (Chen et al., 2003) and investigations in experimental models of the disease have shown that inflammatory responses can trigger nigrostriatal dopaminergic neuronal death (Block et al., 2007; Gao et al., 2011b). Upregulation of inducible nitric oxide synthase and cyclooxygenase-2 containing amoeboid microglia has been observed in the substantia nigra of Parkinson’s disease patients but not in control subjects. Activated glial cells expressing pro-inflammatory cytokines, such as TNF-α, IL-1β and IFN-γ as well as nitric oxide synthase have been reported in the substantia nigra of patients (Knott et al., 2000; Nagatsu, 2002). Moreover, in post-mortem examinations of brains from humans exposed to MPTP, activated microglia were present up to 16 years after exposure, indicating a protracted and ongoing inflammatory response (Langston et al., 1999). Microglia, the resident innate immune cells in the brain, have been implicated as active contributors to neuron damage in neurodegenerative diseases, in which the overactivation and dysregulation of microglia might result in severe and progressive neurotoxic consequences.
There is increasing evidence to suggest that the brain inflammatory response contributes to Parkinson’s disease pathogenesis (Cicchetti et al., 2002), where the loss of these basal ganglia neurons is associated with a glial response composed mainly of activated microglial cells and, to a lesser extent, of reactive astrocytes (Vila et al., 2001). The large population of resident microglia within these structures as compared to other brain regions confers a higher sensitivity to microglia activation-associated death (Machado et al., 2011). The glial response can, under chronic conditions, mediate a variety of deleterious events related to the production of proinflammatory cytokines (Vila et al., 2001). At the molecular level, microglial activation may be triggered by protein aggregation and formation of inclusions arising from mutations (i.e. α-synuclein) or disruption of the ubiquitin-proteasome system, immunological challenges (bacterial or viral infections), or traumatic brain injury. For example, misfolded or aggregated proteins in Lewy bodies of diseased nigral dopaminergic neurons are likely to elicit a self-propelling cycle of microglial activation and increased production of inflammatory mediators in the substantia nigra, thus providing mechanisms for Parkinson’s disease-associated dysfunction to spread to neighbouring neurons (Sulzer, 2007; Zhang et al., 2005).

1.1.3. Etiology of Parkinson’s disease

Although the pathophysiology of Parkinson’s disease has to date been well described, the exact etiology of the condition is still unknown in the majority of cases. It is believed that while genetic variations play a major role in some cases, the majority of cases are due to a combination of environmental and genetic factors working in synergy (Schapira & Jenner, 2011; Warner et al., 2003; Wirdefeldt et al., 2011).
1.1.3.1 Age and gender

Aging is the progressive accumulation of changes with time associated with or responsible for the ever-increasing susceptibility to disease and death which accompanies old age (Harman, 1991). The crude prevalence of sporadic Parkinson’s disease, with and without dementia, is 99.4 per 100,000, increasing from 2.3 per 100,000 for those younger than 50 years to 1,144.9 per 100,000 for those aged 80 years and older (Mayeux et al., 1992). There are several indications of gender differences in Parkinson’s disease. Epidemiological studies have shown that both incidence and prevalence of Parkinson’s disease are 1.5 – 2 times higher in men than in women with studies adjusted for age. It is believed that the effects of oestrogen on the dopaminergic system may play a protective role in women (Miller & Cronin-Golomb, 2010; Saunders-Pullman et al., 2011; Wirdefeldt et al., 2011). In studies incorporating gender which specified age at onset, it was shown to be a mean of two years later in women than men (Twelves et al., 2003). After progression to the clinical phase of the disease, women had better Unified Parkinson’s disease Rating Scale motor scores but a greater prevalence of dyskinesias compared to men (Lyons et al., 1998).

1.1.3.2 Genetic factors

Prior to the turn of the 21st century, it was believed that Parkinson’s disease was sporadic in nature, with no genetic basis. Since then, however, numerous studies have been carried out which have culminated in the discovery of no fewer than 16 genes involved in the etiology of the disease as outlined below (Table 1.1).
<table>
<thead>
<tr>
<th>Locus Name</th>
<th>Gene Symbol</th>
<th>Chromosome Locus</th>
<th>Inheritance</th>
<th>Protein product</th>
<th>Pathology</th>
<th>Onset age</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1</td>
<td>SNCA</td>
<td>4q21</td>
<td>AD</td>
<td>α-synuclein</td>
<td>Nigral degeneration with Lewy bodies</td>
<td>40s</td>
</tr>
<tr>
<td>PARK2</td>
<td>PARK2</td>
<td>6q25.2-27</td>
<td>AR</td>
<td>Parkin</td>
<td>Nigral degeneration without Lewy bodies</td>
<td>20-40</td>
</tr>
<tr>
<td>PARK3</td>
<td>2p13</td>
<td>AD</td>
<td>AD</td>
<td>As yet unknown</td>
<td>Nigral degeneration with Lewy bodies, plaques &amp; tangles</td>
<td>60s</td>
</tr>
<tr>
<td>PARK4</td>
<td>PARK4</td>
<td>4q21</td>
<td>AD</td>
<td>α-synuclein</td>
<td>Nigral degeneration with Lewy bodies, vacuoles in hippocampal neurons</td>
<td>30s</td>
</tr>
<tr>
<td>PARK5</td>
<td>UCHL1</td>
<td>4p14</td>
<td>AD</td>
<td>Ubiquitin carboxyl-terminal hydrolase isozyme L1</td>
<td>No pathology reported</td>
<td>~50</td>
</tr>
<tr>
<td>PARK6</td>
<td>PINK1</td>
<td>1p35-37</td>
<td>AR</td>
<td>Serine / threonine-protein kinase PINK1</td>
<td>No pathology reported</td>
<td>30-40</td>
</tr>
<tr>
<td>PARK7</td>
<td>PARK7</td>
<td>1p38</td>
<td>AR</td>
<td>DJ-1</td>
<td>No pathology reported</td>
<td>30-40</td>
</tr>
<tr>
<td>PARK8</td>
<td>LRRK2</td>
<td>12q12</td>
<td>AD</td>
<td>Leucine-rich repeat serine / threonine-protein kinase 2</td>
<td>Variable α-synuclein and tau pathology</td>
<td>~60</td>
</tr>
<tr>
<td>PARK9</td>
<td>ATP13A2</td>
<td>1p36</td>
<td>AR</td>
<td>As yet unknown</td>
<td>No pathology reported</td>
<td>20-40</td>
</tr>
<tr>
<td>PARK10</td>
<td>PARK10</td>
<td>1p32</td>
<td>AD</td>
<td>As yet unknown</td>
<td>No pathology reported</td>
<td>50-60</td>
</tr>
<tr>
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<td>PARK11</td>
<td>2q34</td>
<td>AR</td>
<td>As yet unknown</td>
<td>No pathology reported</td>
<td>Late</td>
</tr>
<tr>
<td>PARK12</td>
<td>PARK12</td>
<td>7q21</td>
<td>X-Linked</td>
<td>As yet unknown</td>
<td>No pathology reported</td>
<td>Late</td>
</tr>
<tr>
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<td>HTRA2</td>
<td>2p12</td>
<td>AD</td>
<td>As yet unknown</td>
<td>No pathology reported</td>
<td>Late</td>
</tr>
</tbody>
</table>

Table 1.1 Genes that are involved in the etiology of Parkinson’s disease. Modified from (Gasser, 2007; Healy et al., 2004; Healy et al., 2008; Pisan-Ruiz et al., 2004; Saiki et al., 2011; Satake et al., 2009; Shulman et al., 2011; Simon-Sanchez et al., 2009). AD: autosomal dominant, AR: autosomal recessive. No pathology reported: Subjects did not display characteristic Lewy pathology or no data available to date.
1.1.3.2.1 PARK1 - α-synuclein

α-Synuclein, which is encoded by the SNCA gene, is a synuclein protein of unknown function primarily found in neural tissue, making up to 1% of all proteins in the cytosol (Venda et al., 2010). It is predominantly expressed in the neocortex, hippocampus, substantia nigra, thalamus, and cerebellum, as well as in glial cells (Piao et al., 2000). It is normally located in the cell nucleus and presynaptic terminals and its function depends on its location. Presently, the normal function of alpha-synuclein remains poorly understood, although it has been linked with synaptic plasticity and learning, neurotransmitter release and maintenance of synaptic vesicle pools (Burre et al., 2010; Dikiy & Eliezer, 2012; Watson et al., 2009). Normally an unstructured soluble protein, fibrillogenic monomers of α-synuclein form oligomeric intermediates that assemble into fibrils, and finally deposit in Lewy bodies described earlier as being one of the main pathological features of Parkinson’s disease (Schulz & Falkenburger, 2004). α-Synuclein has a strong tendency to self-aggregate in vitro, so increasing its expression would be expected to generate more of these aggregates (Uversky, 2007).

α-Synuclein was the first gene to be linked to Parkinson’s disease (Polymeropoulos et al., 1997) when it was shown that three missense mutations in the α-synuclein gene (A30P, E46K and A53T) in one large Italian family and three unrelated Greek families caused Parkinson’s disease with high penetrance. Duplications and triplications of the wild-type SNCA locus have also been associated with autosomal dominant Parkinson’s disease (Chartier-Harlin et al., 2004; Fuchs et al., 2007). The following year, α-synuclein was found to be the major constituent of Lewy bodies (Spillantini et al., 1997). α-Synuclein duplication is now recognised as a rare cause of familial Parkinsonism, including cases which are phenotypically similar to sporadic Parkinson’s disease, with no atypical
features (Ahn et al., 2008; Chartier-Harlin et al., 2004). Following this, triplication of the SNCA locus was reported in a separate kindred with familial Parkinson’s disease; branches of this family had been previously reported, but found via genealogical methods to be the same. This shows that point mutations along with SNCA multiplication can lead to the disease (Singleton et al., 2003).

Studies have shown that brain tissue from sporadic Parkinson’s disease patients has a higher expression of α-synuclein mRNA compared to controls which suggests that a similar pathogenetic mechanism might be responsible (Chiba-Falek et al., 2006). α-Synuclein has been shown to regulate the production of dopamine in cultured cells through its interaction with tyrosine hydroxylase, the rate-limiting enzyme responsible for converting tyrosine to levodopa in the dopamine synthesis pathway (Perez et al., 2002). Overexpression of α-synuclein in cells reduces the activity of the tyrosine hydroxylase promoter, leading to reduced levels of tyrosine hydroxylase mRNA and protein (Yu et al., 2004). Single and double knockout studies have shown that synucleins are not essential components of the basic machinery for neurotransmitter release but may contribute to the long-term regulation and/or maintenance of presynaptic function. However, data has also shown that α-synuclein knockout mice have reduced learning ability in tests requiring both working and spatial memory, displaying the important role of α-synuclein in the learning process (Chandra et al., 2004; Kokhan et al., 2012). Findings that the level of α-synuclein protein expression directly affects synaptic transmission and neurotransmitter release suggest a mechanism whereby genetic polymorphisms found within regulatory elements controlling α-synuclein gene expression could influence Parkinson’s disease susceptibility (Nemani et al., 2010).
1.1.3.2.2 Other Parkinson’s disease related genetic mutations

1.1.3.2.2.1 PARK2 Mutation

The *PARK2* gene encodes the protein parkin which is a component of the E3 ubiquitin ligase complex which forms part of the ubiquitin-proteasome protein degradation system (Veeriah *et al.*, 2010). Mutations in this gene cause an autosomal recessive, early onset familial form of Parkinson’s disease known as the parkin type of juvenile Parkinson’s disease. Onset of this disease type usually occurs between ages 20 and 40 years although it can be highly variable - even in individuals with the same mutation (Chien *et al.*, 2006). Onset is usually before age 40 years but has been reported in the sixth or seventh decade (Tan *et al.*, 2003). The disease is slowly progressive and disease duration of more than 50 years has been reported (Khan *et al.*, 2003). The parkin type of juvenile Parkinson’s disease is often clinically indistinguishable from sporadic Parkinson’s disease (Lucking *et al.*, 2000). Neuronal loss and sometimes neurofibrillary tangles in the substantia nigra are observed. Absence of cytoplasmic Lewy body inclusions makes this disorder pathologically distinct from classic Parkinson’s disease (Hattori & Mizuno, 2004; Taylor *et al.*, 2002) although a single case with Lewy bodies was reported (Farrer, 2006) and α-synuclein inclusions distinct from Lewy bodies were described in another individual (Sasaki *et al.*, 2004). Penetrance appears to be complete in individuals who have two disease-causing *PARK2* mutations, whereby all individuals carrying these mutations develop Parkinson’s disease. No correlation between missense or truncating *PARK2* mutations and age at onset, clinical presentation, or disease progression has been observed (Lucking et al., 2000).
1.1.3.2.2 PARK6 Mutation

The PARK6 gene encodes the protein PTEN-induced putative kinase 1 (PINK1) which is a serine/threonine-protein kinase. Mutations in this gene cause an autosomal recessive, early onset type of Parkinson’s disease which is often clinically indistinguishable from sporadic Parkinson disease (Valente et al., 2004). Onset usually occurs in the third or fourth decade although again, it can be highly variable even in individuals with the same mutation (Hedrich et al., 2006). The disease is slowly progressive. No correlation between the type of mutation and age at onset, clinical presentation, or disease progression has yet been observed. Individuals with both PARK2 and PARK6 mutations have been reported and suffer very early onset suggesting, that the combination of both genetic mutations accelerates disease onset (Piccoli et al., 2008).

1.1.3.2.3 PARK8 Mutation

Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene causes Parkinson disease type 8 (PARK8). The majority of individuals with LRRK2-related Parkinson’s disease exhibit the hallmark pathological features of the common sporadic form as detailed earlier (Ross et al., 2006). However, LRRK2-related Parkinson’s disease has also been documented with four alternate pathologies including nigral neuronal loss and gliosis without Lewy body inclusions (Adams et al., 2005; Zimprich et al., 2004), neurofibrillary tangles (Gilks et al., 2005), ubiquitin-immunopositive inclusions, named Marinesco bodies, (Paisan-Ruiz et al., 2004) and overexpression of the nuclear protein, TAR-DNA-binding protein-43 (Ross et al., 2006). Immunostaining for the LRRK2 protein has also been reported within Lewy bodies (Giasson et al., 2006). Penetrance of LRRK2 mutations is age dependent but may vary depending on mutation and population ethnicity (Healy et al., 2008). To date, no certain correlations between specific LRRK2 mutations and age at
onset, clinical presentation, or disease progression have been found (Haugarvoll et al., 2008).

Overall, these genetic studies highlight critical cellular proteins and mechanisms for dopamine neuron survival as disrupted in Parkinson's disease. Understanding the genetic variations impacting on dopamine neurons may illuminate other molecular mechanisms involved. Additional candidate genes involved in dopamine cell survival, dopamine synthesis, metabolism and function, energy supply, oxidative stress, and cellular detoxification have been indicated by transgenic animal models and/or screened in human populations with differing results. Genetic variation in genes known to produce different patterns and types of neurodegeneration that may impact on the function of dopamine neurons have shed much light to date on the mechanisms behind the disease (Huang et al., 2004).

1.1.3.3 Environmental Factors

While the genetics of Parkinson’s disease is a relatively new area of research, the link between environmental factors and the disease has been proposed for about the past quarter century (Bharucha et al., 1986; Schoenberg, 1987). As previously iterated, it is currently believed that most cases of Parkinson’s disease are sporadic where the individual develops the disease with no prior family history, with the cause of onset being blamed, at least in part, on exposure to environmental insults. Consistent with the potential for environmental exposures to contribute to the etiology of Parkinson’s disease, is the fact that the disease shows geographic variation in its mortality statistics. Such variation has been reported in Japan (Imaizumi, 1995), Canada (Svenson, 1990; Svenson et al., 1993), and the United States (Lanska, 1997). Also cited as evidence in support of
environmental contributions to this disease, is the fact that Parkinson’s disease occurs in greater frequency in industrialised countries. The Parkinson’s disease prevalence rate is reportedly much lower in China than in the United States (Tanner, 1989). Studies have reported that this may be due to a lower life expectancy compared to Western nations, however, aged matched samples have shown the prevalence in mainland China to be much lower. Long term residents of rural or farming areas of China show an increased association of Parkinson’s disease development, lending support to the idea of pesticide-induced disease development. Chinese women resident in the United States show a lower association compared to United States nationals indicating a predisposed genetic protection (Chan et al., 2004).

1.1.3.3.1 Toxins

1.1.3.3.1.1 MPTP

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a neurotoxin linked to human Parkinsonism. This compound appears to be selectively toxic to the cells in the substantia nigra and is capable of producing virtually all the signs and symptoms of sporadic Parkinson’s disease (Langston et al., 1999; Langston et al., 1984b). The mechanism behind the neurotoxic action of MPTP has been the subject of intense investigation and is relatively well understood. MPTP is a lipophilic protoxin that, following systemic injection, rapidly crosses the blood–brain barrier (Riachi et al., 1989). Once inside the brain, MPTP is converted by monoamine oxidase-B (mostly in glia and serotonergic neurons) into the intermediary, 1-methyl-4-phenyl-2,3, dihydropyridinium (MPDP⁺) before its rapid and spontaneous oxidation to the toxic moiety, 1-methyl-4-phenylpyridinium (MPP⁺) (Chiba et al., 1984). Following its release into the extracellular space, MPP⁺ is taken up via the dopamine transporter into dopaminergic neurons where
cytoplasmic MPP⁺ can trigger the production of reactive oxygen species, which may contribute to its overall neurotoxicity (Javitch et al., 1985). However, the majority of MPP⁺ is eventually accumulated within mitochondria where the key toxic mechanism occurs. Once inside mitochondria, MPP⁺ impairs mitochondrial respiration via inhibition of Complex I of the electron transport chain (Nicklas et al., 1987). This action impairs the flow of electrons along the respiratory chain, leading to reduced adenosine triphosphate production and the generation of reactive oxygen species likely responsible for initiation of cell death-related signalling pathways (Karunakaran et al., 2008), which have been demonstrated *in vivo* following MPTP treatment and may contribute to apoptotic cell death (Jackson-Lewis et al., 1995; Tatton & Kish, 1997).

### 1.1.3.3.1.2 Heavy metals and other chemicals

As far back as 100 years ago, it was noted that high manganese exposure caused Parkinsonian symptoms, although clinically distinct from Parkinson’s disease (Jankovic, 2005). Welders have been of specific interest as they are exposed to manganese-containing fumes, however, two studies based on cohorts of male iron and metal goods manufacturing workers both reported no association between being a welder and either hospitalisation due to basal ganglia related disorders, including Parkinson’s disease, or mortality with a diagnosis of Parkinson’s disease (Fored et al., 2006; McMillan, 2005; Stampfer, 2009). Reports have also been published showing a link between exposure to lead and the disease (Coon et al., 2006). Iron has been believed to play a role in Parkinson’s disease based on its involvement in oxidative stress, and increased iron levels were demonstrated in the substantia nigra of Parkinson’s disease patients compared to controls (Dexter et al., 1989; Mann et al., 1994). Exposure to other metals including copper and zinc may also play a part as alterations in nigral levels of these metals have
also been reported (Barnham & Bush, 2008). Copper and zinc are important as co-factors for the enzyme superoxide dismutase, an enzyme that repairs cells and reduces the damage done to them by superoxide, the most common free radical in the body (Dexter et al., 1992; Dexter et al., 1993). Occupations involving the use of hydrocarbon solvents, such as benzene and toluene, are a risk factor for earlier onset of symptoms of Parkinson’s disease and more severe disease throughout its course (Pezzoli & Canesi, 2012).

1.1.3.1.3 Pesticides

The interest in the relationship between pesticides and Parkinson’s disease was raised in the 1980s, when it was discovered that, as detailed, exposure to MPTP, a substance structurally similar to the herbicide paraquat, resulted in chronic Parkinsonism and degeneration of dopaminergic neurons in humans (Tanner, 1989). While the structural similarities initiated the research interest into paraquat, it was subsequently discovered that despite these similarities, paraquat exerts its deleterious effects on dopaminergic neurons in a manner different from MPTP (Richardson et al., 2005). A multitude of pesticides are on the market and exposure may be widespread through contaminated food and water as well as through aerial spraying and fumigation (McKinlay et al., 2008).

Epidemiological studies have been carried out to examine the association between risk of Parkinson’s disease and exposure to pesticides and herbicides, as well as other measures such as farming, living in rural areas, and consumption of well water (Gorell et al., 1998; Sanyal et al., 2010). Evidence consistently points towards a positive association between pesticide exposure and Parkinson’s disease risk. In most studies, exposure was assessed retrospectively as a broad category, rather than examining specific substances (Kamel et
Exposure to toxins is probable via ingestion or inhalation. Although one possibility is that the final effects are mediated through the blood brain barrier, recent advances in staging Parkinson’s disease strongly suggest that route of entry into the nervous system may in fact be directly through neurons in the gastrointestinal system or the olfactory region over a protracted period of time (Cicchetti et al., 2009). Studies have shown that in experimental animals, developmental exposure to pesticides alone produces minimal neurological or behavioural changes. However, following adult re-challenge, significant decreases in dopamine and nigral cell counts are observed, suggesting that exposure to pesticides produces a state of silent toxicity that is unmasked following adult re-exposure. These findings indicate that exposure to pesticides during the neonatal and postnatal period can produce permanent and progressive lesions of the nigrostriatal dopaminergic system, and enhanced adult susceptibility to these pesticides, suggesting that developmental exposure to neurotoxicants may be involved in the induction of neurodegenerative disorders and/or alter the normal aging process (Fredriksson et al., 1993; Richardson et al., 2006; Thiruchelvam et al., 2002).

Research examining Parkinson’s disease mortality and pesticide exposure in California from 1984 through 1994, reported that mortality was increased in counties using agricultural pesticides after controlling for age, gender, race, birthplace, year of death, and education (Ritz et al., 2000). One ecological study reported high prevalence of Parkinson’s disease in rural agricultural regions in Canada and one of the first reports of an association between pesticides and Parkinson’s disease came in 1978 when a crop duster developed the disease following many episodes of acute and chronic pesticide intoxication (Brown et al., 2006). There have been numerous cases of Parkinson’s disease, particularly of early onset, where the patients have been exposed to either high
acute or chronic doses of pesticides. While it is long believed that pesticides are associated with the onset of Parkinson’s disease, it is only recently that a direct causal link between the two has been established, with users of rotenone or paraquat being 2.5 times more likely to develop the disease (Tanner et al., 2011).

1.1.3.1.3.1 Rothenone

Rothenone is an organic, non-specific pesticide, piscicide and insecticide which is derived from roots of several tropical and subtropical plant species belonging to the genus *Lonchocarpus* or *Derris* (Haag, 1931). Rotenoids, the rotenone-related materials, have been used as crop insecticides since 1848, when they were applied to plants to control leaf-eating caterpillars. They have also been used for centuries in South America to paralyse fish, causing them to surface. *Derris* root has long been used as a fish poison and its insecticidal properties were known to the Chinese well before it was isolated in 1929 (Geoffrey, 1895). While rotenone and its metabolites remain in fish tissue following poisoning, it is considered safe for human consumption as rotenone is very poorly absorbed by the human gastrointestinal tract at the low doses required to kill fish, whereas it is lethal to fish because it readily enters the blood stream through the gills (Chesneau et al., 2009; Wood et al., 2005).

Rothenone is highly lipophilic and thus can freely cross the blood-brain barrier where it has been shown to induce mitochondrial dysfunction by Complex I inhibition. As mentioned earlier, the mitochondrial respiratory chain (Complexes I-V) is the major site of adenosine triphosphate production in eukaryotes, moreover, it is known that this organelle not only generates adenosine triphosphate, but also plays an important role in apoptosis (Green & Reed, 1998). It is now clear that upon apoptotic stimulation,
mitochondria can release several proapoptotic regulators, including cytochrome c (Liu et al., 1996), endonuclease G, and apoptosis-inducing factor, to the cytosol (Susin et al., 1999). These proapoptotic regulators then activate cellular apoptotic programs downstream. Rotenone inhibits the mitochondrial respiratory chain between diphosphopyridine nucleotide and flavine (Baines, 2009). The proton pumping NADH–ubiquinone oxidoreductase catalyses the first step of oxidative phosphorylation, i.e., the transfer of two electrons from NADH to ubiquinone (Prieur et al., 2001). Rotenone is metabolised by the liver where it inhibits the oxidation of nicotinamide adenine dinucleotide (NADH to NAD\(^+\)), and therefore blocks the oxidation by NAD\(^+\) of substrates such as glutamate, \(\alpha\)-ketoglutarate, and pyruvate by binding to the ubiquinone binding site of Complex I (Goodman, 1985). This mechanism of action appears to be similar in both lower and higher organisms and species. Production of reaction oxygen species and nitric oxide by the induced mitochondrial inhibition also leads to the excess formation of peroxynitrite which has been shown to lead to apoptosis and neurodegeneration (Bao & Liu, 2002). Evidence now suggests that defective regulation of microtubules may be responsible for at least some cases of Parkinson’s disease (Choi et al., 2011). It is believed that rotenone may also selectively kill dopaminergic neurons by depolymerising microtubules, leading to a back-up of dopamine and in the transport system. The backed-up dopamine accumulates in the body of the neuron and breaks down, causing a release of toxic free radicals, which destroy the neuron (Feng, 2006).

In experimental models, rotenone induces both loss of nigral dopaminergic neurons and behavioural changes associated with human Parkinson’s disease (Betarbet et al., 2000; Henchcliffe & Beal, 2008). Yet despite decades of laboratory research, only very recently has rotenone been definitively associated with Parkinson’s disease in humans (Tanner et
Previous studies had been inconsistent with some studies showing a positive (Dhillon et al., 2008; Kamel et al., 2007), negative (Tanner et al., 2009) or undetermined association (Kamel et al., 2007). Tanner and colleagues performed the first analysis of pesticides classified by presumed mechanism, rather than by functional categories (e.g., herbicides) or chemical class (e.g., organochlorines). Significant associations of Parkinson’s disease with use of groups of pesticides classified as Complex I inhibitors or as oxidative stressors were found, providing support in humans for findings from decades of experimental work. In particular, Parkinson’s disease was strongly associated with rotenone and paraquat, the two predominant pesticides used to model Parkinson’s disease in the laboratory (Tanner et al., 2011).

1.1.3.3.1.3.2 Paraquat

Paraquat is one of the most widely used herbicides. It is fast-acting and non-selective, killing green plant tissue on contact and in humans can cause acute renal failure, hepatitis, and respiratory failure due to pulmonary inflammation and fibrosis (Neves et al., 2011). Due to its structural similarity to MPP+, paraquat has been postulated to be a potential neurotoxin and cause Parkinson’s disease-like symptoms. It is lipophilic so it can cross the blood-brain-barrier, although poorly (Shimizu et al., 2001). Paraquat is not metabolised but is reduced to an unstable free radical (Bismuth et al., 1987). It is well established that redox cycling is the primary reaction responsible for the toxicity of paraquat (Hassan & Fridovich, 1978). In anaerobic conditions, the paraquat cation is reduced by nicotinamide adenine dinucleotide phosphate dependant microsomal flavoprotein reductase to form the reduced radical. This then reacts with molecular oxygen to reform the paraquat cation and the superoxide ion. Paraquat then continues to cycle from its oxidised to reduced form with the electrons and oxygen (Bonneh-Barkay et
Paraquat is thought to cause cell death by lipid peroxidation or nicotinamide adenine dinucleotide phosphate depletion (Smith, 1987).

Similar to rotenone, until recently (Tanner et al., 2011), there had been conflicting evidence showing a positive (Firestone et al., 2005; Kamel et al., 2007; Tanner et al., 2009) or negative association (Liou et al., 2007; Hertzman et al., 1994) between paraquat exposure and Parkinson’s disease with some research also showing an enhanced effect in individuals exposed to a combination of paraquat and maneb, another general use pesticide (Costello et al., 2009).

1.1.3.4 Gene-environment interactions

As detailed, both genetic and environmental factors have been shown to be causative agents in the onset and progression of Parkinson’s disease in their own right. A widening current belief is that many sporadic cases are due to an interaction and synergistic effect of these factors. The occurrence of Parkinson’s disease-causative genetic defects in apparently sporadic cases of the disease and in healthy carriers, as well as the highly variable onset ages and considerable phenotypic variations in inherited forms underscore a crucial role of inherent gene–environment interactions (Gao et al., 2011a; Vance et al., 2010). The lack of overt dopaminergic nigrostriatal degeneration in most gene-based Parkinson’s disease animal models and the paucity of α-synuclein-containing Lewy body inclusions in most toxin-based models further support the role of gene–environment interplay in the pathogenesis of the disease (Dawson et al., 2010b). The further uncovering of this interaction and the synergistic mechanisms involved may have significant future implications for the treatment of Parkinson’s disease patients.
1.2 Pharmacological treatment of Parkinson’s disease

There is currently no cure for Parkinson’s disease. Several therapies are available to both delay the onset of motor symptoms and to ameliorate such symptoms. All of these therapies are designed to enhance dopamine signalling in the brain either by replacing dopamine, mimicking dopamine, or prolonging the effect of dopamine by inhibiting its breakdown (Fig. 1.5) (Rascol et al., 2011).

![Diagram of pharmacological targets](image)

**Fig. 1.5 Location of pharmacological targets.** Inhibitors of catechol O-methyl transferase (COMT) and dopa decarboxylase act in the periphery to maximize delivery of levodopa across the blood-brain barrier. Levodopa is taken up presynaptically and converted to dopamine before release. Amantadine's site of action is also on the presynaptic dopaminergic neuron, while DAs (and baclofen) work postsynaptically. Anticholinergics such as trihexyphenidyl act within cholinergic neurons. Selegiline and rasagiline slow dopamine catabolism extracellularly.
1.2.1 Dopamine replacement strategies

1.2.1.1 Levodopa

Levodopa therapy is the principal medical treatment for the motor symptoms of Parkinson’s disease (Tomlinson et al., 2010). It successfully provides relief from the main symptoms of the condition and dramatically enhances the quality of life of patients with Parkinson’s disease by improving motor function, mobility and enabling participation in everyday activities (Fahn et al., 2004; Lindahl, 2011). Prior to the use of levodopa, anticholinergics and stereotaxic surgery to perform thalamotomies were used to treat individuals with Parkinson’s disease (Fox et al., 1991). The stereotaxic thalamotomy of the 1950s and 1960s was particularly effective for controlling tremor, somewhat effective for rigidity, and less so for bradykinesia, however, it had no effect on loss of postural reflexes. The procedure did not slow progression of the disease, and elderly patients were prone to develop complications associated with stroke (Fahn, 1993). The disabling symptoms of akinesia and bradykinesia, which had not responded to anticholinergics, did respond to levodopa (Cotzias et al., 1967) and severely disabled patients became more mobile (Cotzias et al., 1969). While motor and central nervous system problems, which will be discussed, are associated with levodopa administration, it has remained the most effective pharmacological treatment of Parkinson’s disease for the past 50 years (Cotzias, 1968; Cotzias et al., 1969; Singh et al., 2007).

Almost 99% of ingested levodopa, when administered by itself, is metabolised peripherally before it can cross into the brain, which necessitates larger amounts of levodopa to achieve the effective central nervous system dosing (Mannisto & Kaakkola, 1990). To overcome this limitation, a combination of carbidopa and levodopa is
administered. Carbidopa is a peripherally-restricted aromatic-l-amino-acid decarboxylase inhibitor which prevents the peripheral conversion of the levodopa to dopamine, allowing more available levodopa to cross the blood-brain barrier, enter the central nervous system and ultimately smaller doses to be used (Fahn, 2008; Robertson et al., 1989).

1.2.1.2 Dopamine agonists

Dopamine agonists directly stimulate the postsynaptic dopamine receptors on the GABAergic neurons in the striatum and thus mimic the action of the neurotransmitter (Schwarz, 2003). When used alone in early Parkinson’s disease, dopamine agonists may reduce symptoms of the disease, especially those that affect motor function, such as stiffness and bradykinesia. Although they are not as effective as levodopa in controlling symptoms, they have the benefit of postponing the need for levodopa therapy (Bonuccelli et al., 2009). This in turn may help delay the onset of levodopa-related motor fluctuations which develop following continued levodopa administration. Four trials have shown that the modern dopamine agonists, cabergoline, pergolide, pramipexole, and ropinirole, produce less dyskinesias compared with levodopa over 3–5 years of therapy (Clarke, 2003). Apomorphine administered via continuous infusion can improve both motor fluctuations and dyskinesias (Colzi et al., 1998; Stocchi et al., 2002). Due to the invasive nature of this therapy (that is, utilising subcutaneous pumps), it is not commonly used. Dopamine agonists are most effective in patients who have not previously been administered levodopa treatment (Talati et al., 2009). However, when levodopa is added to the agonist to maintain adequate symptomatic control of motor symptoms, the risk of motor complications re-emerges (Holloway et al., 2004).
1.2.1.3 Enzyme inhibitors

The rationale for the use enzyme inhibitors in Parkinson’s disease is that inhibition of the enzymes that breakdown dopamine prolongs the availability and action of the neurotransmitter (or exogenous levodopa) in dopaminergic striatal synapses.

1.2.1.3.1 Catechol-O-methyl transferase (COMT) inhibitors

COMT is an enzyme which is involved in the metabolism of catecholamine neurotransmitters including dopamine. 3-Methoxytyramine is a metabolite of dopamine formed by the introduction of a methyl group to dopamine by the enzyme. COMT inhibitors, which inhibit the enzymatic breakdown of dopamine by COMT, are widely used as an addition to levodopa in the treatment of patients with Parkinson’s disease who suffer motor fluctuations (Smith et al., 2005). When levodopa is administered with a DOPA decarboxylase inhibitor, peripheral metabolism occurs primarily by way of COMT, and only about 10% of a given dose gains access to the brain (Mannisto et al., 1990; Olanow & Stocchi, 2004). Administration of levodopa/carbidopa with a COMT inhibitor extends the levodopa plasma elimination half-life by approximately 50–75% and minimises variability in plasma levodopa levels (Nutt et al., 1994). Double-blind, placebo-controlled trials have demonstrated that these pharmacokinetic changes are associated with reduced “off” time, increased “on” time, and enhanced motor scores in fluctuating Parkinson’s disease patients (Brooks & Sagar, 2003; Pahwa et al., 2006; Rinne et al., 1998). They help provide a more stable, constant supply of levodopa, which makes its beneficial effects last longer. COMT inhibitors are always taken in combination with levodopa as they have no beneficial effect by themselves.
1.2.1.3.2 Monoamine Oxidase-B Inhibitors

Monoamine oxidase is a flavin-containing enzyme responsible for the oxidative deamination of endogenous monoamine neurotransmitters including dopamine. The enzyme exists in two forms, monoamine oxidase-A and monoamine oxidase-B, responsible for the deamination of 5-hydroxytryptamine and noradrenaline and the metabolism of dopamine, respectively (Sandler, 1983). Its activity is significantly higher in the brains of patients with Parkinson’s disease. The degeneration processes associated with age and Parkinson’s disease may also be attributed to oxidative stress due to increased monoamine oxidase activity and consequent increased formation of hydrogen peroxide by monoamine oxidase-B. Therefore, monoamine oxidase-B inhibitors may act by both reducing the formation of oxygen radicals and elevating the levels of monoamines in the brain (Foley et al., 2000). Rasagiline and selegiline are the most common monoamine oxidase-B inhibitors available on the market. Both are irreversible inhibitors of the enzyme (Youdim et al., 2001). Rasagiline is approved for the treatment of signs and symptoms of Parkinson’s disease as initial monotherapy and as adjunct therapy to levodopa. As monotherapy, it may reduce Parkinsonian disability and as adjunctive therapy, it may reduce the “off” state and increase the dyskinesias-free “on” state (Rabey et al., 2000). Adding selegiline to levodopa improves established motor fluctuations (Pahwa et al., 2006). It improves Parkinsonian motor symptoms and delays the need for levodopa treatment by several months (Palhagen et al., 2006).

1.2.1.4 Cholinergic Antagonists

The anti-Parkinsonian effect of cholinergic antagonists was discovered in 1867 and for nearly a century, they remained the only drugs available for the symptomatic treatment of Parkinson’s disease. Initially, naturally occurring alkaloid extracts were used. These were
increasingly replaced with synthetic cholinergic antagonist agents from the 1940s on (Katzenschlager et al., 2003). They are competitive antagonists of muscarinic receptors and it is believed that they play a role in correcting the imbalance between the dopaminergic and cholinergic pathways in the striatum following the loss of the nigrostriatal dopamine neurons (Brocks, 1999). It is believed that anticholinergics can correct this imbalance in less advanced forms of Parkinson’s by reducing the degree of neurotransmission mediated by neostriatal acetylcholine, thereby reducing involuntary movements (Olanow et al., 2001). Although few in number (1 – 2% of the total cell population of striatum), the cholinergic interneurons are among the largest striatal cells and have extremely dense axonal arbours. These cells receive prominent synaptic contacts from the substantia nigra, thalamus and cortex, and modulate the activity of the striatal projection neurons and GABAergic interneurons. In the striatum, dopamine inhibits acetylcholine release from the cholinergic interneurons (Pisani et al., 2007). Although this class of medication is fraught with adverse effects, especially in the elderly, notable pharmacotherapeutic benefits can be obtained in the treatment of Parkinson’s disease-related tremor. Because of the potential for side effects, this class of drugs is usually reserved for tremor that is resistant to dopaminergic agents. The most commonly used anticholinergics are trihexyphenidyl, benztropine, and procyclidine (Rezak, 2007).

1.2.2 Limitations of current pharmacological treatments

A major hurdle in the symptomatic treatment of Parkinson’s disease is the high likelihood of the development of adverse reactions (Rowe et al., 2008). Indeed, the evolution of drug induced adverse reactions over time can lead to motor dysfunction as prominent as those caused by the disease itself. These drug-induced adverse reactions will be outlined below.
In addition to the adverse reactions, the main limitation of current symptomatic therapies for Parkinson’s disease is that they provide symptomatic relief only but the disease pathogenesis remains unaltered. The rate of nigral cell death in Parkinson’s disease is not exactly known, but neuroimaging techniques estimate cell death occurs at a rate of approximately 10% per year (Morrish et al., 1998). This ultimately leads to further destruction of the dopaminergic neurons causing increased motor dysfunction as well as decreased effectiveness of symptomatic therapies. Thus, an effective disease-modifying, neuroprotective therapy remains a major unmet clinical need for Parkinson’s disease patients.

1.2.2.1 Levodopa

Despite its major advantages, levodopa has no impact on many of the problems faced by Parkinson’s disease patients. Several motor features, including speech, gait, posture and balance, do not typically respond to levodopa and tend to deteriorate over time despite optimal levodopa therapy (Ho et al., 2008). In addition, levodopa does not improve and indeed tends to aggravate non-motor Parkinsonian symptoms such as hallucinations, cognitive impairment, and orthostatic hypotension (Banerjee et al., 1989; Chaudhuri et al., 2006). Furthermore, most levodopa–treated patients develop motor fluctuations and abnormal movements termed levodopa-induced dyskinesias that tend to worsen with continuing levodopa exposure (Cotzias et al., 1969; Jankovic, 2008; Stocchi et al., 2010). These symptoms are especially common in patients with young-onset Parkinson disease. They are unique to levodopa and are not produced by the other antiparkinsonian drugs to date. As the disease advances, the effect of levodopa begins to wear off and it is reported that dopamine nerve terminals are able to store and release dopamine early in the course of disease but, with more advanced disease and increasing degeneration of dopamine
terminals, the concentration of dopamine in the basal ganglia is much more dependent upon plasma levodopa levels. Plasma levels may fluctuate erratically because of the 90 minute half-life of levodopa and the frequently unpredictable intestinal absorption of this medication. Motor fluctuations are alterations between periods of being "on," during which the patient exhibits a good response to medication, and being "off" during which the patient experiences symptoms of the underlying Parkinsonism. In an "off" state, patients can become very stiff, slow and may be unable to move for a few minutes.

There has also been concern that levodopa might accelerate neuronal degeneration by way of its oxidative metabolism, although this remains a matter of controversy (Parkkinen et al., 2011; Ziv et al., 1997). In vitro studies demonstrate that levodopa can be toxic to cultured dopamine neurons (Melamed et al., 1998), but levodopa is not toxic to dopamine neurons in vivo and might even have neuroprotective effects (Murer et al., 1998). There is no clinical evidence to date to suggest that levodopa adversely alters the rate of Parkinson’s disease progression and most experts consider it highly unlikely that the drug has toxic effects in Parkinson’s disease patients (Agid et al., 2002).

1.2.2.2 Dopamine agonists

One of the main advantages of administering dopamine agonists in the treatment of Parkinson’s disease is their efficacy in treating and preventing motor complications (Nutt & Wooten, 2005). Peripheral adverse events due to apomorphine administration such as nausea, vomiting and orthostatic hypotension are common but can often be controlled by the dopamine D2 receptor antagonist, domperidone, until tolerance emerges within three
to six weeks of initiation of treatment (Poewe & Wenning, 2000). Neuropsychiatric side effects may be seen in some patients, including mild sedation, visual hallucinations, paranoia and hypomania. Marked psychosis rarely develops and can usually be well controlled by clozapine (Pietz et al., 1998). Administering an agonist in combination with levodopa to patients already experiencing motor fluctuations reduces the time spent in the “off” state (Guttman et al., 1997; Lieberman et al., 1997). One of the drawbacks of this treatment regime is that it can cause more dyskinesias. Nonmotor problems such as excessive daytime somnolence, sleep attacks and impulse control disorders have been increasingly recognised among individuals with drug-treated Parkinson’s disease (Paus et al., 2003; Weintraub et al., 2006). Sleep problems can have an impact on driving in young people with Parkinson’s disease and impulse control disorders, pathological gambling and hypersexuality, for example, may have devastating social and economic consequences. While all of these problems may occur with levodopa monotherapy (especially at higher doses), they appear to be significantly more common among patients treated with dopamine agonists and often resolve with discontinuation or reduction of the agonist (Biglan & Ravina, 2007; Chiang et al., 2011; Gallagher et al., 2007). Peripheral dopaminergic side effects (such as orthostatic hypotension) are also more likely with dopamine agonists compared with levodopa. The ergot agonist pergolide, has been voluntarily withdrawn by the manufacturer in the United States because of the risk of cardiac valve fibrosis and patients maintained on this agent in other countries undergo regular echocardiographic assessments (Lees, 2005).

1.2.2.3 COMT inhibitors

The COMT inhibitor tolcapone, can cause severe liver toxicity leading to restrictions in its prescription and the need for regular hepatic monitoring (Watkins, 2000). A sharp
reduction of dopaminergic agents while introducing tolcapone or its abrupt discontinuation may precipitate this potentially lethal complication (Rivest et al., 1999). Most other associated side effects are often mild in severity and typically consist of nausea, anorexia and sleep disorders. Diarrhoea is the adverse effect most often reported (Rezak, 2007). Entacapone has been associated with increased levels of dyskinesias when administered with levodopa (Kaakkola, 2000).

1.2.2.4 Monoamine oxidase-B inhibitors

First generation monoamine oxidase inhibitors (non-selective and irreversible), initially developed and applied in therapy as anti-depressive compounds about fifty years ago, have serious side-effects, including hepatotoxicity and orthostatic hypotension. Increased rates of elevated serum aspartate transaminase and alanine transaminase levels have been noted, indicating liver damage (Cooper, 1989). Selegiline, especially when used in combination with levodopa, can cause anorexia, nausea, dry mouth, dyskinesias, and orthostatic hypotension in patients with Parkinson’s disease, with the latter being most problematic (Volz & Gleiter, 1998). In monotherapy, anorexia, nausea, musculoskeletal injuries, and cardiac arrhythmias occurred more often in patients receiving selegiline compared with those receiving placebo. Hypertensive crisis may occur more frequently in elderly than in younger patients, because cardiovascular systems of the elderly are already compromised by age (Yamada & Yasuhara, 2004b). Acute overdose with monoamine oxidase-B inhibitors cause agitation, hallucinations, hyperpyrexia, hyperreflexia and convulsions (Murphy & Kalin, 1980). Abnormal blood pressure is also a toxic sign and gastric lavage and maintainance of cardiopulmonary function may be required. If an monoamine oxidase-B inhibitor is discontinued abruptly rather than in progressive steps,
symptoms of agitation, irritability, pressured speech, insomnia or drowsiness, psychosis and delirium may result (Alvarez et al., 2007).

1.2.2.5 Cholinergic antagonists

In terms of motor performance, these agents have little influence on reducing bradykinesia or akinesia (Comella and Tanner, 1995). The side effects of cholinergic antagonists often limit their dosing. Side effects such as confusion, hallucinations, drowsiness, agitation and executive dysfunctions are common (Bedard et al., 1999; Ehrt et al., 2010; Starkstein, 2010). Effects on memory have also been documented with an increased propensity to develop dementia (Comella and Tanner, 1995). Furthermore, abrupt withdrawal leads to precipitation of acute Parkinsonian symptoms (Horrocks et al., 1973).

1.2.3 Development of novel pharmacological therapies

The preceding discussion has highlighted the limitations of the current pharmacological therapies for Parkinson’s disease, including inducing symptomatic relief only with no alteration of the disease pathogenesis and lack of protection of the nigrostriatal neurons, as well as a plethora of adverse drug reactions and reduced effectiveness with continued disease progression. This has prompted the search for novel and alternative pharmacological targets and neuroprotective therapies (Schapira, 2005). In this context, there are data to suggest a benefit from glial cell line-derived neurotrophic factor, neuroimmumophlin ligands, minocycline, Coenzyme Q10, creatine, reduced glutathione, adenosine A2A receptor antagonists as well as glutamate release inhibitors and prodrugs. In order to strengthen the pharmacological activity of antiparkinsonian drugs, enhancing their penetration of the blood–brain barrier, different approaches are possible. Among
these, the prodrug approach appears promising, and many prodrugs have been prepared in an effort to optimize physicochemical characteristics. In addition, novel therapeutic strategies based on formulations linking dopaminergic drugs with neuroprotective agents, increasing striatal levodopa levels and offering sustained release of the drug without any fluctuation of brain concentration, offer promising avenues for development of other effective new treatments for Parkinson’s disease (Gibson et al., 2012; Sozio et al., 2012; Van der Schyf & Geldenhuys, 2011).

Thus, without the development of novel therapeutics to overcome these limitations, the prognosis for Parkinson’s disease patients remains rather bleak. A major goal of preclinical Parkinson’s disease research is to develop novel disease-modifying, neuroprotective therapies which could slow or halt the underlying neurodegenerative process and ameliorate the disease symptoms. To facilitate this, improved animal models of Parkinson’s disease must be developed which replicate its classical features and which can be used to test the effectiveness of novel experimental therapies (Emborg, 2004). In the next section, the current models of Parkinson’s disease will be described.

1.3 Preclinical models of Parkinson’s disease

1.3.1 Requirements of a relevant animal model

To understand better the pathophysiology of Parkinson’s disease and to develop novel therapies for improved symptomatic management, it is important to have relevant models of the disease, in which new pharmacological agents and treatment strategies can be assessed before clinical trials are initiated (Shimohama et al., 2003). In order to be both a) a relevant model of human Parkinson’s disease, and b) a relevant test subject for new therapies, the ideal animal model should have a number of characteristic features. First,
there should be a normal complement of dopaminergic neurons at birth, more than 50% of which are then selectively, gradually and measurably lost in adulthood. Second, the model should display relevant spontaneous motor deficits. Third, it should show the characteristic Lewy body neuropathology in the form of α-synuclein accumulation. For a model to be effective and relevant, these features need to be incorporated in a progressive and stable manner without causing overt peripheral or non-specific toxicity. Finally, it should have a relatively short disease course of a few months, allowing rapid and less costly screening of therapeutic agents (Beal, 2001; Emborg, 2004). The validity of animal models is evaluated by three sets of criteria: predictive validity (capacity of the model to predict some event of the clinical condition), face validity (phenomenological similarities between the model and the clinical condition) and construct validity (some similarities between the mechanisms underlying animal behaviour and psycho- or neurobiological mechanisms underlying the clinical condition). The criterion for face validity is that the model should simulate the etiology, biochemistry, symptomatology and treatment of the clinical condition being modeled. However, some of these aspects are also included in other validity criteria (e.g., treatment in predictive validity and biochemistry in construct validity) and others are difficult to assess due to clinical uncertainty (e.g., etiology of psychiatric disorders) (Skalisz et al., 2002; Willner, 1986).

1.3.2 Historical overview of Parkinson’s disease models

It has been 50 years since dopamine was identified as a neurotransmitter and the use of reserpine to deplete stores of monoamines in the brain was shown to produce hunched immobility in rodents that could be reversed by administration of levodopa (Carlsson et al., 1957). Although quite a crude pharmacological mimic of the neurochemistry of Parkinson’s disease, this model was instrumental in first demonstrating the therapeutic
efficacy of what still remains the gold-standard treatment for Parkinson’s disease. Reserpine interferes with the storage mechanisms of monoamines in synaptic vesicles (Miller et al., 1999; Spina & Cohen, 1989). Thus, its systemic injection provokes a depletion of brain monoamines that, depending on the number of injections, can last for several weeks (Bezard et al., 1998b; Tolwani et al., 1999). Reserpinisation is still used to produce a rodent model of Parkinson’s disease, although the features are transient and no pathological changes are induced. It is easy and quick to induce, and it does alter all the monoamines affected in Parkinson’s disease (Dawson et al., 2000; Peixoto et al., 2005). Conversely, α-methyl-para-tyrosine depletes brain dopamine by inhibiting tyrosine hydroxylase. As with reserpine, α-methyl-para-tyrosine causes a rapid depletion of brain dopamine and other catecholamines, which lasts only during the period of the drug administration schedule (Bezard & Przedborski, 2011).

It was the discovery in the late 1960s of the toxicity of 6-hydroxydopamine toward catecholaminergic neurons that opened the era of toxin-based models of Parkinson’s disease (Sachs & Jonsson, 1975; Ungersted, 1968; Ungerstedt & Arbuthnott, 1970). The stereotaxic injection of 6-hydroxydopamine into the substantia nigra, medial forebrain bundle or striatum, or peripheral administration in neonatal rats induced degeneration of the nigrostriatal pathway and striatal dopamine depletion, thus mimicking events occurring in Parkinson’s disease (Ungerstedt et al., 1970).

The next breakthrough in Parkinson’s disease animal modelling came in the late 1970s with the discovery of the ability of MPTP to produce selective nigral cell degeneration and Parkinsonism in humans following its systemic administration (Davis et al., 1979; Langston et al., 1983). The resemblance of MPTP-induced Parkinson’s disease to sporadic Parkinson’s disease was so striking that researchers quickly tested this
compound in various animal species and showed that MPTP administration was able to reproduce most but not all of the clinical and pathological hallmarks of Parkinson’s disease in monkeys (Langston et al., 1984b; Mitchell et al., 1989; Nomoto et al., 1985) and at least the degeneration of dopaminergic neurons in mice (Heikkila et al., 1984), but not in rats, which were found to be resistant to this toxin (Chiueh et al., 1984). The subsequent unravelling of its mechanism of action and the identification of MPP⁺ as the active moiety opened up new ways of inducing nigrostriatal degeneration (Singer et al., 1988). The toxicity of MPTP to dopaminergic cells in non-human primates provided the first effective primate model of Parkinson’s disease (Langston et al., 1984a). The realisation that MPTP produced nigrostriatal tract degeneration through the targeting of mitochondrial Complex I led to the search for other mitochondrial toxins that might be used to model Parkinson’s disease. The best known of the models to emerge from this is the rotenone model of Parkinson’s disease, but since its first introduction (Betarbet et al., 2000; Heikkila et al., 1985) it has continued to provoke much debate.

In more recent years, since the unequivocal association of α-synuclein mutations with familial Parkinsonism (Kruger et al., 1998; Polymeropoulos et al., 1997), a number of new animal models of Parkinson’s disease have been generated by both transgenesis (Dawson et al., 2010b; Fernagut & Chesselet, 2004; Fleming et al., 2005) and viral gene transfer (Kirik et al., 2003a; Kirik & Bjorklund, 2003b; Kirik et al., 2002).

The next section examines the main models developed over the past 50 years (Table 1.2) in terms of advances they have made to the field as well as their main advantages and limitations.
<table>
<thead>
<tr>
<th>Model</th>
<th>Nigrostriatal degeneration</th>
<th>Motor impairments</th>
<th>α-Synucleinopathy</th>
<th>Advantages</th>
<th>Limitations</th>
<th>Key References</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-OHDA (into nigra or MFB)</td>
<td>Rapid dose-dependent loss of DA neurons</td>
<td>Robust spontaneous and drug-induced behavioural phenotypes</td>
<td>None</td>
<td>Full DA depletion of NS pathway, mimics late-stage PD, test therapeutic treatments</td>
<td>Not progressive, no Lewy inclusions, no extra-nigral pathology</td>
<td>(Deumens et al., 2002; Kirik et al., 1998)</td>
</tr>
<tr>
<td>6-OHDA (into striatum)</td>
<td>Circumscribed loss of DA neurons at injection site</td>
<td>Robust spontaneous and drug-induced behavioural phenotypes</td>
<td>None</td>
<td>Progressive DA loss, produces incomplete lesions that mimic PD</td>
<td>Strong striatal glial reaction, no Lewy inclusions, no extra-nigral pathology</td>
<td>(Deumens et al., 2002; Kirik et al., 1998)</td>
</tr>
<tr>
<td>MPTP</td>
<td>Rapid dose-dependent loss of DA neurons</td>
<td>Akinesia, bradykinesia, rigidity of the limb and trunk, postural abnormalities</td>
<td>None</td>
<td>Inhibits Complex I activity, test therapeutic treatments, systemic administration possible</td>
<td>Not progressive, no Lewy inclusions, not reliable in rats, reproducibility issues</td>
<td>(Langston et al., 1999; Schober, 2004)</td>
</tr>
<tr>
<td>Transgenic</td>
<td>No consistent loss</td>
<td>Variable between models with many displaying no obvious impairment</td>
<td>α-synuclein in DA neurons</td>
<td>Mitochondrial abnormalities, gliosis, loss of motor neurons, α-synuclein aggregate formation, early stage modelling</td>
<td>Poor DA phenotypes, variable motor impairments</td>
<td>(Dawson et al., 2010b)</td>
</tr>
<tr>
<td>Viral gene transfer</td>
<td>Variable loss of NS neurons</td>
<td>Impairment in subset of animals</td>
<td>Robust α-synuclein expression</td>
<td>Suitable in rats, capable of targeting DA neurons, lasting expression</td>
<td>Slow progression, variable DA loss and motor impairments</td>
<td>(Kirik et al., 2003b; Low &amp; Aebischer, 2011)</td>
</tr>
<tr>
<td>Paraquat</td>
<td>Nigral cell loss, little or no measurable change in striatal DA innervation</td>
<td>Reduced locomotor activity, stereotypic and rotational behaviours</td>
<td>None</td>
<td>Inconsistent DA loss, no Lewy inclusions, no extra-nigral pathology</td>
<td>(Manning-Bog et al., 2002; Nistico et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>Rotenone (chronic pump)</td>
<td>Dose-dependent loss of NS neurons</td>
<td>Bilateral impairment of motor function</td>
<td>α-synuclein in DA neurons</td>
<td>Inhibits Complex I activity, progressive cell loss, extra-nigral pathology</td>
<td>Large variations in animal sensitivity, varied motor response, high peripheral toxicity and mortality</td>
<td>(Betarbet et al., 2000; Ferrante et al., 1997)</td>
</tr>
<tr>
<td>Rotenone (intracerebral)</td>
<td>Time- and dose-dependent reduction of DA levels.</td>
<td>Pronounced contralateral rotation</td>
<td>None</td>
<td>Inhibits Complex I activity, less toxic than systemic rotenone</td>
<td>Large variations in animal sensitivity, no Lewy inclusions</td>
<td>(Heikkila et al., 1985; Moreira et al., 2011)</td>
</tr>
</tbody>
</table>

Table 1.2 Overview of current Parkinson’s disease models. DA: dopamine; MFB: medial forebrain bundle; NS: nigrostriatal
1.3.3 Established models

1.3.3.1 The 6-hydroxydopamine model

The characterisation of the hydroxylated analogue of dopamine, 6-hydroxydopamine, as a toxin that can induce degeneration of dopaminergic neurons in the nigrostriatal tract has led to it being a widely used tool to induce Parkinsonism in rodents (Ungerstedt, 1968). 6-Hydroxydopamine does not efficiently cross the blood–brain barrier and so requires direct injection into the brain. This is one of its main limitations as specialised stereotaxic surgical instruments and training are required. Unilateral lesions of the nigrostriatal tract are almost invariably employed since bilateral lesions result in marked adipsia and aphagia, rendering intense nursing necessary for the maintained welfare and survival of the animals (Sakai & Gash, 1994), although some studies have utilised bilateral partial lesions (Amalric et al., 1995). 6-hydroxydopamine is injected into the nigrostriatal tract at one of three locations: into the substantia nigra, into the medial forebrain bundle or the striatum (Paille et al., 2010). Following its injection, it is taken up into the dopaminergic neurons via the dopamine transporter. Given that 6-hydroxydopamine also shows high affinity for the noradrenaline transporter, systemic injection of the noradrenaline transporter inhibitor, despiramine, given 30–60 min before 6-hydroxydopamine, ensures improved specificity of the toxin for dopaminergic neurons by blocking uptake by noradrenergic neurons (Luthman et al., 1989).

Although the exact mechanism behind its toxicity is still subject to investigation, current understanding is that, once inside dopaminergic neurons, 6-hydroxydopamine initiates degeneration through a combination of oxidative stress and mitochondrial respiratory dysfunction. The 6-hydroxydopamine model also mimics many of the biochemical features of Parkinson’s disease, including reduced levels of striatal dopamine and tyrosine
hydroxylase. Since 6-hydroxydopamine produces a dose-dependent degeneration of the nigrostriatal tract, animals bearing a full or marked (>90%) lesion can be produced following administration of high amounts of 6-hydroxydopamine into each of the three sites, though in practice, injections into either the medial forebrain bundle or substantia nigra are most often used to produce ‘full’ lesions (Truong et al., 2006; Yuan et al., 2005). Injection of 8 µg 6-hydroxydopamine into either the medial forebrain bundle or substantia nigra reliably produces over 90% degeneration of cells in the substantia nigra but only 40% loss of cells in the ventral tegmental area (Carman et al., 1991). The rapid nature of the degeneration is far removed from the slowly progressive nature of Parkinson’s disease since nigral cell death commences within 12 h of 6-hydroxydopamine injection, is maximal around 6 days and remains stable for at least 4 weeks post lesion (Jeon et al., 1995; Ungerstedt, 1968; Zuch et al., 2000).

Accompanying this marked degeneration are a number of robust spontaneous and drug-induced behavioural phenotypes. So while the rapid nature of cell death means this model is open to criticism when investigating novel neuroprotective agents, it provides a stable baseline against which to monitor the efficacy of symptomatic agents (Cenci et al., 2002; Linazasoro, 2004). The most widely used locomotor assessments of unilaterally lesioned rats are the circling responses induced by the systemic injection of either the mixed D₁/D₂ agonist apomorphine or amphetamine. Through its ability to induce dopamine release, amphetamine creates an imbalance in dopamine transmission which can be detected with as little as a 50% loss of dopaminergic neurons (Barneoud et al., 2000; Hefti et al., 1980; Hudson et al., 1993). Administration of amphetamine causes excessive release of dopamine from the intact striatum compared to the damaged striatum leading to the emergence of a rotational effect whereby the lesioned animal will rotate in the direction
contralateral to the lesion side. Administration of apomorphine induces stimulation of supersensitive receptors in the lesioned striatum leading to a rotational effect whereby the lesioned animal will rotate in the direction ipsilateral to the lesion side. 6-hydroxydopamine-lesioned rats with ‘full’ lesions also display forelimb akinesia that can be measured using a range of non-invasive tests such as the adjusted Stepping Test and Cylinder Test (Austin et al., 2010; Grealish et al., 2008; Kirik et al., 1998; Olsson et al., 1995; Schallert et al., 2000).

Partial lesions induced by 6-hydroxydopamine have also been produced in some cases by reducing the dose injected into the substantia nigra or medial forebrain bundle. Although the degree of degeneration is more variable with reduced doses of 6-hydroxydopamine, in general, a dose of around 6 µg injected into either the medial forebrain bundle or substantia nigra is sufficient to produce around 70% loss of nigral cells and striatal dopamine depletion within two weeks (Costa et al., 2001; Visanji et al., 2006). However, the behavioural deficits obtained from animals bearing a partial lesion of this nature have turned out to be less reliable. While animals bearing a partial lesion do produce ipsiversive rotations in response to amphetamine, this response may be seen in as few as half the animals tested (Hefti et al., 1980). In addition, animals bearing lesions of less than 80% do not show robust deficits in the Cylinder Test (Hefti et al., 1980). Therefore, this model can only be reliably used for assessing the histological and neurochemical benefits of potential neuroprotective agents, although the rapid progression of cell death is still a limitation for such studies. A more slowly developing partial lesion of the nigrostriatal pathway has been achieved by administering 6-hydroxydopamine into the striatum, which produces striatal terminal damage within 1 day of injection, whilst nigral cell loss is minimal at one week, reaching a maximum within two to three weeks.
(Blandini et al., 2007; Kirik et al., 1998; Sauer & Oertel, 1994). Amphetamine-induced rotation is consistently observed, but the intensity of rotation does not change significantly across a wide range of nigral cell loss (between 50% and 90%) (Barneoud et al., 2000; Kirik et al., 1998; Lee et al., 1996), so, although of use in pre-screening for a partial lesion, amphetamine-induced rotation will not reliably detect functional improvements even in animals where a marked degree of neuroprotection has been achieved. Also, apomorphine, which is generally considered to only produce rotation when 90% or more of striatal dopamine content is lost, may produce rotations in animals with partial lesions (Blandini et al., 2007; Cadet & Zhu, 1992; Kirik et al., 1998; Lee et al., 1996; Przedborski et al., 1995).

With the exception of the antimuscarinic treatments, all the drugs in clinical use today have shown efficacy in the 6-hydroxydopamine lesion models, thus supporting their predictive validity (Eden et al., 1991; Fukuzaki et al., 2000; Johnson et al., 1976; Schmidt et al., 2008).

The 6-hydroxydopamine lesion model resembles Parkinson’s disease in a number of key areas. It has construct validity, combining mitochondrial dysfunction, oxidative stress and inflammation as found in the human condition and face validity, combining the associated nigrostriatal pathology and forelimb akinesia. However, the model does not capture all features of the illness. In Parkinson’s disease, pathological change occurs in many brain areas outside of the basal ganglia such as the locus coeruleus and raphe nuclei, and this is not recapitulated in the 6-hydroxydopamine model. In most forms of the 6-hydroxydopamine model, cell death occurs far more rapidly than in Parkinson’s disease, and, although this is less so following intrastriatal 6-hydroxydopamine administration, this model shows marked variability in the size of lesion and behavioural readouts.
produced. The presence of intracellular proteinous aggregates resembling Lewy bodies remains to be established, and the unilateral nature of the lesion does not mimic that of Parkinson’s disease.

1.3.3.2 The MPTP model

MPTP, as described, is commonly used for inducing both rodent and primate models of Parkinson’s disease based on its ability to induce persistent Parkinsonism in humans. Investigations in non-human primates identified that selective destruction of the dopaminergic neurons of the nigrostriatal pathway was the pathological basis behind the motor deficits observed (Chiueh et al., 1984).

The discovery of the ability of MPTP to induce Parkinsonism in humans led to an opportunity to use systemic toxin administration to produce a model of Parkinson’s disease in primates with a high degree of construct validity (Burns et al., 1983; Davis et al., 1979; Langston et al., 1983; Langston et al., 1984b). This had not been achieved before and those models previously available involved surgical approaches to destroy the nigrostriatal pathway or the use of 6-hydroxydopamine toxicity, as just described (Olanow et al., 1996). The repeated systemic administration of MPTP by intraperitoneal, subcutaneous and intravenous administration in doses that vary with species and route over three to five days leads to the onset of a Parkinsonian syndrome almost immediately and certainly within a few days of commencing treatment (Langston et al., 1984a; Mogi et al., 1998). It consists of akinesia, bradykinesia, rigidity of the limb and trunk and postural abnormalities which form cardinal symptoms of Parkinson’s disease as they occur in the human condition. However, classical rest tremor is not commonly observed; rather postural tremor is present. MPTP has a particular efficacy in destroying
dopaminergic neurons in the primate substantia nigra which is not seen in most lower species, with the exception of mice. The reason for this peculiar sensitivity is not well understood, but it may relate to the persistence of its metabolite, MPP\(^+\), in the brain for long periods of time compared with the rapid clearance that occurs in other species (Herkenham et al., 1991). It may also reflect a higher sensitivity of primate nigral dopaminergic neurons to toxins such as MPTP, since Parkinson’s disease appears to be a syndrome that specifically affects humans. Many species, including rats, are insensitive to the toxic effects of MPTP, possibly due to the relatively rapid clearance of MPP\(^+\) (Johannessen et al., 1985). However, specific strains of mice, notably black C57 are sensitive to MPTP and have enabled development of the MPTP mouse model of Parkinson’s disease (Sonsalla & Heikkila, 1988). The reason for the disparity between mice strains is not fully understood, however, it has been reported that strains which have been shown to be resistant to the effects of MPTP, such as BALB/c, become sensitive to the effects of the compound as age progresses (Filipov et al., 2009). The underlying processes involved are not fully understood however data suggests that sensitivity to MPTP is age dependent and that mice from an MPTP-resistant strain lose their resistance as they age.

The MPTP-treated mouse has some clear advantages over the 6-hydroxydopamine lesion model. Being systemically active, MPTP administration does not require the type of skilled stereotaxic surgery that production of a 6-hydroxydopamine lesion requires. The systemic injection also produces a bilateral degeneration of the nigrostriatal tract, more reflective of that seen in Parkinson’s disease. The MPTP model also mimics many of the known biochemical features of Parkinson’s disease (Gudehithlu et al., 1991; Hadjiconstantinou et al., 1985). Further downstream in the basal ganglia, extracellular
glutamate levels have been shown to be elevated in the substantia nigra of MPTP-treated mice, a rise associated with the induction of programmed cell death (Meredith et al., 2009), whilst glutathione levels are significantly reduced (Ferraro et al., 1986) as in Parkinson’s disease itself. In further support of the construct validity of this model, inflammatory markers are elevated in the striatum and substantia nigra of MPTP-treated mice (Hebert et al., 2003; Kurkowska-Jastrzebska et al., 1999), which occurs as a result of reactive microgliosis in Parkinson’s disease. The MPTP model does, however, have some clear disadvantages over the 6-hydroxydopamine model, particularly in terms of reproducibility and the range of behavioural assessment data which can be obtained. Mice are far less sensitive to MPTP than primates, and the higher doses required can be acutely lethal as a result of the peripheral neuro- or cardiotoxicity induced (Jackson-Lewis & Przedborski, 2007).

The pattern of cell death produced by MPTP in animals is similar to that seen in humans, with the substantia nigra affected more than the ventral tegmental area (German et al., 1988; Hung & Lee, 1996; Sundstrom et al., 1990), and chronic infusion may also induce loss of noradrenergic cells in the locus coeruleus, further resembling the clinical picture (Fornai et al., 2005). However, in all cases, the cell death is rapid in onset, with first signs appearing within 12 – 72 hours, and is maintained for up to 28 days (Jackson-Lewis et al., 1995; Novikova et al., 2006; Tatton et al., 1997), although striatal dopamine depletion may show signs of recovery when using acute or sub-acute MPTP dosing paradigms (Lau & Meredith, 2003). As noted for the 6-hydroxydopamine model, this rapidity of cell death is not reflective of the disease itself and is an obvious weakness of this model. Care should also be taken in studies where assessment of the effects of MPTP are limited to measurement of striatal dopamine content, as MPTP can exert a reserpine-like effect and
deplete catecholamines with recovery over the following two months (Hallman et al., 1985).

Controversy still surrounds the issue of whether MPTP-treated mice exhibit Lewy body-like inclusions. In one of the earliest studies examining this phenomenon, whilst very few inclusions were noted three weeks post-chronic MPTP treatment, by 24 weeks, several of the remaining tyrosine hydroxylase-positive nigral neurons contained α-synuclein- and ubiquitin-immunoreactive inclusions, though these did not resemble classical Lewy bodies found in the disease (Fornai et al., 2005; Meredith et al., 2002).

Where behavioural deficits are displayed, they have been shown to be reversed by some of the drugs in clinical use today, confirming a certain degree of predictive validity of some MPTP models for assessing symptomatic agents. For example, levodopa and the dopamine agonists bromocriptine, cabergoline and pramipexole reverse these behavioural deficits (Fredriksson et al., 1990; Rozas et al., 1998; Viaro et al., 2010), whilst the monoamine oxidase-B inhibitor selegiline, the COMT inhibitor tolcapone, and amantadine have been shown to potentiate the effects of levodopa in these mice. The effects of apomorphine are again more varied, with some studies showing no effect (Rozas et al., 1998), yet others showing reversal of hypokinesia (Fornai et al., 2005). The MPTP mouse model has also been able to predict the efficacy of non-dopaminergic agents, such as the A₂A antagonist, istradefylline (Shiozaki et al., 1999). In clinical trials, istradefylline was generally well tolerated and reduced “off” time as assessed by home diaries. Severity of dyskinesias was unchanged, but “on” time with dyskinesias increased (Hauser et al., 2003). The model is also expected to predict the ability of agents to provide protection or repair against degeneration in the MPTP-treated primate, especially given they share a common inducer. This is borne out by some studies, such as
that showing the ability of 5-HT$_{1A}$ agonists to protect against MPTP-induced degeneration in both mice and primates (Bezard et al., 2006).

1.3.3.3 Clinical developments from established models

The development of experimental models has allowed for improved symptomatic treatment of the motor symptoms of Parkinson’s disease since the initial discovery of dopamine depletion in the caudate-putamen. Their use has led to the introduction of novel dopamine agonist drugs into therapy, however their effectiveness in assessing nondopaminergic treatments has been lacking. The unilateral 6-hydroxydopamine–lesioned rat became the universal standard model for assessing the activity of potential anti-Parkinsonian drugs. With some exceptions, it is a relatively good predictive tool of the subsequent effect of dopaminergic drugs in humans (Dawson et al., 2002) on the dopamine-related motor features of the disease.

The value of experimental models of Parkinson’s disease has not translated when attempting to discover neuroprotective therapies however. There has not been the same success or predictability of clinical response. The prevailing reason for the poor preclinical to clinical translation is due to the models themselves. The best animal models for Parkinson’s disease would provide a gradual onset of pathophysiological symptoms and only after manifestation of symptoms would a drug or neuroprotective agent be administered to test for effectiveness (Meissner et al., 2004) Therapeutic strategies that slow or stop the neurodegenerative process of Parkinson’s disease are expected to have a major impact on its treatment. To date, however, no drug has been established to have a definitive neuroprotective effect and none has been approved for a neuroprotective indication (Rascol et al., 2011; Stocchi & Olanow, 2003; Ybot-Gorrin et al., 2011).
Current models are often limited by their acute character (i.e. a single systemic or local infusion of the toxin) that triggers an immediate degeneration of the dopaminergic neurons that does not necessarily reflect the ongoing degenerative process in Parkinson’s disease patients (Willis et al., 2004). Chronic progressive models of Parkinson’s disease are better suited to assess putative neuroprotective effects of any given substance. Moreover, as described, in humans, the onset of Parkinson’s disease symptoms occurs when nigral neuronal death exceeds 50–60% and nerve terminal death in the striatum exceeds 70–80% (Bernheimer et al., 1973). Current animal experiments do not reconcile this observation because the tested agent is usually applied prophylactically (i.e. weeks before the neurotoxin) or together with the neurotoxin. Thus, to assess neuroprotective in a similar setting to clinical trials, the tested substance should be applied after the loss of nigral dopaminergic neurons exceeds the critical threshold responsible for the onset of Parkinson’s disease symptoms (Meissner et al., 2004). This issue is rarely taken into account in the experimental design process. One example that underlines the lack of preclinical validation of putative neuroprotective agents based on a clinically driven design is provided by a study showing that iron chelation prevents MPTP-induced neurotoxicity in vivo (Kaur et al., 2003). The conclusion that iron chelation might be an effective therapy for the treatment of Parkinson’s disease remains debatable because mice were pretreated for 8 weeks before the MPTP, inducing the degeneration of the substantia nigra neurons, was applied. The planning of many studies to show neuroprotective effects of treatment strategies should assessed to ensure clinical relevance.

While the MPTP non-human primate model was developed to study Parkinson’s disease progression and assess neuroprotective drugs, its translational qualities are limited (Bezard et al., 2001). This model is useful because it allows the tested drug to be
administered from a given threshold of lesion, however, this model uses a neurotoxin that elicits its toxic effects in a manner that is known to be different from sporadic Parkinson’s disease neurodegeneration. While researchers rely heavily on 6-hydroxydopamine and MPTP as well as newer models to provide insight into disease mechanisms, there is controversy as to which model best represents the progressive nature of Parkinson’s disease and whether either established model can demonstrate the important distinction between preclinical and clinical disease states. As with MPTP, demonstrating specific and progressive dopaminergic cell loss with 6-hydroxydopamine has been disappointing with many protocols. In addition, some of these animal models cannot be used to study many of the clinical manifestations of the disease and some of the behavioural effects they induce, such as rotations, have little to no relevance to the human condition (Maetzler et al., 2009). When assessing behavioural changes in rodent models, it is important to keep in mind that although the neuroanatomical components underlying motor control may be similar for humans and rodents, the manifestation of these motor deficits may be expressed differently between species. Rodents and nonhuman primates are an important resource for the study of Parkinson’s disease, but the limitations of these models must be kept in mind when interpreting results. Nonhuman primate models are anatomically, physiologically, and behaviourally more similar to humans, but they are rarely used because of cost and ethical concerns. Rats and mice are widely used for modelling Parkinson’s disease, but no toxin or genetic model to date completely reproduces the pathophysiology seen in humans (Potashkin et al., 2010).
1.3.4 Newer models

1.3.4.1 Transgenic models

With the identification of genetic mutations associated with Parkinson’s disease, there has been a concomitant development of many transgenic animal models overexpressing or knocking out up to sixteen different genes. Attempts to produce α-synuclein-based models by overexpression of the normal or mutated human protein have led to a variety of abnormalities in the brain and spinal cord, including mitochondrial abnormalities, gliosis, loss of motor neurons, α-synuclein aggregate formation and some functional abnormalities in the nigrostriatal system, but there has not been any consistent reports of loss of nigral dopaminergic neurons (Dawson et al., 2010b). LRKK2 transgenic mice similarly display dopaminergic dysfunction and some behavioural deficits that are levodopa responsive but no nigral cell degeneration (Lin et al., 2009). The models of autosomal-recessive Parkinson’s disease, based on knockout of parkin (Dawson & Dawson, 2010a; Perez & Palmiter, 2005), PINK1 (Kitada et al., 2007) or DJ-1 (Goldberg et al., 2005) genes, despite showing the expected mitochondrial dysfunction and subtle abnormalities in dopaminergic transmission such as reduced evoked striatal dopamine release, also failed to replicate nigral pathology.

Since mutations in α-synuclein are 100% penetrant and penetrance appears to be complete in individuals who have two disease-causing mutations in Parkin, PINK1, or DJ-1, it is puzzling why there is no meaningful neurodegeneration of dopaminergic neurons in mouse models of Parkinson’s disease. Genetic background may not be the sole factor accounting for the resistance of dopaminergic neurons to degeneration. For instance, overexpression of α-synuclein via viruses is capable of eliciting neurodegeneration of dopaminergic neurons (Kirik et al., 2003b; Lo Bianco et al., 2002).
All the promoters used to drive expression of α-synuclein in transgenic models primarily drive expression in neurons, with very little expression in glia (Chesselet, 2008; Chesselet et al., 2008). Adeno-associated virus, which was used in the α-synuclein viral models, drives expression in both neurons and glia (Kirik et al., 2003b; Lo Bianco et al., 2002). Thus, it is possible that α-synuclein may need to be expressed in both neurons and glia to elicit neurodegeneration of dopaminergic neurons, with both non-cell-autonomous and cell-autonomous processes contributing to the death of the neurons.

An alternative hypothesis to explain why dopaminergic neurons are resistant to degeneration in these animal models, is that environmental factors might contribute to the neurodegeneration in humans. Consistent with this idea is the observation that toxins such as the mitochondrial Complex I inhibitors exacerbate neurodegeneration in Drosophila and C. elegans LRRK2 transgenic models (Ng et al., 2009; Saha et al., 2009). In addition, in some transgenic α-synuclein mice and PINK1 or DJ-1 deficient mice, dopaminergic neurons are more susceptible to the toxic effects of MPTP (Haque et al., 2008; Kim et al., 2005; Song et al., 2004). Arguments have been made that perhaps the reason dopaminergic neurons do not degenerate in the various vertebrate models is that there are compensatory mechanisms that prevent the loss of dopaminergic neurons during the lifespan of the mouse (Song et al., 2004).

The study of current parkin, PINK1, and DJ-1 knockouts may be useful in understanding the earliest abnormalities in the nigrostriatal dopaminergic system that occur due to these mutations. They might also be particularly useful for understanding the molecular mechanisms underlying compensation. In a similar manner, current LRRK2 transgenic models may be most useful for studying early derangements in the nigrostriatal dopaminergic system. Some transgenic α-synuclein animal models also have progressive
sensorimotor anomalies that are due to dopaminergic dysfunction (Chesselet et al., 2008). Thus, many of the models are most useful for understanding processes that precede degeneration. However, the models have failed to provide extensive phenotypic information about affected dopaminergic neurons, and there has been relatively poor characterisation of the effects in non-dopaminergic neurons. Moreover, these models have not been used to study cell-autonomous versus non-cell-autonomous neurodegeneration.

The mouse α-synuclein transgenic models with degeneration are valuable tools to study general toxicity and mechanisms of α-synuclein pathology as well as testing therapeutic strategies. Unfortunately, there has been only a paucity of neuroprotection trials in the α-synuclein mice. These studies confirm that maintaining or converting α-synuclein into its monomeric state and enhancing its degradation are therapeutic (Gupta et al., 2008). The majority of the α-synuclein transgenic models may also be useful for understanding mechanisms of degeneration in non-dopaminergic systems. Moving towards conditional knockouts or viral vector-mediated delivery of transgenes in the adult might yield better models, and there is already some evidence to support this.

### 1.3.4.2 Viral gene transfer models

The adeno-associated virus (AAV) –based vector system has gained attention as a potentially useful alternative to the more commonly used retroviral and adenoviral vectors for human gene therapy. Recombinant AAV (rAAV) is a promising gene delivery vehicle for gene therapy. Their cloning capacity of 4.5 kb can accommodate a variety of cDNAs (Rabinowitz et al., 2002). Furthermore, no diseases associated with AAV have been reported in either human or animal populations (Monahan & Samulski, 2000). The properties that make AAV an ideal vector for gene therapy include its ability to infect
both dividing and non-dividing cells and the longevity of expression in tissues such as brain, skeletal muscle, and liver. Nine primate AAV serotypes have been characterised in the literature and are designated as AAV types 1–9 (Bartel et al., 2012; Koerber et al., 2009). There is divergence in homology and tropism for various AAV serotypes (Bantel-Schaal et al., 1999; Muramatsu et al., 1996). Findings have shown that recombinant AAV5 (rAAV5) can transduce both neuronal cells and glial cells in a highly efficient manner, while rAAV2 preferentially transduces neuronal cells (Monahan et al., 2000).

The use of rAAV vectors to overexpress both normal and mutated human α-synuclein in the adult rodent and primate brain represents an important way to model Parkinson’s disease in rodents because this approach produces a rapid degeneration of nigrostriatal neurons, a feat not yet reproduced by genetic mutations in mice or rats (Kirik et al., 2003b). Furthermore, viral gene delivery revealed the ability of overexpression of wild-type α-synuclein to induce nigrostriatal pathology (Kirik et al., 2002), a finding in agreement with evidence in familial forms of Parkinson’s disease (Lee & Trojanowski, 2006). Several types of viral vectors have been used, primarily lentiviruses and adeno-associated viruses (Kirik et al., 2002; Klein et al., 2002; Lauwers et al., 2003; Mochizuki et al., 2006; St Martin et al., 2007). Because viral vector delivery requires stereotactic injections within or near the site of the neuronal cell bodies in the substantia nigra, rats are generally used, although the model has been reproduced in mice (St Martin et al., 2007).

Overexpression of human wild-type α-synuclein in the rat midbrain leads to Parkinson’s disease-like neurodegeneration in the nigrostriatal pathway, readily observable from about eight weeks after injection of a single infusion of an AAV-α-synuclein vector into the substantia nigra (Kirik et al., 2003b; Kirik et al., 2002). Immunohistochemical
staining against tyrosine hydroxylase has shown reduced dopaminergic innervation in the striatum in the injected side compared to the intact side. Intra-cytoplasmic inclusions immunoreactive for tyrosine hydroxylase are present in dopaminergic dystrophic terminals and there is a substantial loss of tyrosine hydroxylase-positive neurons in the substantia nigra on the injected side compared to the contralateral side, while the neurons in the ventral tegmental area are largely spared. Immunohistochemical staining against human α-synuclein has shown overexpression of α-synuclein in the midbrain dopaminergic neurons and in the striatal axonal terminals (Gorbatyuk et al., 2008; Kirik et al., 2002; Yamada et al., 2004a).

As previously iterated, the presence of ongoing inflammation may contribute and hasten the progression of Parkinson’s disease. This is supported by evidence of activated microglia, accumulation of cytokines and oxidative damage in post-mortem Parkinson’s disease brains (Alam et al., 1997; Floor et al., 1998). A-Synuclein protein is prone to aggregate and form intermediate species under stress conditions such as oxidative stress. Moreover, α-synuclein is known to interact with dopamine or its toxic metabolites leading to the formation of oligomers or protofibrils. These intermediates are suggested to be toxic to the cell, unless they are degraded by the endogenous pathways or neutralized by forming insoluble fibrillar inclusions (Conway et al., 2001). In the AAV-α-synuclein model, the levels of α-synuclein in nigral dopaminergic neurons are elevated to levels severalfold above normal and increased formation of toxic intermediates is proposed to be a key player in the induction of the inflammatory response, the formation of inclusions and aggregates, and cell death (Golovko et al., 2009).

Impairments in motor function have been observed in rats unilaterally intranigraly infused with AAV-α-synuclein. Although often the mean performance of the AAV-α-
synuclein-injected animals does not significantly differ from controls animals in either the amphetamine-induced rotation test, the Cylinder Test or the Stepping Test, clear impairments are observed in a subset of α-synuclein-overexpressing animals (Kirik et al., 2003b). While the AAV-α-synuclein model incorporates many of the desired features of a Parkinson’s disease model, the variability of motor impairment induction represents a clear limitation of this model.

1.3.4.3 Pesticide models

As detailed, recent epidemiological and experimental studies indicate that exposure to environmental agents, including a number of agricultural chemicals, may contribute to the pathogenesis of this disorder. Parkinson’s disease models utilising pesticides have been developed and refined over the past 25 years.

1.3.4.3.1 Paraquat and Maneb

Due to its structural similarity to MPP⁺, as described, paraquat has been postulated to be a potential neurotoxin and cause Parkinson’s disease-like symptoms. Paraquat can cross the blood-brain-barrier (although poorly) and so it can be administered both systemically and intracerebrally (Thiruchelvam et al., 2000a). Systemic dose administration of paraquat tends to range from 3–30 mg kg⁻¹ day⁻¹. Animals treated with 10 mg kg⁻¹ day⁻¹ paraquat once a week for three weeks show degeneration of dopaminergic neurons in the substantia nigra but it does not deplete striatal dopamine (McCormack et al., 2002) or cortical dopaminergic transmission (Ossowska et al., 2005). This may, however, have been due to an upregulation of tyrosine hydroxylase, as examined by these groups, and not a lack of depletion of striatal dopamine itself. The effect of systemic paraquat on behaviour has also been assessed and intraperitoneal administration of paraquat at doses
between 5-20 mg kg\(^{-1}\) can have the effect of significantly reducing locomotor activity as well as inducing stereotypic and rotational behaviours (Brooks et al., 1999; Chanyachukul et al., 2004). The accumulation of \(\alpha\)-synuclein after repeated doses of paraquat has also been documented (Brooks et al., 1999). Cell line experiments have shown that paraquat is not transported via the dopamine transporter and that it does not impair dopamine uptake (Ramachandiran et al., 2007).

Many of the studies carried out administered paraquat in conjunction with maneb, which is a manganese ethylene(bis)dithiocarbamate fungicide used in the control of early and late blights on potatoes and tomatoes and many other diseases of fruits, vegetables, field crops, and ornamentals. The pesticides are used together as it is believed that Parkinson’s disease is the result of different insults acting at the same time and that the results of these studies would be more relevant etiologically (Betarbet et al., 2002). Exposure to the two pesticides together produces greater effects on the dopaminergic system than either of the chemicals by themselves (Thiruchelvam et al., 2000b). After administration of 10 mg kg\(^{-1}\) of paraquat and 30 mg kg\(^{-1}\) of maneb, a significant degeneration of dopaminergic neurons in the substantia nigra after six weeks was noted (Martine Saint-Pierre, 2006). Twice-weekly intraperitoneal administration of 8 mg kg\(^{-1}\) paraquat and 24 mg kg\(^{-1}\) maneb combination caused a reduction of spontaneous locomotor activity and motor incoordination in mice (Li et al., 2005). Mice aged 3, 5 and 18 months treated with a combination of the chemicals show deficits in behavioural activity. The deficits in motor coordination and locomotor activity are sustained post treatment in the animals ages 3 and 5 months but the symptoms worsen in the animals aged 18 months. In line with this, the greatest reduction in dopamine metabolites and dopamine turnover is observed in the treatment group of 18 month old animals (Thiruchelvam et al., 2003). The systemic
administration of these chemicals has led to high levels of peripheral toxicity and relatively high mortality rates. In one study, following two doses of paraquat 10 mg kg\(^{-1}\) and maneb 30 mg kg\(^{-1}\), 52% of animals developed fatal lung injury (Martine Saint-Pierre, 2006). Paraquat has been shown to accumulate in lung tissue, where free radicals are formed producing diffuse alveolitis, followed by extensive pulmonary fibrosis (Dinis-Oliveira et al., 2006; Uversky, 2004). Due to the fact that paraquat does not easily cross the blood brain barrier and accumulates in peripheral tissue, a chronic systemic dose that is selectively neurotoxic has yet to be determined.

In an attempt to overcome this toxicity, paraquat has also been administered intracerebrally in some studies. Following unilateral intranigral infusion of paraquat (1–5 \(\mu\)g) there was a lasting and irreversible dose-dependent depletion of striatal dopamine in the side of the lesion and it also caused rotational behaviour in rats contralateral to the lesioned side in response to apomorphine administration (Liou et al., 2001; Liou et al., 1996).

1.3.4.3.2 Rotenone

The first study to claim that rotenone caused Parkinson’s disease-like symptoms in experimental animals was in 1985 when Heikkila and colleagues showed that intracerebral infusion of the mitochondrial toxin caused damage to the dopaminergic nigrostriatal pathway (Heikkila et al., 1985). This route of exposure was neglected following this finding for the next 20 years. In the interim, Ferrante and colleagues first showed that it caused selective damage to the striatum and globus pallidus following systemic administration (Ferrante et al., 1997). Since then both systemic and intracerebral administration of rotenone has been used in studies with varying results both in the
damage caused and the survival rates in the animals used. Systemic daily injections of rotenone have typically been administered in doses of 1.0-5.0 mg kg\(^{-1}\) day\(^{-1}\) over periods of 10-60 days with the main route of administration being intraperitoneal. Studies have shown that with low dose administration of rotenone (1.5 mg kg\(^{-1}\) day\(^{-1}\)) over 60 days there is an increase in nitric oxide levels and also development of akinesia and rigidity (Bashkatova et al., 2004). Nitric oxide plays important roles in neurotransmitter release, neurotransmitter reuptake, neurodevelopment, synaptic plasticity, and regulation of gene expression. However, excessive production of nitric oxide following a pathologic insult such as rotenone administration can lead to neurotoxicity (Parathath et al., 2006). Over a similar period, doses of 1.5-2.5 mg kg\(^{-1}\) day\(^{-1}\) have been shown to cause depletion of dopamine in the posterior striatum and substantia nigra and reduced tyrosine hydroxylase-immunoreactivity in the caudate putamen after intraperitoneal administration. Over that period, dose-dependent catalepsy was also observed (Alam & Schmidt, 2002; Biehlmaier et al., 2007). While the chronic systemic administration model by daily injection evokes some of the desired behavioural dysfunction and histological hallmarks, its validity is called to question due to the high mortality rates and systemic toxicity, such as widespread liver softening & necrosis, gastrointestinal dysfunction and loss of muscle mass, which accompany it (Lapointe et al., 2004). In doses above 2.0 mg kg\(^{-1}\) day\(^{-1}\), mortality rates above 50% have been reported along with systemic organ toxicity and significant weight loss which is some cases lead to the premature sacrificing of the animals (Richter et al., 2007; Yong Yang, 2005).

Another approach to systemic administration of rotenone is chronic infusion where studies have been carried out for up to two months. Osmotic pumps have been implanted to deliver the drug subcutaneously (Fleming et al., 2004; Milusheva et al., 2005; Panov et
al., 2005; Sherer et al., 2003a); through the femoral vein (Amie et al., 2006; Hoglinger et al., 2003) or the jugular vein (Betarbet et al., 2000; Fleming et al., 2004). As with the chronic intraperitonal injections, the doses have generally varied from 1.5-5.0 mg kg\(^{-1}\) day\(^{-1}\). The results from these studies have been variable and again dose dependent mortality is observed. It was also observed that the strain of animal used can play a role in the results found with Sprague-Dawley rats being more susceptible than Lewis rats following systemic administration (Betarbet et al., 2000). The mechanisms involved in the strain dependency are not fully understood, however, it suggests that genetic factors may modulate the toxicity of rotenone, a scenario possibly similar to the human condition. Studies have shown that a dose of 2.0 mg kg\(^{-1}\) day\(^{-1}\) disrupts motor behaviour while not causing any of the pathological hallmarks of the disease (Fleming et al., 2004), while others have shown that the same dose can cause highly selective nigrostriatal dopaminergic lesions and also the formation of α-synuclein positive cytoplasmic aggregates in the nigral neurons (Sherer et al., 2003c). While there is a dose-dependent decrease in tyrosine hydroxylase staining and increase in motor deficits, there is also a dose-dependent decrease in survival rates. Peripheral organ toxicity and weight loss are also often seen with increasing rotenone doses (Ferrante et al., 1997; Fleming et al., 2004; Ravenstijn et al., 2008; Zhu et al., 2004). Low doses of 1.5 mg kg\(^{-1}\) day\(^{-1}\) have shown 100% survival rates after 28 days of treatment, however, with only about 20–30% decrease in tyrosine-hydroxylase staining in the substantia nigra (Amie et al., 2006).

1.3.5 The intracerebral rotenone model

As a method of trying to reduce peripheral toxicity and induce a unilateral depletion of dopamine, some recent studies have administered rotenone intracerebrally. An initial study examining the effect of rotenone on the dopaminergic system showed that direct
stereotaxic infusion of rotenone into the medial forebrain bundle damaged the nigrostriatal pathway, marked by reduced dopamine levels in the striatum (Heikkila et al., 1985). Stereotaxic infusion did not cause selective dopaminergic depletion as serotonin levels in the striatum were reduced also. Acute doses (0.5-12 µg) of the toxin have been injected into the substantia nigra and median forebrain bundle and have resulted in a significant decrease in Complex 1 activity along with a significant time- and dose-dependent reduction of dopamine levels (Klein et al., 2011; Norazit et al., 2010; Rojas et al., 2009; Saravanan et al., 2005; Sindhu et al., 2006; Sindhu et al., 2005; Swarnkar et al., 2011; Xiong et al., 2009). These depletions occurred in a progressive manner (Saravanan et al., 2005). Added to the depletion of dopamine is the fact that intranigral administration of rotenone also causes behavioural deficits in treated rats, such as pronounced contralateral rotation (Saravanan et al., 2006; Sindhu et al., 2006; Sindhu et al., 2005). However, a full assessment of the behavioural deficits associated with rotenone infusion has not been carried out to date. The nigrostriatal degeneration caused by intracerebral injection appears to be durable as the effects are not reversed over time (Antkiewicz-Michaluk et al., 2004), however these models failed to reproduce the extensive pathological involvement seen in Parkinson’s disease. Moreover, α-synuclein expression in the dopaminergic neurons was not noted in all but one of these studies (Norazit et al., 2010; Saravanan et al., 2005; Sindhu et al., 2005). As rotenone is being directly administered to the brain, the propensity to cause systemic damage is greatly reduced and so mortality rates are much decreased.

1.3.6 Development of more relevant models

To summarise the main message of the previous paragraphs, modelling Parkinson’s disease remains a major challenge for preclinical researchers as existing models fail to
reliably recapitulate all of the classic features of the disease, namely, the progressive emergence of a bradykinetic motor syndrome with underlying nigrostriatal \( \alpha \)-synuclein protein accumulation and nigrostriatal neurodegeneration. One limitation of the existing models is that they are normally induced by a single neuropathological insult even though the human disease is thought to be multifactorial with genetic and environmental factors contributing to the disease pathogenesis. Until improved preclinical models are developed with improved construct, face and predictive validity, the prognosis for Parkinsonian patients remains bleak. Moreover, without an effective treatment, Parkinson’s disease remains a significant economic burden globally. Thus, development of a relevant model that will facilitate the development of an effective disease-modifying, neuroprotective therapy will not only improve the life expectancy of the patients themselves, as well as the quality of life of their carers, but will also significantly reduce the economic cost to health services internationally.
1.4 Aim of this thesis

Thus, the aim of this thesis is to develop and characterise a novel rat model of Parkinson’s disease that recapitulates the main neuropathological and motor impairments of the human condition.

Of the existing models, the two that show most promise are the rotenone model and the AAV-α-synuclein viral gene transfer model. The systemic rotenone model has incorporated the triad of classic features of the human condition but is associated with serious toxicity issues that may be overcome by intracerebral delivery. The AAV-α-synuclein model also recapitulates the classic features of Parkinson’s disease but is limited by high variability.

Therefore, the first series of experiments will focus on further development and characterisation of the unilateral intracerebral rotenone model of Parkinson’s disease. The second series of experiments will focus on developing a dual-hit environmental/genetic model in which rotenone is administered to rats overexpressing α-synuclein using the AAV vector. If a novel model can be developed which consolidate the classic feature of the human condition, this will yield an invaluable new tool for Parkinson’s disease researchers which should facilitate the development of novel therapies for this debilitating movement disorder.
Chapter 2: Materials and methods

2.1 Ethical statement

All procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC) under Irish Cruelty to Animals Act licences issued to Mr. Pádraig Mulcahy (licence number: B100/4064) and Dr. Eilís Dowd (licence number: B100/3827), and were approved by the Animal Care and Research Ethics Committee of the National University of Ireland, Galway (approval number: 012/08). Any experiments involving the administration of viral vectors to animals were completed under a licence from the Irish Environmental Protection Agency (GMO register number: 290).

List of materials used

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## Materials and methods

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### Chapter 2: Materials and methods

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### 2.2 Animal husbandry

A total of 243 male Sprague Dawley rats (6 weeks of age and weighing 230-250 g at experiment commencement) sourced from Charles River, UK were used in this body of research. This strain was chosen due to their known susceptibility to rotenone. Rats were maintained on a 12 hour light:dark cycle (lights on at 08:00h) under regulated temperature (21±2 °C) and humidity (30-50%) conditions. Rats were housed (4 per cage) in plastic bottom cages (50.5 x 13 x 24 cm) with a wire grid lid and wood shavings as bedding. 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 body 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, whereby animals were identified by number alone and not by treatment group for the duration of the experimental period.

### 2.3 Global experimental design

The overall aim of this project was to use the disease-associated pesticide, rotenone, and/or virally-mediated overexpression of the disease-associated protein, α-synuclein to develop a robust and reliable rat model of Parkinson’s disease that incorporates the triad of classic features of the human condition, namely, progressive motor dysfunction, nigrostriatal neurodegeneration and α-synucleinopathy.

In Chapter 3, efforts to establish the appropriate dose and infusion site for intracerebral administration of rotenone are detailed. Specifically, we sought to determine the dose and site that would induce motor dysfunction and nigrostriatal neurodegeneration without any overt local toxicity in the brain. We then went on to assess the effects of increasing doses of intracerebral rotenone on motor function, nigrostriatal degeneration, neurotransmitter levels and α-synucleinopathy. These experiments allowed us to determine whether direct intracerebral administration of the Parkinson’s disease-associated pesticide is a valid approach to modelling the human condition in the rat.

In Chapter 4, the effect of systemic administration of the pesticide on rats overexpressing human α-synuclein is examined. In these experiments, α-synuclein was overexpressed in the rat brain using AAV vectors and rotenone was administered chronically using intraperitoneal injections or subcutaneous osmotic minipumps. These experiments
allowed us to determine whether combining genetic and environmental insults is a valid approach to modelling Parkinson’s disease in the rat.

Finally, in Chapter 5, the effect of intracerebral infusion of rotenone on rats overexpressing human α-synuclein is examined. In these experiments, α-synuclein was again overexpressed in the rat brain using AAV vectors and, rotenone was infused into either the nerve terminals at the level of the striatum or the cell bodies at the level of the substantia nigra. Ultimately, these experiments allowed the modelling Parkinson’s disease by combining genetic and environmental risk factors without the toxicity that is associated with systemic administration of rotenone.

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

### 2.4 AAV preparation

#### 2.4.1 GFP and α-synuclein plasmid procurement

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

#### 2.4.2 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 37°C in a humidified 5% CO₂ 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 37°C, 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 supernatant 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 $2 \times 10^4$ cells cm$^{-2}$ in a T-175 flask. For viral transfection, HEK 293T cells were grown and passaged until a total of 25-40 T-175 flasks had reached 60-70% confluence.

2.4.3 Preparation of plasmid DNA

Competent Dh5alpha *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 -80°C (Appendix 1.1.1). Preparation of glycerol stocks of bacteria allows for long-term storage at -80°C without compromising viability of cells by reducing the harmful effect of ice crystals on bacteria which can damage cells by dehydration caused 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 37°C. Single colonies were picked and added to 2 ml nutritionally rich lysogeny broth (LB) containing 50 mg ml$^{-1}$ ampicillin and incubated overnight at 37°C 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 34°C while shaking. Bacterial cells were then harvested by centrifugation at 6000 x g for 15 min at 4°C. Plasmid isolation was performed using QIAGEN® Plasmid Gigaprep Kit (QIAGEN) according to the manufacturer’s instructions. The pellet was air-dried for 30 min and DNA was redissolved in 10 ml sterile TE buffer.
2.4.4 Plasmid quantification

Plasmid DNA quantification was determined by spectrophotometric measurement of the absorption at 260 nm using a NanoDrop® sample quantification system. Plasmids were stored at -20°C (Appendix 1.1.2).

2.4.5 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 calcium phosphate precipitation (Appendix 1.1.3). Briefly, T-175 cell culture flasks, 60-70% confluent with HEK 293T cells were split into 15 cm round cell culture dishes (one flask 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 plasmid DNA per 10 dishes by calcium phosphate precipitation in 5% DMEM. 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 centrifuged (5000 x g for 10 min), supernatant discarded and lysis buffer added to lyse the 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 37°C for 1 hour. For purification, the virus was concentrated using iodixanol gradient ultracentrifugation followed by affinity chromatography purification using Q-sepharose columns. Further concentration of the virus via centrifugal filter units resulted in a final viral volume of 250-400 µl. Viral titres were established using real time PCR, expressed as DNAse-resistant particles (drp) µl⁻¹. Viruses were aliquoted and stored at -80°C.
2.5 Surgery

2.5.1 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 the 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 polythene tubing (0.28 mm ID) filled with distilled water. 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 (with the exception of AAV solutions which were kept on dry 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) (Fig. 2.1A), while the striatum was targeted at 4 sites using stereotaxic coordinates AP +1.3, ML ±2.7; AP +0.4, ML ±3.1; AP -0.4, ML ±4.3; AP -1.3, ML ±4.7 (from bregma) and DV -5.0 (below dura) (Fig. 2.1B). All rotenone (or corresponding vehicle) infusions were 3 µl in volume at an infusion rate of 1 µl min\(^{-1}\) and allowed 2 min for diffusion (Appendix 1.2.1). All AAV infusions were 2 µl in volume at an infusion rate of 1 µl min\(^{-1}\) and allowed 2 min for diffusion. Following infusions, the incision was sutured and the rats were allowed to recover before returning to their home cage.
Chapter 2: Materials and methods

Fig. 2.1. Location of nigral and striatal injection sites. Schematic of a sagittal section of the rat brain, highlighting the nigrostriatal pathway (blue) and the mesolimbocortical pathway (purple) and showing the site of (A) intranigral and (B) 4 site intrastriatal infusions. ACC= Nucleus Accumbens, PFC= Prefrontal Cortex, SN= Substantia Nigra, STR= Striatum, VTA= Ventral Tegmental Area.

2.5.2 Osmotic minipump implantation surgery

Subcutaneous implantation of osmotic minipumps (Model 2ML4, Alzet, UK) was conducted under isofluorane anaesthesia (5% in oxygen for induction; 2% in oxygen for maintenance) as per manufacturer’s instructions (Appendix 1.2.2). The back of the animal was shaved at the implantation site. A mid-scapular incision of 2 cm was made adjacent to the site for pump placement. A haemostat was inserted into the incision and the jaws opened and closed in order to create a pocket for the pump. To allow some free movement of the pump, the pocket made was about 1 cm longer than the pump. To minimize interaction between the compound delivered and the healing of the incision, the filled pump was placed into the pocket with the delivery portal side inserted first. The wound was then closed using wound clips and the animal was allowed to recover before returning to their home cage.
2.6 Behavioural tests of motor impairment

2.6.1 Corridor Test

The Corridor Test (Dowd et al., 2005; Fitzsimmons et al., 2006) relies on the phenomenon of contralateral sensorimotor neglect where rats with unilateral dopaminergic lesions ignore food on their contralateral side and almost exclusively eat from their ipsilateral side. Rats were habituated to the apparatus in an empty corridor (150 cm long, 24.5 cm high and 7 cm wide) for 5 min. The task was carried out in an adjacent corridor in which lids containing 2-3 Coco Pops® were placed in adjacent pairs every 10 cm, allowing room at each end for the rat to turn (Fig. 2.1A). The number of ipsilateral and contralateral retrievals made by each rat was counted, and the task was complete when the rat had made 20 retrievals (maximum per trial) or had spent 5 min in the corridor. The number of retrievals made by each rat from the ipsilateral and/or contralateral sides was expressed as the percentage of the total retrievals made.

2.6.2 Stepping Test

The Stepping test (Olsson et al., 1995) measures forelimb kinesis which is impaired on the contralateral side by unilateral dopaminergic lesions. The test was carried out by the experimenter who held the rat in a comfortable position with one forepaw restrained and the other forepaw making contact with the table surface. The rat was then allowed to make adjusting steps sideways along the edge of a table over a distance of 90 cm in 5 s in a forehand and backhand direction (Fig. 2.1B). This was then repeated for the other forepaw. The number of adjusting steps made in each direction with each forepaw was counted, and data were expressed as the number of ipsilateral or contralateral steps in either the forehand or backhand direction. It was important that the experimenter
habituated each rat thoroughly to this test before data collection and that both experimenter and rat were comfortable during the test, as a stressful environment could lead the rat to make steps in an attempt to escape which could lead to a false positive result.

2.6.3 Whisker Test

The vibrissae-elicited forelimb placement test (Schallert et al., 2000) measures sensorimotor integration, which is impaired on the contralateral side by unilateral dopaminergic lesions. This test involved the experimenter supporting the rat in both hands in mid air with 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 his forepaw on the table edge (Fig. 2.1C). This was repeated 10 times with each forepaw. The number of forelimb placings on each side was counted, and the data were expressed as the number of ipsilateral or contralateral forelimb placings. As for the Stepping Test, the experimenter was required to habituate the rat thoroughly to the handling involved in this test to allow accurate interpretation of the data. It was important that both the experimenter and the rat were comfortable performing the test in order to avoid the rat making attempts to escape from the experimenter’s hold leading to false positive results.
Fig. 2.1 Photographs of rats performing the Corridor, Stepping and Whisker Tests. In the Corridor Test, the rat is allowed to retrieve Coco Pops® from either side of its body (A). During 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, while the contralateral vibrissae are brushed against the corner of a table. This stimulus elicits a reflex response in the rat where the rat reaches forward and places the free forelimb on the table (C).

2.6.4 Locomotion tracking

EthoVision® (Noldus Information Technology, Netherlands) video tracking software was used to record animal behaviours in a defined enclosure. A closed circuit camera detected and recorded the pre-defined areas into which the animals were singly placed. The video signal was digitised by a frame grabber (EasyGrab® v.5.619, Euresys, Belgium). The system detected the movements of the animal by referencing the change in the image against a freeze frame image of the arena without the animal. For this set of experiments, dark coloured sawdust was used in order to better distinguish the arena from the white coloured Sprague Dawley rat. monitoring system is a steel structure set on wheels which measures 5’ x 6’ x 2’. The system allows recording of locomotor activity in an environment which is somewhat similar to the homecage rack. The structure is divided into four separate and adjacent chambers measuring 45cm x 27cm x 40cm to allow monitoring of up to four animals at the same time. Above each of the cages were four adjustable surveillance cameras connected to a DVD recorder. Lighting in this environment was kept as close to normal home cage as possible by using dimmer switches on the lamps. As the four different cages could be viewed at the same time using
a split screen computer monitor they could all be tracked using EthoVision simultaneously. In the present experiment, distance travelled was measured over a 1 hr period.

2.7 Histology

2.7.1 Tissue processing

Rats were deeply anaesthetised with pentobarbital (100 mg kg$^{-1}$ i.p., Vétoquinol, Dublin, Ireland) and transcardially perfused with 100 ml of ice-cold heparinised saline (5000 units l$^{-1}$, Wockhardt, Wrexham, UK) followed by 150 ml of 4% paraformaldehyde (pH 7.4). Their brains were removed and placed into 4% paraformaldehyde for post-fixation for 24 hours prior to transfer to 25% sucrose. After a minimum of 48 hours equilibration in sucrose solution, 30-40 μm serial sections of the fixed brains were cut using a freezing sledge microtome (Bright, Cambridgeshire, England).

2.7.2 Immunohistochemistry

Free-floating immunohistochemistry was carried out as described previously (Grealish et al., 2008) (Appendix 1.3). All incubations were carried out in a volume of 1 ml.

For immunoperoxidase-based staining, following quenching of endogenous peroxidise activity for 5 min (using a solution of 3% hydrogen peroxide/10% methanol in distilled water), sections were washed 3 x 5 min 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, pH 7.4). Sections were then incubated overnight at room temperature in the appropriate primary antibody (in 1% normal serum and TBS containing 0.2% Triton-X, pH 7.4). The sections were washed 3 x 10 min 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 with TBS before incubating in a streptavidin-biotin-horseradish peroxidase solution for 2 hours (Vector, Peterborough, UK). Further washes with TBS (3 x 10 min) and tris-non saline (TNS) (3 x 5 min) were carried out before immunolabelling was revealed by incubating the sections in a 0.5% solution of diaminobenzidine tetrahydrochloride (DAB) in TNS containing 30% hydrogen peroxide. Sections were mounted on gelatine-coated microscope slides, dehydrated in an ascending series of alcohols (5 min in each of 50%, 70%, 100% and 100% ethanol), cleared twice in xylene (5 min 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.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Marked Population</th>
<th>Source and catalogue number</th>
<th>Host</th>
<th>Reactivity</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-synuclein</td>
<td>α-synuclein protein</td>
<td>Abcam (AB36615)</td>
<td>Mouse</td>
<td>Human</td>
<td>1:1000</td>
</tr>
<tr>
<td>α-synuclein</td>
<td>α-synuclein protein</td>
<td>Abcam (AB1903)</td>
<td>Mouse</td>
<td>Rat/Human</td>
<td>1:1000</td>
</tr>
<tr>
<td>GFP</td>
<td>GFP reporter protein</td>
<td>Invitrogen (A6455)</td>
<td>Rabbit</td>
<td></td>
<td>1:1000</td>
</tr>
<tr>
<td>Tyrosine hydroxylase</td>
<td>Catecholamine neurons</td>
<td>Millipore (MAB318)</td>
<td>Mouse</td>
<td>Rat</td>
<td>1:1000</td>
</tr>
<tr>
<td>Biotinylated secondary antibody</td>
<td></td>
<td>Vector (BA2001)</td>
<td>Horse</td>
<td>Mouse</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Table 2.1 List of primary and secondary antibodies used in this project.
2.7.3 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.

2.7.3.1 Substantia nigra cell body quantification

To quantify the survival of dopaminergic neurons in the substantia nigra after rotenone and/or AAV-α-synuclein treatment, tyrosine hydroxylase immunopositive (TH-positive) cells were counted (Fig. 2.2) in three coronal photomicrographs through the structure (AP distance from bregma (mm): -6.0, -6.4, -6.8). The number of TH-positive cell bodies in the substantia nigra ipsilateral and contralateral to the lesion was counted according to boundaries defined by Kirik et al. (1998).
For each section, counts were performed on both the left and right substantia nigra.

2.7.3.2 Striatal terminal density and volume quantification

For quantification of rotenone and/or AAV-α-synuclein-induced nigrostriatal terminal loss, the optical density of tyrosine hydroxylase immunostaining (Fig. 2.3) in three coronal photomicrographs through the striatum (AP distance from bregma (mm): +0.7, −0.3, −0.7) was analysed using ImageJ software (ImageJ v1.41o, National Institute of Health, USA). For quantification of AAV-α-synuclein-induced α-synuclein expression in the striatum, the optical density of α-synuclein immunoreactivity in the striatum was quantified in a single coronal section (AP distance from bregma (mm): −0.3). For all optical density analyses, the mean gray value of both the intact and lesioned striata was measured, together with the mean background gray 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=log₁₀(255/mean gray value)).

Fig. 2.2 Screen grab of Image J software used to count 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 substantia nigra.
Final optical densities were calculated as the difference in staining between the immunostained striata and unstained corpus callosum. For striatal volumetric analysis, ImageJ software was again used. The remaining striatal tissue was defined in a manner similar to that depicted in Fig. 2.3 whereby the border of 3 coronal sections per rat were outlined and the area calculated with reference to graticule images of known size.

Fig. 2.3 Screen grab of Image J software used to quantify optical density of staining in the striatum. Images were converted to greyscale before analysis. Yellow border depicts the area to be measured. For each section, measurements were taken of the left and right sides as well as a non-stained background measurement.

2.7.3.3 Quantification of α-synuclein positive inclusions

The number of α-synuclein immunopositive cell bodies and the area of α-synuclein immunoreactivity in the ventral mesencephalon were quantified using Image J. For each animal, photomicrographs were taken from one representative ventral mesencephalic section (AP distance from bregma (mm): −6.4) using a Nikon SMZ800 microscope with a DXM1200C digital camera. The number of α-synuclein positive cell bodies in the ventral
mesencephalon ipsilateral to the lesion were counted according to defined boundaries. ImageJ quantification was carried out similar to that of substantia nigra cell bodies as in Fig. 2.2 The medial border was defined by a vertical line passing through the cerebral aqueduct and the lateral border was defined by a line running perpendicular to the medial border just ventral the cerebral aqueduct.

2.7.3.4 Quantification of tyrosine hydroxylase positive dystrophic neurites

The number of TH-positive dystrophic neurites in the striatum were counted using ImageJ. For each animal, photomicrographs were taken from one representative striatal section (Fig. 2.4) at the same level spanning the rostrocaudal axis of the striatum 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.
Fig. 2.4 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 infused striatum.

2.8 HPLC

Striatal concentrations of dopamine, noradrenaline and 5-HT in the intact and lesioned striata were determined using HPLC with electrochemical detection as previously described (Connor et al., 1997). In brief, striatal tissue samples were briefly sonicated in 1 ml of mobile phase (0.1 M citric acid, 0.1 M sodium dihydrogen phosphate, 0.1 mM EDTA, 1.4 mM octane-1-sulphonic acid, 10% (v/v) methanol in distilled water, pH 2.8) containing the N-methyl 5-hydroxytryptamine (2 ng/20 μl) as the internal standard. Homogenates were centrifuged at 15,000 × g for 15 min and 20 μl of the resultant supernatant was injected onto a reverse-phase column (LI Chrosorb RP-18, 25 cm × 4 mm internal diameter, particle size 5 μm) for separation of catecholamines (flow rate 1 ml min⁻¹). Neurotransmitter concentrations were quantified by
electrochemical detection (Shimadzu) and chromatograms were generated using a Merck-Hitachi D-2000 integrator.

Striatal concentrations of GABA were determined using reverse-phase HPLC with fluorometric detection as described previously (Rea et al., 2005). In brief, tissue was sonicated and centrifuged as described above and GABA concentrations in the supernatant were determined off-line by pre-column derivatization with o-phthaldialdehyde/mercaptoethanol reagent (50 mg o-phthaldialdehyde (Sigma) dissolved in 1 ml methanol and added to 99 ml 0.5 mol l$^{-1}$ NaHCO$_3$ (pH 9.5) containing 10 µl 2-mercaptoethanol), and separation by reverse-phase HPLC with fluorometric detection. Samples (50 µl) were derivatized with 30 µl o-phthaldialdehyde/mercaptoethanol reagent, mixed and allowed to react for 2 min. Then 50 µl of the reaction mixture was injected by a Gilson 401C autosampler (Gilson, Villiers le Bel, France) onto the HPLC apparatus. The derivatisation mixture was separated using an isocratic mobile phase and measured by fluorometric detection. The mobile phase consisted of 70 mm di-sodium hydrogen phosphate, 400 µm EDTA, 0.15% (v/v) tetrahydrofurane and methanol (30% (v/v)). The pH of the mobile phase was adjusted to 5.26 with phosphoric acid. The HPLC system consisted of a Supercosil LC-18-DB column (150 × 4.6 mm, particle size 3 µm; Supelco Inc., Bella Fonte, PA, USA), a Gynkotec 300C high precision pump (flow rate 0.95, or 1.00 ml min$^{-1}$; Gunkotec, Germering, Germany) and a JASCO FP-1520 fluorometric detector (excitation $\lambda = 350$ nm, emission $\lambda = 450$ nm; Jasco Corporation, Tokyo, Japan).

2.9 Statistical analyses

Statistical analyses were carried out using either SPSS or Statistica software packages. Data were assessed for normality and equality of variance using Shapiro-Wilk and
Bartlett’s tests, respectively. Data are expressed as mean ± standard error of the mean. An unpaired \( t \)-test was used to compare the means of two independent groups and a paired \( t \)-test was used to compare dependent groups. 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 Dunnett’s or Newman-Keuls tests where appropriate. Analyses were deemed to be significant at \( p<0.05 \) in all cases.
Chapter 3: Development and characterisation of a novel Parkinson’s disease model induced by direct intracerebral infusion of rotenone

3.1 Introduction

It has been 50 years since the first demonstration that the dopamine precursor, levodopa, was capable of reversing the motor dysfunction associated with Parkinson's disease (Birkmayer & Hornykiewicz, 1998; Carlsson et al., 1957). Shortly thereafter, the first report of levodopa-associated motor side effects, termed dyskinesias, was published (Cotzias et al., 1969). In the intervening decades, despite substantial research into other potential drug targets, levodopa has remained the most effective treatment for the Parkinsonian motor disorder despite its associated side effects and the fact that it is simply a symptomatic, and not disease-modifying, treatment. One of the main reasons that has been suggested for the paucity of drug discovery for Parkinson's disease is the lack of a relevant animal model of the disease that incorporates the main neuropathological and symptomatic features of the human condition (Biglan et al., 2007; Kieburtz & Ravina, 2007; Linazasoro, 2004; Meredith et al., 2008; Willis et al., 2004). As described earlier in more detail, these include accumulation of the α-synuclein protein into intracellular Lewy bodies, nigrostriatal neurodegeneration and the bradykinetic motor syndrome, with all of these occurring in a progressive manner (Samii et al., 2004). The animal models that are currently in use for preclinical drug discovery trials in Parkinson's disease have succeeded in recapitulating some, but not all of these features (Beal, 2001; Dauer & Przedborski, 2003). However, one of the most promising models to have emerged in recent years is one in which the pesticide rotenone is administered systemically to experimental animals (Greenamyre et al., 2003; Perier et al., 2003).
Unlike some other neurotoxins used to model Parkinson's disease (e.g. MPTP/6-hydroxydopamine) which, by virtue of their dependence on the dopamine transporter, are selective for dopamine neurons, rotenone is not a selective dopaminergic neurotoxin. Despite this, in recent years, systemic administration of rotenone to rats has emerged as a relevant model of human Parkinsonism (Greenamyre et al., 2003; Perier et al., 2003). Systemic rotenone administration has been shown to induce α-synucleinopathy (Uversky et al., 2001), relatively selective dopaminergic neurodegeneration (Sherer et al., 2003c) and motor dysfunction (Alam et al., 2002; Zhu et al., 2004). However, one of the major drawbacks associated with this model is that it is associated with significant systemic organ toxicity (Ferrante et al., 1997; Lapointe et al., 2004), body weight loss (Alam & Schmidt, 2004b; Greene et al., 2009) and mortality rates of up to 50% (Antkiewicz-Michaluk et al., 2003; Ferrante et al., 1997). In addition to the selective effects of rotenone on the dopaminergic system, the repetitive systemic infusions cause a number of side effects, such as non-specific interference with mitochondrial respiration processes in the body resulting in multi organ damage atypical for Parkinson’s disease (Hoglinger et al., 2003; Lapointe et al., 2004). Thus, studies have investigated intracerebral infusion as an alternative route to administer rotenone.

One potential way to overcome the organ toxicity associated with systemic rotenone administration is to deliver the toxin directly to the brain using standard stereotaxic techniques. Interestingly, a report of dopaminergic neurotoxicity induced by intracerebral administration of rotenone to rats preceded the first report of systemic administration by over 10 years (Heikkila et al., 1985), and this was followed by more recent investigations by Mohanakumar and colleagues (Saravanan et al., 2005; Sindhu et al., 2006; Sindhu et al., 2005). This group have shown that injection of rotenone into the cell bodies (in the
substantia nigra) and axons (at the level of the medial forebrain bundle) of the nigrostriatal neurons leads to their degeneration. It is clear from these studies that this novel approach to modelling Parkinson’s disease in the laboratory rat merits further characterisation and validation. In particular, it is not yet known whether injection of rotenone into the nigrostriatal terminals (at the level of the striatum) can also induce nigrostriatal degeneration, or whether intracerebral rotenone causes α-synucleinopathy, as well as a motor syndrome relevant to human Parkinsonism (thus far only effects on spontaneous and drug-induced postural/turning biases have been reported (Sindhu et al., 2006; Sindhu et al., 2005).

Therefore, the aim of this chapter is to develop and characterise the intracerebral rotenone model of Parkinson’s disease by determining the effect of administration of the pesticide on body weight, spontaneous motor behaviour, nigrostriatal integrity and α-synuclein expression.

### 3.2 Methods

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

### 3.3 Experimental design

This chapter will detail the results of three separate studies. The first is a pilot study completed to determine an approximate dose of rotenone that could induce motor dysfunction without any gross structural damage. Having established the appropriate dose, the second pilot study was completed to determine the best site for administration of rotenone along the nigrostriatal neurons, that is, into the cell bodies in the substantia nigra.
or into the terminals in the striatum. Finally, after establishing the best site of administration, the main study was completed to assess the responsivity of motor function, nigrostriatal integrity and α-synuclein expression to increasing doses of rotenone.

### 3.3.1 Pilot study to determine the appropriate intracerebral rotenone dose

Male Sprague Dawley rats were unilaterally infused into the substantia nigra with rotenone (0.03-36 µg, n=5-8 rats per group) or vehicle (50-80% DMSO in saline, n=3-6 rats per group). The effect of unilateral intranigral rotenone administration on motor function was assessed in the Stepping Test of forelimb akinesia. Rats were then sacrificed by transcardial perfusion 16-21 days after infusion and the structural integrity of the midbrain was assessed in tyrosine hydroxylase immunostained sections using a qualitative rating scale where a score of 0 indicated no structural damage to the midbrain, a score of 1 indicated mild structural damage to the midbrain, and a score of 2 indicated severe structural damage to the midbrain.

### 3.3.2 Pilot study to determine the appropriate intracerebral rotenone site

Having established an appropriate intracerebral dose of rotenone, male Sprague Dawley rats were then unilaterally infused into the substantia nigra or striatum with the pesticide (nigra: 0.3 µg at a single site; striatum: 0.3 µg at 4 rostro-caudal sites (1.2 µg in total), n=6 rats per group) or vehicle (50% DMSO in saline, n=6 rats per group). Due to the size of the striatum, infusions were made at four sites along its rostro-caudal axis to produce a more uniform distribution of the toxin. The effect of unilateral intranigral or intrastriatal...
rotenone administration on motor function was assessed in the Corridor Test of contralateral neglect, the Stepping Test of forelimb akinesia and the Whisker Test of sensorimotor integration. Rats were then sacrificed by transcardial perfusion 21 days post-infusion and the effect of rotenone on nigrostriatal integrity was assessed using quantitative tyrosine hydroxylase immunohistochemistry.

3.3.3 Main study to establish the dose-related effects of intracerebral rotenone

Having established the appropriate dose and site of rotenone for intracerebral infusion, a dose-response study was then completed to assess the effect of intracerebral rotenone on the triad of classic Parkinson’s disease features (i.e. motor dysfunction, nigrostriatal degeneration and α-synucleinopathy). Forty four male Sprague Dawley rats were used, weighing 239 ± 5 g (average ± SEM, range: 232-249 g) at the start of testing. Baseline performance in the Corridor, Stepping and Whisker Tests was established over one week pre-operatively. Rats were then divided into performance-matched groups and received rotenone or control infusions into the striatum at four points along its rostro-caudal axis to yield 6 final groups as indicated in Table 3.1. Post-operative behavioural testing was carried out over a five week period following which the rats were sacrificed by transcardial fixation and assessed for quantitative tyrosine hydroxylase and α-synuclein immunohistochemistry. A separate cohort of twenty six rats was administered the same rotenone or control infusions (n=4-5 per group) and were sacrificed by decapitation 5 weeks after surgery for HPLC analyses.
Table 3.1 Final groups used in dose-response study for behavioural and histological analysis. Rats were divided into 6 performance-matched groups and were given rotenone or control infusions into the striatum at 4 points along its rostro-caudal axis to yield the final groups indicated in the table. Vehicle was DMSO, Cremophor® and saline in a ratio of 1:1:18.

3.4 Results

3.4.1 Pilot study to determine the appropriate intracerebral rotenone dose

To determine an appropriate dose of rotenone for intracerebral administration, increasing doses of the pesticide were infused into the rat substantia nigra in increasing concentrations of DMSO (n=3-6 per group). The effect of the rotenone and DMSO solutions on the structural integrity of the midbrain as well as on motor function in the Stepping Test is shown in Fig. 3.1. Higher doses of rotenone (1-36 µg) as well as higher concentrations of DMSO (70-80%) caused gross structural damage to the midbrain and induced substantial impairments in contralateral stepping performance. However, a dose of 0.3 µg in 50% DMSO did not affect the structural integrity of the midbrain but it did induce impairments in the Stepping Test (Group, $F_{(3,23)}=3.98$, $P<0.05$, post-hoc Dunnett’s confirmed 0.3 µg dose induced stepping impairments relative to 50% DMSO vehicle group at $P<0.01$). Overall, this pilot study revealed that infusion of 0.3 µg of rotenone into the substantia nigra was sufficient to induce motor impairments without gross structural damage, and that 50% DMSO was not toxic to this region.
Fig. 3.1 Pilot study to determine the appropriate dose of rotenone for intracerebral administration. The effect of increasing doses of rotenone and increasing concentration of DMSO (n=3-6 per group) on the structural integrity of the midbrain (A and C) and on motor function in the Stepping Test (B). Section in C represents a score of 2 in qualitative rating scale employed with the inset being a magnified version. Rotenone at 0.03, 0.1 and 0.3 \(\mu\)g dissolved in 50% DMSO; 1, 3 and 6 \(\mu\)g dissolved in 70% DMSO; 9, 18, 27 and 36 \(\mu\)g dissolved in 80% DMSO. Data is shown as mean ± s.e.m. **\(P<0.01\) vs. 50% DMSO vehicle by one-way ANOVA with post-hoc Dunnett’s test. One-way analysis performed comparing dose of rotenone to DMSO concentration it was dissolved in. Scale bar = 1mm Rot: rotenone.

3.4.2 Pilot study to determine the appropriate intracerebral rotenone site

Having established that unilateral intranigral infusion of 0.3 \(\mu\)g of rotenone in 50% DMSO could induce contralateral motor dysfunction without affecting the gross structural integrity of the midbrain, we then sought to assess the effect of the pesticide on body weight, motor function and nigrostriatal degeneration after intranigral or intrastriatal infusion.
3.4.2.1 Neither intranigral nor intrastriatal rotenone infusion is detrimental to the rats’ general health

Unilateral intranigral or intrastriatal rotenone in 50% DMSO did not induce mortality in any of the rats. Moreover, infusion at neither of these sites affected the body weight of the animals and rotenone-infused rats gained the same amount of weight as vehicle-infused rats (Fig. 3.2).

Fig. 3.2 Intranigral or intrastriatal rotenone infusion does not affect the rats’ body weights. Unilateral intranigral or intrastriatal infusion of rotenone had no detrimental effect on the body weight of the rats. Data is shown as mean ± s.e.m. XY graph on left represents data over the full study period while the bar chart on the right represents collapsed data from the post-operative period only. Dashed line represents day of infusion surgery. SN: substantia nigra, STR: striatum, Surg: surgery

3.4.2.2 Intrastriatal but not intranigral rotenone induces motor dysfunction

When rotenone was unilaterally infused into the substantia nigra or striatum, the motor function of the rats on the ipsilateral side was not affected (data not shown). However, the effect of the pesticide on contralateral motor function in the Corridor Test, the Stepping Test and the Whisker Test is shown in Fig. 3.3. Interestingly, although the previous pilot study indicated that infusion of 0.3 μg of rotenone into the substantia nigra
may cause motor impairments, this was not evident in this pilot study in any of the behavioural tests used. In contrast, 4-site intrastriatal infusion caused significant impairments in all 3 tests. In the Corridor Test, intrastriatal infusion of rotenone significantly impaired the rats’ ability to retrieve food from the contralateral side of their bodies (Group, $T(9)=3.15, P<0.05$), in the Stepping test, it caused a significant decrease in contralateral forelimb adjusting steps (Group, $T(9)=3.62, P<0.01$), while in the Whisker test, it significantly reduced the number of vibrissae-elicited contralateral forelimb placings (Group, $T(9)=2.37, P<0.05$). Another interesting observation from this pilot study was that the 4-site intrastriatal infusion of 50% DMSO vehicle in itself had a tendency to induce mild motor dysfunction.

![Graphs showing the effects of rotenone infusion on contralateral retrieval, backhand, and whisker test performance.](image)

**Fig. 3.3 Intrastriatal but not intranigral rotenone infusion induces contralateral motor dysfunction.** Unilateral intrastriatal but not intranigral infusion of rotenone affected the rats’ contralateral performance in the Corridor (A), Stepping (B) and Whisker (C) Tests. Data is shown as mean ± s.e.m of collapsed data from the post-operative period only. *$P<0.05$, **$P<0.01$ vs. appropriate control group by Student’s $t$-test. SN: substantia nigra, STR: striatum.

### 3.4.2.3 Intracerebral rotenone induces nigrostriatal degeneration

Once behavioural testing was completed, rats were sacrificed for quantitative tyrosine hydroxylase immunohistochemical staining (Day 21 post-rotenone infusion) to assess the impact of rotenone on nigrostriatal integrity. Analysis of immunostained sections revealed that unilateral intracerebral infusion of rotenone induced a loss of tyrosine...
hydroxylase immunostaining at the site of infusion (Fig. 3.4). Thus, intranigral infusion induced a loss of cell bodies from the substantia nigra but did not affect the terminals in the striatum, whereas, in contrast, intrastriatal infusion induced a loss of nigrostriatal terminals from the striatum but did not affect the cell bodies in the substantia nigra.
Chapter 3: Direct intracerebral infusion of rotenone

Fig. 3.4 Intracerebral rotenone infusion induces nigrostriatal degeneration at the site of administration. Intranigral infusion of rotenone caused a loss of tyrosine hydroxylase immunoreactive cell bodies from the substantia nigra (A), whereas intrastriatal infusion of rotenone caused a loss of tyrosine hydroxylase immunoreactive terminals from the striatum (B). Data is shown as mean ± s.e.m. *P<0.05 vs. appropriate control group by Student’s t-test. Scale bar: Substantia nigra = 0.5 mm; Striatum = 1 mm. TH-ir: tyrosine hydroxylase immunoreactivity, SN: substantia nigra, STR: striatum.

3.4.3 Main study to establish the dose-related effects of intracerebral rotenone

As the previous pilot study had established that administration of 1.2 µg rotenone at 4 sites along the rostro-caudal axis of the striatum (4 x 0.3 µg) could induce motor deficits and partial deafferentation of the striatum but not nigral cell body death, we then sought to further characterise this approach to modelling Parkinson’s disease by assessing the
effect that increasing doses of the pesticide had on body weight, motor function, nigrostriatal degeneration and α-synucleinopathy. As we also observed that the vehicle used in the previous study (50% DMSO) had a tendency to affect motor function when infused intrastriatally, we also tested a novel vehicle composed of DMSO, Cremophor® and saline in a 1:1:18 ratio (i.e. 5% DMSO). A similar vehicle (1:1:18 of ethanol:Cremophor®:saline) is used routinely in our (Walsh et al., 2010) and other laboratories for dissolution of lipid soluble cannabinoid drugs. Therefore, this led us to test the DMSO, Cremophor® and saline solution for dissolution and administration of rotenone.

3.4.3.1 The novel vehicle used had no overt detrimental effects

As outlined above, in this experiment, rotenone was dissolved and administered in a mixture of DMSO, Cremophor® and saline (in a 1:1:18 ratio, respectively). As we were unable to find any reports of previous use of this vehicle for direct intracerebral infusion, we sought to determine if it had any detrimental effects in its own right by including a separate saline control group in the study. As can be seen from Figs. 3.5-3.9 below, there was no significant effect of the vehicle used when compared with saline on either the rats’ body weights (Fig. 3.5, Saline vs. Vehicle, $t_{(9)}=0.29, P=0.78$, ns), motor performance (Fig. 3.6, Corridor: Saline vs. Vehicle, $t_{(9)}=0.31, P=0.76$, ns; Fig. 3.7, Stepping: Saline vs. Vehicle, $t_{(9)}=1.33, P=0.21$, ns; Fig. 3.8, Whisker: Saline vs. Vehicle, $t_{(9)}=0.43, P=0.67$, ns) or nigrostriatal integrity (Fig. 3.9, Striatum: Saline vs. Vehicle, $t_{(9)}=0.26, P=0.80$, ns; Substantia nigra: Saline vs. Vehicle, $t_{(9)}=0.48, P=0.63$, ns). Thus, DMSO:Cremophor® (polyethoxylated castor oil emulsifying agent):saline (1:1:18) is an effective and safe vehicle for dissolution of and intracerebral administration of rotenone.
3.4.3.2 Intracerebral rotenone is not detrimental to the rats’ general health

Intrastriatal rotenone did not induce mortality in any of the rats. Moreover, it did not have any effect on the body weight of the animals, and all rotenone-treated groups continued to gain weight at the same rate as the control groups (Fig. 3.5, Time, $F_{(8,280)}=838.69$, $P<0.0001$; Group, $F_{(5,35)}=2.19$, $P=0.09$, ns).

Fig. 3.5 Intrastriatal rotenone infusion does not affect the rats’ body weights. Unilateral intrastriatal infusion of rotenone had no detrimental effect on the body weight of the rats (n=5-8 per group). The XY plot on the left depicts the data collected over the course of the study, while the bar chart on the right represents collapsed data from the post-operative period only. Data is shown as mean ± s.e.m. Rot: rotenone; Surg: surgery.

3.4.3.3 Intracerebral rotenone induces motor dysfunction

Unilateral intrastriatal infusion of rotenone caused a dose-dependent impairment in contralateral motor function in the Corridor Test of contralateral neglect (Fig. 3.6), the Stepping Test of forelimb akinesia (Fig. 3.7) and the Whisker Test of sensorimotor integration (Fig. 3.8). In the Corridor Test (Fig. 3.6), intrastriatal administration of the two highest doses of rotenone led to a significant preference for the ipsilateral side of the body (Group, $F_{(5,35)}=4.58$, $P<0.01$; post-hoc Newman Keuls confirmed that 3.6 μg and 10.8 μg rotenone groups were higher than all other groups at $P<0.05$; analysis restricted to the post-operative period), and a significant impairment in the rats’ ability to retrieve
food from the contralateral side (Group, $F_{(5,35)}=4.87, P<0.01$; post-hoc Newman Keuls confirmed that 3.6 $\mu$g and 10.8 $\mu$g rotenone groups were less than all other groups at $P<0.05$; analysis restricted to the post-operative period). In the Stepping (Fig. 3.7) and Whisker (Fig. 3.8) Tests, intrastriatal administration of the highest dose of rotenone significantly reduced the number of contralateral backhand forelimb adjusting steps and vibrissae-elicited contralateral forelimb placings, respectively (Stepping: Group, $F_{(5,35)}=3.16, P<0.05$; Whisker: Group, $F_{(5,35)}=3.78, P<0.01$, post-hoc Newman Keuls confirmed that 10.8 $\mu$g rotenone group was less than control groups at $P<0.05$; analysis restricted to the post-operative period). These behavioural impairments were evident from the first post-operative week and they did not worsen over the 5 weeks of post-operative testing.
Fig. 3.6 Intrastriatal rotenone infusion induces spontaneous contralateral motor dysfunction in the Corridor Test. Unilateral intrastriatal infusion of rotenone dose-dependently decreased the rats’ contralateral retrievals (B) in the Corridor Test with a resultant increase in ipsilateral retrievals (A). The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-operative period only. Data are shown as mean ± s.e.m. *P<0.05 vs. control groups by one-way ANOVA with post-hoc Newman Keuls. Rot: rotenone; Surg: surgery.
3.7 Intrastriatal rotenone infusion induces spontaneous contralateral motor dysfunction in the Stepping Test. Unilateral intrastriatal infusion of rotenone dose-dependently affected the rats’ contralateral performance in the Stepping Test (C & D) but not in the rats’ ipsilateral performance (A & B). The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-operative period only. Data are shown as mean ± s.e.m. **P<0.01 vs. vehicle control group by one-way ANOVA with post-hoc Newman Keuls. Rot: rotenone, Surg: surgery
3.8 Intrastriatal rotenone infusion induces spontaneous contralateral motor dysfunction in the Whisker Test. Unilateral intrastriatal infusion of rotenone dose-dependently affected the rats’ contralateral performance (B) in the Whisker Test but not the rats’ ipsilateral performance (A). The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-operative period only. Data are shown as mean ± s.e.m. *P<0.05 vs. control groups by one-way ANOVA with post-hoc Newman Keuls. Rot: rotenone, Surg: surgery

3.4.3.4 Intracerebral rotenone induces nigrostriatal degeneration

Once behavioural testing was completed, rats were sacrificed for quantitative tyrosine hydroxylase immunohistochemical staining (Day 37 post-rotenone infusion) to assess the impact of rotenone on nigrostriatal integrity. Unilateral intrastriatal infusion of rotenone caused a dose-dependent degeneration of the nigrostriatal neurons as revealed by quantitative tyrosine hydroxylase immunohistochemical staining (Fig. 3.9). In the striatum (Fig. 3.9A) the two highest doses of rotenone caused a significant decrease in the density of tyrosine hydroxylase immunoreactivity on the infused side (Group, $F_{(5,35)}=18.74$, $P<0.0001$; post-hoc Newman Keuls confirmed that 3.6 µg and 10.8 µg were less than all other groups at $P<0.01$). In the substantia nigra (Fig. 3.9B), the highest
dose of rotenone caused a small but significant decrease in the number of tyrosine hydroxylase immunoreactive cell bodies on the infused side (Group, $F_{(4,36)}=3.10, P<0.05$; post-hoc Newman Keuls confirmed that 10.8 µg was less than the combined control group at $P<0.05$).

![A) B) Image](image_url)

**Fig. 3.9 Intrastriatal rotenone infusion induces nigrostriatal degeneration.** Unilateral intrastriatal infusion of rotenone caused a widespread and dose-dependant dopaminergic deafferentation of the ipsilateral striatum as indicated by a loss of tyrosine hydroxylase immunoreactivity (A). It also caused a modest loss of nigrostriatal cell bodies as indicated by a loss of tyrosine hydroxylase immunoreactive cells from the substantia nigra (B). *P<0.05; **P<0.01; ***P<0.001 vs. control groups by one-way ANOVA with post-hoc Newman Keuls. Data are shown as mean ± s.e.m. Scale bar: A = 1 mm; B = 0.5 mm. TH-ir: tyrosine hydroxylase immunoreactivity, Rot: rotenone.
3.4.3.5 Intracerebral rotenone does not affect α-synuclein expression

To determine if intrastriatal rotenone could induce changes in the expression of α-synuclein in the rat brain, quantitative immunohistochemical staining was completed (Fig. 3.10). However, there was no evidence that rotenone induced any changes in expression of the α-synuclein protein in either the striatum (Fig. 3.10A, Group, $F_{(5,35)}=0.22$, $P=0.95$, ns) or the substantia nigra (Fig. 3.10B, Group, $F_{(5,35)}=0.19$, $P=0.96$, ns).

![Image](https://example.com/image1.png)

**Fig. 3.10 Intrastriatal rotenone infusion does not affect α-synuclein expression.** Unilateral intrastriatal infusion of rotenone did not induce any changes in ipsilateral α-synuclein expression in either the striatum (A) or the substantia nigra (B) as indicated by quantitative α-synuclein immunohistochemistry. Photos are representative of vehicle and highest infused rotenone dose. Inserts are high magnification of the substantia nigra. Data are shown as mean ± s.e.m. Scale bar = 0.5 mm. α-syn-ir: α-synuclein immunoreactivity; Rot: rotenone
3.4.3.6 Selectivity of intracerebral rotenone administration

To determine the selectivity of rotenone for the dopaminergic neurons, the effect of the pesticide on striatal volume and striatal neurotransmitter content was assessed. Volumetric analysis revealed that the two highest doses of rotenone led to loss of striatal volume on the ipsilateral side (Saline: 95±5%; Vehicle: 99±2%; 0.4 µg rotenone: 90±2%; 1.2 µg rotenone: 86±4%; 3.6 µg rotenone: 65±7%; 10.8 µg rotenone: 58±7%; **P<0.01, ***P<0.001 vs. control groups by one-way ANOVA with post-hoc Newman Keuls). At a neurochemical level (Fig. 3.11), HPLC analysis of the content of various neurotransmitters in the intact and lesioned striata revealed that unilateral infusion of rotenone into the striatum caused dopamine depletion on the lesioned side (Fig. 3.11A. Side, $F_{(1,21)}=32.05, P<0.0001$; post-hoc Newman Keuls confirmed a loss of dopamine on the lesioned side at 1.2 µg and 3.6 µg rotenone) but did not have any effect on the levels of either noradrenaline (Fig. 3.11B. Side, $F_{(1,21)}=1.57, P=0.22$, ns) or GABA (Fig. 3.11 C. Side, $F_{(1,21)}=0.08, P=0.78$, ns).

**Fig. 3.11 Intrastriatal rotenone selectively depletes striatal dopamine content.** Unilateral intrastriatal infusion of rotenone caused depletion of dopamine (A) but not noradrenaline (B) or GABA (C) from the ipsilateral (lesioned) striatum. Data are shown as mean ± s.e.m. *P<0.05 vs. intact side by one-way ANOVA with post-hoc Newman Keuls. DA: dopamine; NA: noradrenaline. Rot: rotenone
3.5 Discussion

The overall aim of the experiments described in this chapter was to develop and characterise a novel model of Parkinson’s disease by direct infusion of the organic pesticide, rotenone, into the rat brain. After a series of pilot studies to establish the appropriate dose and intracerebral infusion site, in the main study, rats received a 4-site intrastriatal infusion of increasing doses of rotenone, and the effect of the pesticide on motor function, nigrostriatal integrity and α-synuclein expression was assessed. We found that intracerebral infusion of rotenone was capable of replicating some, but not all, of the classic features of human Parkinson’s disease. Specifically, infusion of the pesticide into the rat striatum induced impairments in spontaneous motor function as well as partial degeneration of nigrostriatal terminals and cell bodies. However, this approach to modelling Parkinson’s disease was not without its limitations: firstly, the behavioural impairments induced by the pesticide did not emerge in a progressive manner, secondly, there was no evidence that rotenone induced any changes in endogenous α-synuclein expression, and thirdly, intrastriatal infusion of rotenone caused a loss of striatal parenchyma. Thus, in terms of our aim to develop and characterise a novel model, we have shown that intracerebral infusion of rotenone can certainly model some, but not all, of the features of the human disease (Mulcahy et al., 2011).

Before embarking on the main study, we first completed a series of pilot experiments to establish the appropriate dose of rotenone for intracerebral infusion. The range of doses we used was quite wide and this reflects the range of doses (0.5–12 μg) used in previous studies (Alam et al., 2004a; Antkiewicz-Michaluk et al., 2004; Heikkila et al., 1985; Klein et al., 2011; Norazit et al., 2010; Rojas et al., 2009; Saravanan et al., 2005; Sindhu
et al., 2005; Xiong et al., 2009). Initial studies showed that higher doses of rotenone were extremely toxic and caused destruction of tissue around the injection site as has been subsequently reported (Klein et al., 2011; Rojas et al., 2009). The main finding of this series of pilot studies was that lower doses of rotenone were capable of inducing motor dysfunction without any gross structural damage. However, the exact threshold for rotenone-induced structural damage was difficult to establish in this pilot study due to the toxic effects of the DMSO vehicle itself (this will be discussed in more detail below).

In our second series of pilot experiments, we compared the effects of intranigral and intrastriatal infusion of rotenone on motor function and nigrostriatal integrity. We found that infusion of rotenone caused nigrostriatal degeneration at the site into which it was infused. With regards to nigral infusion, although it caused a loss of tyrosine hydroxylase immunopositive cell bodies, it did not cause any loss of terminal fibre density or motor impairments in the tests used. This is probably due to well-established compensatory mechanisms in the remaining nigrostriatal neurons such as terminal sprouting and upregulation of tyrosine hydroxylase with increased dopamine synthesis, and is known to happen in the human condition as well as in other models of Parkinson’s disease (Agid et al., 1973; Anglade et al., 1995; Zigmond et al., 1990). When rotenone was infused into the striatum, however, it led to impairments in spontaneous contralateral motor function and reductions in striatal fibre density. The extent of striatal degeneration observed the highest dose is comparable to a similar high dose of 4 x 7 μg 6-hydroxydopamine (Kirik et al., 1998). This occurred without degeneration of cell bodies in the substantia nigra. While the exact cause of this is not known, we believe that the dose and time frame used may have played a role. While the dose (0.3 μg x 4) of rotenone used was high enough to cause destruction of the dopaminergic terminals it may not have been high enough to
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retrogradely kill the associated cell bodies. The time required for die-back of the neurons to occur may also have been longer than that allowed in this study (21 days). However, our overall finding from this pilot study was that intrastriatal infusion of rotenone showed promise and warranted further investigation.

When taken together, a number of other aspects of the two initial studies merit discussion at this stage. One finding from these early studies is the toxicity associated with the use of DMSO as a vehicle. This was evident from the structural damage observed in the first pilot study as well as the behavioural data gathered in the second pilot study. Although this toxic effect of DMSO after direct intracerebral administration is well established (Authier et al., 2002; Castro et al., 1995; Garcia-Garcia et al., 2005; Hanslick et al., 2009), it continues to be used and reported as a vehicle for intracerebral rotenone administration (Klein et al., 2011; Paul et al., 2010; Swarnkar et al., 2010; Swarnkar et al., 2011). As this was clearly not satisfactory, it led us to develop a novel vehicle for dissolution and delivery of rotenone to the rat brain. The 1:1:18 combination of DMSO, Cremophor and saline we developed is based on the 1:1:18 combination of ethanol, Cremophor, and saline widely used by researchers for delivering lipid soluble cannabinoid drugs to the brain (Fride et al., 2006; Morera-Herreras et al., 2010; Rubino et al., 2008; Walsh et al., 2010). To assess this vehicle, we compared it to a saline control in the main study and found it to be both safe and effective for delivery of rotenone to the rat brain. Another interesting observation from the two pilot studies was finding that intranigral infusion of 0.3 µg of rotenone caused motor dysfunction in the first study but did not in the second study. This is not entirely surprising as rotenone is known to induce variable and somewhat inconsistent lesions in the rat (Betarbet et al., 2000). Indeed some studies have shown that the same dose of rotenone administered by the same route can
fail to induce any pathological hallmarks in some studies (Fleming et al., 2004) while causing highly selective nigrostriatal dopaminergic lesions and α-synuclein aggregates in another study (Sherer et al., 2003c). The reason why rotenone is so variable is not known though it has been reported that 2-3% of Sprague Dawley rats are resistant to the effects of rotenone. The variability may also be due to the fact that in some animals with decreased dopaminergic levels there is an enhancement of striatal tyrosine hydroxylase immunoreactivity (Kim et al., 2006; Richter et al., 2007). The variability in individual response to rotenone toxicity, even though it requires the use of larger experimental groups, provides an opportunity to identify mechanisms involved in protection or susceptibility of dopaminergic neurons to mitochondrial compromise and pesticide exposure.

Having completed our pilot studies, we then embarked on the main study to assess the responsivity of motor function, nigrostriatal integrity and α-synuclein expression to increasing doses of rotenone. One of the main limitations of the systemic rotenone model has been the high levels of toxicity and mortality which are reported following its use (Betarbet et al., 2002; Betarbet et al., 2000; Greene et al., 2009; Panov et al., 2005). Therefore, one of the initial aims of this research was to determine if intracerebral infusion of rotenone could overcome this toxicity. In this main study, no change in the rats’ general health was observed and there was no significant difference in the body weight of rats infused with any dose of rotenone when compared with control infusions. Thus, because intracerebral rotenone administration had no overall detrimental effect on the rats’ general health, this represents major fiscal, logistical and ethical advantages of this intracerebral model over the systemic models.
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Although previous studies have investigated the behavioural effects of direct intracerebral rotenone administration (Sindhu et al., 2006; Sindhu et al., 2005), these studies have focused on drug-induced rotational asymmetry. Therefore, in the present study, we chose to focus instead on the effects of intracerebral rotenone on spontaneous motor functions which bear more relevance to the human Parkinsonian state. We found that intracerebral infusion of rotenone across the rostro-caudal axis of the striatum induced a dose-dependent deficit in contralateral neglect, contralateral sensorimotor integration, and contralateral forelimb kinetics as assessed by the Corridor, Whisker and Stepping Tests respectively. These tests of lateralised motor function have not been assessed previously in a rotenone model of Parkinson’s disease. The behavioural effects observed here were not transient in nature and remained stable over the course of the five week study. However, a progressive decline in motor function was not observed. The fact that behavioural impairments did not manifest in a progressive manner is not a distinct feature of the intracerebral rotenone model however as it is also common in other well established neurotoxic models of Parkinson’s disease (Beal, 2001; Betarbet et al., 2002; Schober, 2004).

Previous studies have reliably shown that rotenone, when administered systemically (Ferrante et al., 1997; Sherer et al., 2003c) or indeed into the substantia nigra (Saravanan et al., 2005; Xiong et al., 2009) causes nigrostriatal degeneration. It was found here that intrastriatal infusion of increasing doses rotenone can also induce degeneration of the nigrostriatal neurons as revealed by quantitative immunohistochemistry and HPLC. Interestingly, the degeneration at the level of the terminals was more pronounced than the degeneration at the level of the cell bodies. This indicates that intrastriatal rotenone-induced mitochondrial inhibition can induce widespread dopaminergic deafferentation of

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the striatum without widespread retrograde degeneration of the cell bodies. We had speculated that a longer time frame following infusion might lead to increased cell body loss, however, this was only seen following the infusion of the highest dose of rotenone.

Although the neurochemical analyses suggested a selective effect of rotenone on the dopaminergic system with sparing of the GABAergic and noradrenergic systems, volumetric analyses indicated that high doses of the pesticide did induce loss of striatal parenchyma. This is not that surprising since rotenone, unlike other dopamine transporter-dependent neurotoxins used to model Parkinson’s disease, is reportedly not a selective dopaminergic neurotoxin (Cannon et al., 2009; Sherer et al., 2003b), and even such “selective” catecholaminergic neurotoxins can be toxic to intrinsic striatal neurons when infused directly into the striatum (Kirik et al., 1998; Walsh et al., 2010). Moreover, this loss of striatal parenchyma is not restricted to preclinical models as it is also found in the human brain from the early stages Parkinson’s disease and becomes more pronounced in patients who were older and had longer disease duration (Bonneville et al., 2005; Krabbe et al., 2005; Tinaz et al., 2010).

Previous studies have shown that systemic administration of rotenone in animal models can cause cerebral α-synucleinopathy (Betarbet et al., 2000; Hoglinger et al., 2003; Sherer et al., 2003c; Uversky et al., 2001), but this has not yet been reliably reported for intracerebral administration. In the present study, we found no evidence that intrastriatial administration of the pesticide could induce any changes in endogenous α-synuclein expression either in the nigrostriatal terminals or cell bodies. The reason that systemic, but not intracerebral, rotenone administration can induce α-synucleinopathy is not clear, but it is possible that one or more of the rotenone metabolites might be responsible for
this neuropathological feature (Caboni et al., 2004) even though the parent compound has been shown to be responsible for the nigrostriatal degeneration.

In summary, this study has shown that administration of the organic Parkinson’s disease-associated pesticide rotenone directly into the rat striatum shows promise as an approach to modelling the human condition (Mulcahy et al., 2011). It causes deficits in spontaneous motor function relevant to the human condition such as limb kinesis, sensorimotor integration and sensory neglect. It also induces degeneration of nigrostriatal neurons at both the terminal and cell body level and, from a neurochemical perspective, is selective for striatal dopamine. Moreover, it is not toxic to the rats’ overall health, thus presenting major advantageous over the systemic rotenone model. However, this model is not without some limitations. Firstly it induces behavioural dysfunction that does not progressively worsen over time, secondly, despite being selective for striatal dopamine at a neurochemical level, it induces a loss of striatal volume, and finally, it does not induce any changes in α-synuclein expression. Thus, although this intracerebral model may be associated with limitations, it is advantageous over the systemic model and other neurotoxin models for researchers investigating the role of the environmental agritoxins in the etiology of Parkinson’s disease, particularly in light of recent epidemiological studies (Tanner et al., 2011). Further studies in this thesis will attempt to address and overcome the associated limitations.
Chapter 4: Development and characterisation of a novel Parkinson’s disease model induced by systemic administration of rotenone combined with intranigral administration of AAV-α-synuclein

2 4.1 Introduction

As detailed previously, systemic administration of rotenone to experimental animals has recently emerged as a promising approach to modelling human Parkinson’s disease (Alam et al., 2002; Sherer et al., 2003c; Uversky et al., 2001; Zhu et al., 2004). However its widespread adoption into preclinical studies is limited by systemic organ toxicity and high mortality rates (Alam et al., 2004a; Antkiewicz-Michaluk et al., 2003; Ferrante et al., 1997; Greene et al., 2009; Lapointe et al., 2004). In the previous chapter, we attempted to overcome this toxicity by directly infusing the pesticide into the rat brain, and, although this approach was capable of modelling some of the features of the human Parkinson’s disease, it too was associated with inherent limitations (Mulcahy et al., 2011). In an attempt to address these limitations, in this chapter, we will revisit the systemic rotenone model, and investigate whether chronic systemic administration of a sub-threshold, non-toxic dose combined with virally-induced nigral α-synuclein overexpression can induce Parkinsonian features in rats.

Attempting to model Parkinson’s disease by combining environmental and genetic risk factors is a valid approach as the disease is multifactorial in nature and cannot be ascribed to a unitary etiology. Indeed, it is probable that Parkinson’s disease is the result of the net interactions of multiple risk factors encountered over the lifetime, which, in addition to those promoting risk, such as exposure to pesticides and other environmental toxins (Tanner et al., 2011), geographical location (Imaizumi, 1995; Svenson et al., 1993), head
trauma (Goldman et al., 2006), age, race, gender (Van Den Eeden et al., 2003) and genetic predisposition (Sato & Hattori, 2011), would also include factors that have shown to be protective against Parkinson’s disease, such as caffeine and cigarette smoking (Gorell et al., 1999; Ross & Petrovitch, 2001). Such a multifactorial etiology is consistent with the clinical presentation of Parkinson’s disease as it exhibits marked heterogeneity with respect to signs and symptoms that manifest, the age of onset, and the rate of progression. Therefore, to allow the disease to be modelled in a manner which more closely resembles the human condition, a multi-hit paradigm incorporating different known risk factors may be more appropriate. The nigrostriatal system, in particular, may be readily able to compensate for the effects of an individual environmental or genetic factor acting alone. However, when the system is exposed to multiple factors it may no longer be able to homeostatically regulate itself, thereby leading to sustained or cumulative damage.

Therefore, the aim of this chapter is to determine whether a multi-hit model incorporating both environmental and genetic insult exposure could induce a more relevant and robust model of Parkinson’s disease without the limitations identified in the previous chapter. To that end, we sought to characterise the effect of low dose systemic administration of rotenone on rats with targeted overexpression (induced by AAV vectors) of α-synuclein in the nigrostriatal system.

4.2 Methods

All methods have been described in more detail in Chapter 2. One exception in this chapter, however, is that quantification of behavioural impairments as well as tyrosine hydroxylase and α-synuclein immunohistochemistry is reported in absolute values. This
was necessary as, even though AAV-\(\alpha\)-synuclein was unilaterally infused, rotenone was administered systemically and thus would have a bilateral effect on the nigrostriatal system. The contralateral side to the AAV infusion site could not be considered a true intact control.

### 4.3 Experimental design

This chapter will detail the results from two main studies. The first main study was carried out to establish the effect of combined intranigral AAV-\(\alpha\)-synuclein and twice-weekly intraperitoneal rotenone administration on motor function, nigrostriatal degeneration and \(\alpha\)-synucleinopathy. The second main study was carried out to establish the effect of combined intranigral AAV-\(\alpha\)-synuclein and continuous subcutaneous infusion of rotenone (using osmotic minipumps) on motor function, nigrostriatal degeneration and \(\alpha\)-synucleinopathy. Both of these main studies were preceded by pilot studies to establish the appropriate sub-threshold, non-toxic dose of rotenone for intraperitoneal administration or continuous subcutaneous infusion, respectively.

#### 4.3.1 Pilot study to determine a sub-toxic rotenone dose for intraperitoneal injection

In order to ascertain a tolerable dose of systemically administered rotenone, male Sprague Dawley rats were injected intraperitoneally with doses of rotenone ranging from 0–1 mg kg\(^{-1}\) week\(^{-1}\) (n = 4-6 per group). This was maintained over a 12 week period as this was the planned duration of the subsequent main study with the AAV-\(\alpha\)-synuclein model. Body weight was monitored as a measure of general animal health for the duration of the study. As in indirect indicator or nigrostriatal toxicity, locomotor activity was assessed using EthoVision tracking software once every two weeks, as described in section 2.6.4,
over the course of the study where the total distance moved by each animal over a one hour period was recorded.

4.3.2 Main study to determine the effect of combined intranigral AAV-α-synuclein and low dose systemic (intraperitoneal) rotenone

Having established a dose of rotenone which was sub-threshold for toxicity, male Sprague Dawley rats were unilaterally infused with 2 µl of either AAV-α-synuclein or AAV-GFP. The viral titers were $2.93 \times 10^{10}$ drp (DNase-resistant particles) µl$^{-1}$ for AAV-α-synuclein and $1.07 \times 10^{10}$ drp µl$^{-1}$ for AAV-GFP. This was followed by twice-weekly intraperitoneal injections of rotenone ($1.0 \text{ mg kg}^{-1}$) or vehicle (1:1:18 combination of DMSO, Cremophor and saline, respectively), both delivered at 1 ml kg$^{-1}$ resulting in 4 groups as per Table 4.1. The effect of unilateral intranigral AAV-α-synuclein and systemic rotenone administration on motor function was assessed in the Corridor Test of contralateral neglect, the Stepping Test of forelimb akinesia and the Whisker Test of sensorimotor integration. Rats were then sacrificed by transcardial perfusion 13 weeks following initial AAV infusion and the combined effect of rotenone administration on nigrostriatal integrity was assessed using quantitative tyrosine hydroxylase immunohistochemistry as well as quantitative α-synuclein immunohistochemistry.
Table 4.1 Final groups used in this study. Rats were unilaterally infused with AAV-α-synuclein or AAV-GFP into the substantia nigra and this was followed by twice-weekly intraperitoneal injections of rotenone (1 mg kg⁻¹) or vehicle (DMSO, Cremophor® and saline in a 1:1:18 ratio).

<table>
<thead>
<tr>
<th>Group</th>
<th>Intranigral infusion</th>
<th>Intraperitoneal injection</th>
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<tr>
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<tr>
<td>Rotenone</td>
<td>AAV-GFP</td>
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<tr>
<td>α-Synuclein</td>
<td>AAV-α-synuclein</td>
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<td>Combined</td>
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<td>Rotenone</td>
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4.3.3 Pilot study to determine a sub-toxic rotenone dose for subcutaneous infusion

In order to determine a sub-toxic dose of rotenone for continuous subcutaneous infusion, male Sprague Dawley rats were implanted with osmotic minipumps for delivery of different doses of rotenone (2-5 mg kg⁻¹ day⁻¹ for 2-4 weeks). Body weight was monitored as a measure of general animal health for the duration of the study.

4.3.4 Main study to determine the effect of combined intranigral AAV-α-synuclein and low dose systemic (continuous subcutaneous) rotenone

Having established a dose of rotenone which was sub-threshold for toxicity, male Sprague Dawley rats were divided into two performance matched groups for unilateral intranigral infusion of 2 µl of AAV-GFP or AAV-α-synuclein. The viral titers were 2.93×10¹⁰ drp µl⁻¹ for AAV-α-synuclein and 1.07×10¹⁰ drp µl⁻¹ for AAV-GFP Post-virus motor function was assessed at weeks 4, 8 & 12 in the Corridor, Stepping and Whisker Tests. Thirteen weeks after virus infusion, rats were further sub-divided into performance-matched groups and received rotenone (2.5 mg kg⁻¹ day⁻¹) or vehicle (DMSO:PEG (1:1)) infusion via subcutaneous osmotic minipump, thus yielding 4 final
groups as indicated in Table 4.2. Post-rotenone behavioural testing was carried out over a further 4 week period following which rats were sacrificed by transcardial fixation and processed for quantitative tyrosine hydroxylase and α-synuclein immunohistochemistry.
Chapter 4: Combined AAV-α-synuclein and systemic rotenone

Table 4.2. Final groups used in this study. Rats were unilaterally infused with AAV-α-synuclein or AAV-GFP into the substantia nigra and 13 weeks later this was followed by subcutaneous implantation of osmotic minipumps delivering rotenone (2.5 mg kg⁻¹ day⁻¹) or vehicle (DMSO:PEG in 1:1 ratio).

<table>
<thead>
<tr>
<th>Group</th>
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<th>Subcutaneous infusion</th>
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<tr>
<td>Rotenone</td>
<td>AAV-GFP</td>
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<tr>
<td>α-Synuclein</td>
<td>AAV-α-synuclein</td>
<td>Vehicle</td>
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<td>Combined</td>
<td>AAV-α-synuclein</td>
<td>Rotenone</td>
<td>8</td>
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4.4 Results

4.4.1 Determination of the effect of combined AAV-α-synuclein and low dose systemic (intraperitoneal) rotenone

In this study, twice-weekly intraperitoneal injections of rotenone were administered for a period of 12 weeks following AAV infusion and the impact of the single and combined treatment on body weights, motor function, nigrostriatal integrity and α-synuclein expression were assessed. This was preceded by a pilot study to establish a sub-threshold, non-toxic dose of rotenone for intraperitoneal administration.

4.4.1.1 Pilot study to determine a sub-toxic rotenone dose for intraperitoneal injection

Over the course of this pilot study, there were no signs of ill-health in the rotenone-injected rats – they gained weight at a normal rate and there was no difference in body weight between groups (Fig. 4.1A, Time, $F_{(31,465)}=283.80$, $P<0.0001$; Group x Time,
Moreover, none of the doses of rotenone led to any changes in the rats’ locomotor activity (Fig. 4.1B, Group, $F_{(3,15)}=0.69$, $P=0.60$, ns; Group x Time, $F_{(15,75)}=0.47$, $P=0.95$, ns). The reason for the high level of locomotor activity during the first exposure is unknown, however, as the animals were in a novel arena there may have been an increase in exploratory behaviour. We therefore concluded that a once weekly dose of 1.0 mg kg$^{-1}$ was subthreshold for both peripheral and nigrostriatal toxicity. Since there was no evidence of any toxicity in this pilot study, for the main study we chose to implement a twice-weekly rather than a once weekly injection regime. This was done in an attempt to induce neuropathological effects without causing acute peripheral side effects.

Fig. 4.1 Low dose systemic rotenone infusion does not affect the rats’ body weight or locomotor activity. Low dose intraperitoneal rotenone had no detrimental effect on body weight (A) or the locomotor activity (B) of the rats. The dashed line depicts the point at which injections began. Data are shown as mean ± s.e.m. Rot: rotenone.

4.4.1.2 The effect of combined AAV-α-synuclein and low dose systemic rotenone on the rats’ body weights

Administration of neither the environmental nor genetic insults alone caused any mortality or ill-health over the course of the study and all animals continued to gain weight at the same rate as the Control group (Fig. 4.3; Group, $F_{(3,29)}=0.71$, $P=0.42$, ns;
Group x Time, $F_{(63,693)}=1.30$, $P=0.06$, ns). Furthermore, animals in the Combined group followed a similar pattern showing that administration of combined insults is not detrimental to their general health.

Fig. 4.3 Combined environmental and genetic insults do not affect the rats’ body weights. Twice-weekly low dose intraperitoneal rotenone (1.0 mg kg$^{-1}$) and/or AAV-α-synuclein administration had no detrimental effect on the body weight of the rats. The XY plot on the left depicts the data collected over the course of the study, while the bar chart on the right represents data from the final day of the study. The dashed line depicts the point at which the viruses were infused and intraperitoneal injections began. Data are shown as mean ± s.e.m. Surg: surgery.

4.4.1.3 AAV-α-synuclein induces motor dysfunction which is not exacerbated by low dose systemic rotenone

In the motor tests used, twice-weekly intraperitoneal administration of low dose rotenone (1.0 mg kg$^{-1}$) alone had no impact on either ipsilateral or contralateral motor function (Fig. 4.4 – 4.6). In its own right, unilateral intranigral infusion of AAV-α-synuclein caused impairments in contralateral motor function in the Corridor Test of contralateral neglect and the Stepping Test of forelimb akinesia but not the Whisker Test of sensorimotor integration (Fig. 4.4 – 4.6). However, the most important finding of the present behavioural study was that low dose systemic rotenone administration did not significantly enhance the AAV-α-synuclein-induced impairments in motor function. Thus, in the Corridor Test (Fig. 4.4), although AAV-α-synuclein infusion significantly
impaired the rats’ ability to retrieve food from the contralateral side of their body this was not exacerbated by rotenone administration (Group, $F_{(3,29)}=4.49, P<0.05$; post-hoc Newman Keuls confirmed α-Synuclein and Combined groups were less than Control group). In the Stepping Test (Fig. 4.5), infusion of AAV-α-synuclein alone caused a significant decrease in the number of adjusting steps made in the contralateral backhand direction, but this was not worsened by the rotenone treatment (Group, $F_{(3,29)}=3.27, P<0.05$; post-hoc Newman Keuls confirmed α-Synuclein and Combined groups were less than Control group). In the Whisker Test (Fig. 4.6), neither the genetic nor environmental insult, either alone or combined, induced any impairment in vibrissae-elicited forelimb placing.
Fig. 4.4 Systemic rotenone administration does not exacerbate AAV-α-synuclein induced impairments in the Corridor Test. Animals infused with AAV-α-synuclein developed an ipsilateral bias (A) and a contralateral decline (B) over the study period but this was not exacerbated by twice-weekly low dose intraperitoneal rotenone (1.0 mg kg⁻¹). The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the final test session. Data are shown as mean ± s.e.m. *P<0.05 vs. control group by one-way ANOVA with post-hoc Newman Keuls. Surg: surgery
4.5 Systemic rotenone administration does not exacerbate AAV-α-synuclein induced impairments in the Stepping Test. Animals infused with AAV-α-synuclein developed an impairment in contralateral motor function (C & D) over the study period but this was not exacerbated by twice-weekly low dose intraperitoneal rotenone (1.0 mg kg$^{-1}$). The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the final test session. Data are shown as mean ± s.e.m. *$P<0.05$ vs. control group by one-way ANOVA with post-hoc Newman Keuls. Surg: surgery.
4.6 Neither systemic rotenone nor unilateral AAV-α-synuclein administration affected the rats’ performance in the Whisker Test. Neither rotenone, AAV-α-synuclein nor combined administration of both insults affected the rats’ ipsilateral performance (A) or contralateral (B) performance in the Whisker Test. The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the final test session. The dashed line depicts the point of AAV infusion. Data are shown as mean ± s.e.m. Surg: surgery

4.4.1.4 AAV-α-synuclein induces nigrostriatal degeneration which is not exacerbated by low dose systemic rotenone

Once behavioural testing was completed, rats were sacrificed for quantitative immunohistochemical staining (Week 12 post-AAV infusion and initiation of i.p. rotenone administration) to assess the impact of rotenone on nigrostriatal integrity. Systemic administration of rotenone (1.0 mg kg⁻¹ i.p.) twice-weekly, alone did not lead to any loss of nigrostriatal cell bodies from the substantia nigra or terminals from the striatum as revealed by quantitative tyrosine hydroxylase immunohistochemical staining.
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(Fig. 4.7). In contrast, unilateral intranigral infusion of AAV-α-synuclein caused nigrostriatal degeneration at the level of the cell bodies and terminals (Fig. 4.7). However, importantly, combining low dose systemic rotenone administration with unilateral intranigral AAV-α-synuclein did not significantly enhance the AAV-α-synuclein-induced degeneration in either the substantia nigra (Group, $F_{(3,29)}=4.22, P<0.05$; post-hoc Newman Keuls confirmed α-Synuclein and Combined groups were less than Control group) or striatum (Group, $F_{(3,29)}=3.68, P<0.05$; post-hoc Newman Keuls confirmed α-Synuclein and Combined groups were less than Control group). Tyrosine hydroxylase stained sections were also analysed for the presence of dystrophic neurites (Fig. 4.8) as α-synuclein-induced dystrophy of nigrostriatal terminals is a feature of the AAV-α-synuclein model (Kirik et al., 2002). As expected, we found significantly increased level of dystrophic neurites in the striatum but the level of neuronal dystrophy was not enhanced by co-administration of rotenone (Group, $F_{(3,29)}=10.41, P<0.0001$; post-hoc Newman Keuls confirmed α-Synuclein and Combined groups were greater than Control group).
**Fig. 4.7** Systemic rotenone administration does not exacerbate AAV-α-synuclein induced nigrostriatal degeneration. Animals infused with AAV-α-synuclein displayed loss of ipsilateral nigrostriatal cells bodies (A) and a deafferentation of the ipsilateral striatum (B) as indicated by a loss of tyrosine hydroxylase immunoreactivity. However, this was not exacerbated by twice-weekly low dose intraperitoneal rotenone (1.0 mg kg\(^{-1}\)). *P*<0.05; vs. control group by one-way ANOVA with post-hoc Newman Keuls. Data are shown as mean ± s.e.m. TH-ir: tyrosine hydroxylase immunoreactivity; OD: optical density. Scale bar: A) = 1.0 mm; B) = 0.5 mm.
Fig. 4.8 Systemic rotenone administration does not exacerbate AAV-α-synuclein induced neuritic dystrophy. Immunohistochemistry for tyrosine hydroxylase revealed significant neuritic dystrophy in the ipsilateral striatum in animals infused with AAV-α-synuclein but this was not enhanced by rotenone administration. ***P<0.001; vs. control group by one-way ANOVA with post-hoc Newman Keuls. Data are shown as mean ± s.e.m. Scale bar: 0.05 mm.

4.4.1.5 AAV-α-synuclein induces α-synuclein expression which is not exacerbated by low dose systemic rotenone

Systemic administration of rotenone (1.0 mg kg\(^{-1}\) i.p.) twice-weekly, alone did not lead to any change in α-synuclein expression as revealed by quantitative α-synuclein expression immunohistochemical staining (using an antibody that cross-reacts with both human and rat α-synuclein protein) (Fig. 4.9). As expected, unilateral intranigral AAV-α-synuclein infusion induced expression of the human α-synuclein protein in the rat brain on the ipsilateral side (Fig. 4.9). However, neither nigral (Group, \(F_{(3,32)}=10.34, \ P<0.0001\); post-hoc Newman Keuls confirmed α-Synuclein and Combined groups were greater than Control group at \(P<0.01\)) nor striatal (Group, \(F_{(3,32)}=9.52, \ P<0.001\); post-hoc Newman Keuls confirmed α-Synuclein and Combined groups were greater than Control group at
P<0.01) expression of α-synuclein was enhanced when combined with rotenone administration.

**Fig. 4.9 Combined environmental and genetic insults does not exacerbate AAV-α-synuclein induced α-synuclein expression.** Unilateral administration of AAV-α-synuclein into the rat substantia nigra caused significant expression of α-synuclein in the nigrostriatal pathway at the level of the cell bodies in the substantia (A), as well as in the nigra terminals in the striatum (B). However, there was no evidence that administration of systemic rotenone affected the level of α-synuclein expression indicated by quantitative α-synuclein immunohistochemistry. Images in A are representative of striatal staining in each group while images in B are representative are representative of the infused VM staining in each group. **P<0.01; vs. control group by one-way ANOVA with post-hoc Newman Keuls. Data is shown as mean ± s.e.m. α-syn-ir: α-synuclein immunoreactivity; OD: optical density. Scale bar: A = 1.0 mm; B = 0.5 mm.
4.4.2 Determination of the effect of combined AAV-α-synuclein and low dose systemic (continuous subcutaneous) rotenone

The above study did not reveal any impact of twice-weekly intraperitoneal injections of rotenone (1.0 mg kg$^{-1}$) on AAV-α-synuclein-induced motor dysfunction, nigrostriatal degeneration and dystrophy, or α-synuclein expression, when administered over a 12 week period following intranigral infusion of the virus. Therefore, we then considered that an alternative approach might better reveal the effect of combining the environmental and genetic risk factors. Thus, in this study, osmotic minipumps delivering rotenone were subcutaneously implanted 13 weeks after intranigral infusion of the virus to assess if the pesticide could impact on the Parkinsonian features when administered to rats with a high background of α-synuclein expression. This was preceded by a pilot study to establish a sub-threshold, non-toxic dose of rotenone for subcutaneous infusions.

4.4.2.1 Pilot study to determine a sub-toxic rotenone dose for subcutaneous infusion

Over the course of this pilot study, rats implanted with osmotic minipumps releasing 1.5 or 2 mg kg$^{-1}$ day$^{-1}$ of rotenone continued to gain weight (Fig. 4.11). However, the rat implanted with a minipump releasing 5 mg kg$^{-1}$ day$^{-1}$ of rotenone lost weight progressively over the 3 weeks following implantation. We therefore opted to take a dose of 2.5 mg kg$^{-1}$ day$^{-1}$ forward to the main study.
4.11 Effect subcutaneous infusion of rotenone on body weight. Subcutaneous implantation of osmotic minipumps releasing rotenone at doses up to 2.0 mg kg$^{-1}$ day$^{-1}$ did not induce any overt toxicity, and animals continued to feed and gain weight as normal. However, the rat that received 5.0 mg kg$^{-1}$ day$^{-1}$ of rotenone started to lose weight 2 weeks after implantation and had to be sacrificed prematurely. Dashed lines represent the days of minipump implantation (Day 6) and removal (Day 34). Rot: rotenone.

4.4.2.2 The effect of combined AAV-α-synuclein and continuous subcutaneous infusion of rotenone on the rats’ body weights

Infusion of AAV-α-synuclein did not have any impact on the rats’ general health and they continued to gain weight over the entire 17 weeks of the study (Fig. 4.12). In contrast, rotenone-infused rats began to lose weight at steady rate from the second week after osmotic minipump implantation (Group x Time, $F_{(24,272)}=11.86$, $P<0.0001$) until the end of the study (Group, $F_{(3,30)}=3.57$, $P<0.05$; post-hoc Newman Keuls confirmed Combined group was less than Control and α-Synuclein groups at $P<0.05$). Despite this weight loss, all rotenone-infused rats (with one exception) survived until the natural study endpoint (17 weeks after intranigral virus injection and 4 weeks after minipump implantation). In 12 of the 19 rotenone-infused animals, a build up of undigested food
was observed in the stomach and intestines, in line with previous reports of rotenone-induced decreases in gastrointestinal motility (Greene et al., 2009). This was a qualitative assessment carried out during transcardial perfusion. The stomachs of rotenone infused animals were observably much larger than non-rotenone infused animals and tightly packed with rat chow. Because the rats survived until the natural study endpoint, this allowed us to assess the impact of 4-week continuous rotenone infusion on Parkinsonism in rats with a high nigrostriatal load of α-synuclein.

**Fig. 4.12 Continuous subcutaneous infusion of rotenone induces loss of weight in infused rats.** Unilateral administration of AAV-α-synuclein into the rat substantia nigra did not affect the body weight of the rats. However, rats infused systemically with rotenone began to lose weight from the second week after pump implantation. The XY plots on the left depict the data collected over the course of the study, while the bar chart on the right represents the collapsed data from the final study day only. *P<0.05; vs. control group by one-way ANOVA with post-hoc Newman Keuls. Data are shown as mean ± s.e.m. Surg: surgery

**4.4.2.3 AAV-α-synuclein induces motor dysfunction which is exacerbated by continuous systemic infusion of rotenone on the contralateral side only**

In its own right, unilateral intranigral infusion of AAV-α-synuclein caused contralateral motor impairments in the Corridor Test of contralateral neglect, the Stepping Test of forelimb akinesia and the Whisker Test of sensorimotor integration (Fig. 4.13-4.15). Similarly, in its own right, continuous systemic infusion of rotenone (2.5 mg kg\(^{-1}\) day\(^{-1}\)
for 4 weeks) also caused motor dysfunction in all tests on both the ipsilateral and contralateral sides (Fig. 4.13-4.15). However the most important finding of the present study was the additive effect of the genetic and environmental insults on the contralateral side of the rats’ bodies only.

In the Corridor Test (Fig. 4.13), the impairments in contralateral retrieval induced by either AAV-α-synuclein or rotenone were exacerbated by combining the two insults (Group, \(F_{(3,30)}=40.76, P<0.0001\); post-hoc Newman Keuls confirmed significant motor dysfunction in the Combined group relative to the α-Synuclein and Rotenone groups on this side). Importantly, this additive effect was not seen on the ipsilateral side (Group, \(F_{(3,30)}=155.40, P<0.0001\); post-hoc Newman Keuls confirmed that the Combined group did not differ from the Rotenone group). Similarly, in the Stepping Test (Fig. 4.14), the impairments in contralateral backhand stepping induced by either AAV-α-synuclein or rotenone were exacerbated by combining the two risk factors (Group, \(F_{(3,30)}=32.01, P<0.0001\); post-hoc Newman Keuls confirmed significant motor dysfunction in the Combined group relative to the α-Synuclein and Rotenone groups on this side). As with the Corridor Test above, this additive effect was not seen on the ipsilateral side in this test (Group, \(F_{(3,30)}=17.11, P<0.0001\); post-hoc Newman Keuls confirmed that the Combined group did not differ from the Rotenone group). A similar pattern was also seen in the Whisker Test (Fig. 4.15) where the impairments in contralateral vibrissae-elicited forelimb placings induced by either AAV-α-synuclein or rotenone were exacerbated by combining the two together (Group, \(F_{(3,30)}=30.18, P<0.0001\); post-hoc Newman Keuls confirmed significant motor dysfunction in the Combined group relative to the α-Synuclein and Rotenone groups on this side). As in the other tests, this additive effect was
not seen on the ipsilateral side (Group, \( F_{(3,30)}=31.13, P<0.0001; \) post-hoc Newman Keuls confirmed that the Combined group did not differ from the Rotenone group).

**Fig. 4.13.** Exacerbation of AAV-\(\alpha\)-synuclein-induced motor dysfunction by continuous subcutaneous infusion of rotenone on the contralateral side only. Effect of unilateral intranigral infusion of AAV-\(\alpha\)-synuclein and continuous systemic infusion of rotenone (2.5 mg kg\(^{-1}\) day\(^{-1}\) for 4 weeks) on ipsilateral (A) and contralateral (B) motor performance in the Corridor Test. Although the genetic and environmental insults affected motor function in their own right, combining them together only exacerbated motor dysfunction on the contralateral side indicating an additive effect of the insults on this side. The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-operative period only. Data is shown as mean ± s.e.m. ***\(P<0.001\) vs. Control; ##\(P<0.01\) vs. Rotenone; +\(P<0.05\), +++\(P<0.001\) vs. \(\alpha\)-Synuclein by one-way ANOVA with post-hoc Newman Keuls. Surg: surgery.
**Fig. 4.14 Exacerbation of AAV-α-synuclein-induced motor dysfunction by continuous subcutaneous infusion of rotenone on the contralateral side only.** Effect of unilateral intranigral infusion of AAV-α-synuclein and continuous systemic infusion of rotenone (2.5 mg kg\(^{-1}\) day\(^{-1}\) for 4 weeks) on ipsilateral forehand (A), ipsilateral backhand (B), contralateral forehand (C) and contralateral backhand (D) stepping in the Stepping Test. Although the genetic and environmental insults affected motor function in their own right, the additive effect of the two was only observed on the contralateral side. The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-operative period only. Data is shown as mean ± s.e.m. *P<0.05, **P<0.01, ***P<0.001 vs. Control; ###P<0.001 vs. Rotenone; †P<0.05, ‡‡P<0.001 vs. α-Synuclein by one-way ANOVA with post-hoc Newman Keuls. Surg: surgery.
Fig. 4.15 Exacerbation of AAV-α-synuclein-induced motor dysfunction by continuous subcutaneous infusion of rotenone on the contralateral side only. Effect of unilateral intranigral infusion of AAV-α-synuclein and continuous systemic infusion of rotenone (2.5 mg kg⁻¹ day⁻¹ for 4 weeks) on A) ipsilateral and B) contralateral motor performance in the Corridor Test. Although the genetic and environmental insults affected motor function in their own right, combining them together only exacerbated motor dysfunction on the contralateral side indicating an additive effect of the insults on this side. The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-operative period only. Data is shown as mean ± s.e.m. *P<0.05, ***P<0.001 vs. Control; ##P<0.01 vs. Rotenone; ++P<0.01, +++P<0.001 vs. α-Synuclein by one-way ANOVA with post-hoc Newman Keuls. Surg: surgery.
4.4.2.4 Unilateral intranigral AAV-α-synuclein and systemic rotenone administration leads to a progressive decline in motor function.

One of the most important features of human Parkinson’s disease which has proven difficult to model in laboratory animals is the progressive nature of the movement disorder. In the present study, one of the significant features of the data is the relatively slow and protracted emergence of the motor dysfunction in the Corridor Test in the Combined group (Fig. 4.13B). Thus, in this test, intranigral injection of AAV-α-synuclein and systemic rotenone administration induced a progressive decline in contralateral motor function that emerged over a period of weeks (Group x Time, $F_{(21,217)}=10.11$, $P<0.0001$. Post-hoc testing confirmed a progressive decline in contralateral retrieval over the 4 weeks after initiation of rotenone administration in the Combined group ($P<0.05$ vs. Control by Week 2 after minipump implantation, $P<0.0001$ vs. Control by Week 4 after minipump implantation)).

4.4.2.5 AAV-α-synuclein induces nigrostriatal degeneration on the ipsilateral side which is exacerbated by continuous systemic infusion of rotenone

Once behavioural testing was completed, rats were sacrificed for quantitative immunohistochemical analysis (Week 17 post-AAV infusion and week 4 post-minipump implantation). Quantitative tyrosine hydroxylase immunohistochemical staining revealed that, in its own right, unilateral intranigral infusion of AAV-α-synuclein caused nigrostriatal degeneration on the injected side (Fig. 4.16). In contrast, continuous systemic infusion of rotenone (2.5 mg kg$^{-1}$ day$^{-1}$ for 4 weeks) did not have any impact on
nigrostriatal integrity when injected alone. However, the most important finding of this immunohistochemical study was that nigrostriatal degeneration at the level of the nigrostriatal terminals is worsened in rats following combined treatment. Thus, in the striatum, the loss of nigrostriatal terminals induced by AAV-α-synuclein was exacerbated by combining the two insults (Group, \( F_{(3,30)} = 21.23, P < 0.0001 \); post-hoc Newman Keuls confirmed significant terminal loss in the Combined group relative to the α-Synuclein and Rotenone groups on this side). This combined effect was not seen in the substantia nigra where combining the genetic and environmental insults did not exacerbate the AAV-α-synuclein-induced cell body loss (Group, \( F_{(3,30)} = 19.64, P < 0.0001 \); post-hoc Newman Keuls confirmed that the Combined group did not differ from the α-Synuclein group). Tyrosine hydroxylase stained sections were also analysed for the presence of dystrophic neurites (Fig. 4.17) as α-synuclein-induced dystrophy of nigrostriatal terminals is a feature of the AAV-α-synuclein model (Kirik et al., 2002). As expected, we found significantly increased level of dystrophic neurites in the striatum but the level of neuronal dystrophy was not enhanced by co-administration of rotenone (Group, \( F_{(3,29)} = 10.41, P < 0.0001 \); post-hoc Newman Keuls confirmed α-Synuclein and Combined groups were greater than Control group).
Fig. 4.16 Exacerbation of AAV-α-synuclein-induced nigrostriatal degeneration by continuous subcutaneous infusion of rotenone. Effect of unilateral intranigral infusion of AAV-α-synuclein and continuous systemic infusion of rotenone (2.5 mg kg\(^{-1}\) day\(^{-1}\) for 4 weeks) on tyrosine hydroxylase immunopositive nigrostriatal terminals (A) and cell bodies (B). Although α-synuclein affected nigrostriatal integrity in its own right, combining it with rotenone exacerbated nigrostriatal terminal loss on the ipsilateral side indicating an additive effect of the insults on this side. Data is shown as mean ± s.e.m. *P<0.05, ***P<0.001 vs. Control; #P<0.05 vs. Rotenone; +P<0.05 vs. α-Synuclein by one-way ANOVA with post-hoc Newman Keuls. TH-ir: tyrosine hydroxylase immunoreactivity, OD: optical density. Scale bar: A = 1.0 mm; B = 0.5 mm.
Fig. 4.17 Systemic rotenone administration does not exacerbate AAV-α-synuclein induced neuritic dystrophy. Immunohistochemistry for tyrosine hydroxylase revealed significant neuritic dystrophy in the ipsilateral striatum in animals infused with AAV-α-synuclein. Rotenone administration did not exacerbate this effect when combined with AAV-α-synuclein infusion. ***P<0.001; vs. control groups by one-way ANOVA with post-hoc Newman Keuls. Data are shown as mean ± s.e.m. Scale bar: 0.1 mm.

4.4.2.6 AAV-α-synuclein induces α-synuclein expression on the ipsilateral side which is not exacerbated by continuous systemic infusion of rotenone

To determine if unilateral intranigral AAV-α-synuclein or rotenone infusion could induce any changes in the expression of α-synuclein in the rat brain, quantitative immunohistochemical staining for α-synuclein was completed using an antibody that cross-reacts with both rat and human α-synuclein (Fig. 4.18). As expected, unilateral intranigral infusion of AAV-α-synuclein caused a significant increase in the expression of human α-synuclein in the substantia nigra and the striatum but this was not enhanced when combined with rotenone infusion (Striatum: Group, $F_{(3,35)}=14.63, P<0.0001$; Substantia nigra: Group, $F_{(3,35)}=18.74, P<0.0001$; post-hoc Newman Keuls confirmed greater α-synuclein expression in the α-Synuclein and Combined groups relative to the Control and Rotenone groups).
Fig. 4.18 No exacerbation of AAV-α-synuclein-induced α-synuclein expression by continuous subcutaneous infusion of rotenone. Effect of unilateral intranigral infusion of AAV-α-synuclein and continuous systemic infusion of rotenone (2.5 mg kg⁻¹ day⁻¹ for 4 weeks) on α-synuclein expression in the ventral mesencephalon (A) and striatum (B). As expected, AAV-α-synuclein induced α-synuclein expression on the ipsilateral side. This was not enhanced by combining it with rotenone. Data is shown as mean ± s.e.m. ***P<0.001 vs. Control by one-way ANOVA with post-hoc Newman Keuls. SYN-ir: α-synuclein immunoreactivity, OD: optical density Scale bar: A = 1.0 mm; B = 0.5 mm.
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4.5 Discussion

This overall aim of the experiments described in this chapter was to develop and characterise a novel multi-hit model of Parkinson’s disease by exposing rats to both genetic and environmental risk factors that are relevant to the human condition. Because the previous chapter revealed that direct intrastriatal infusion of rotenone into the rat brain was associated with serious limitations (including a failure to induce any \(\alpha\)-synucleinopathy), in this chapter, we investigated whether chronic systemic administration of a sub-threshold dose of rotenone combined with virally-induced nigral \(\alpha\)-synuclein overexpression could induce Parkinsonism in rats. To this end, we used two different experimental approaches – one in which rotenone (1.0 mg kg\(^{-1}\)) was administered by twice-weekly intraperitoneal injection for 13 weeks after unilateral intranigral AAV-\(\alpha\)-synuclein administration, and one in which the pesticide was administered by continuous subcutaneous infusion (2.5 mg kg\(^{-1}\) day\(^{-1}\)) for 4 weeks from weeks 13-17 after unilateral intranigral AAV-\(\alpha\)-synuclein administration. We found that the twice-weekly intraperitoneal administration regime had no impact whatsoever on AAV-\(\alpha\)-synuclein induced \(\alpha\)-synuclein expression, nigrostriatal degeneration or motor dysfunction. However, in contrast, continuous subcutaneous infusion of the pesticide to rats with a high nigrostriatal load of \(\alpha\)-synuclein exacerbated AAV-\(\alpha\)-synuclein-induced nigrostriatal degeneration and motor dysfunction. Thus, this novel multi-hit model is one of the only preclinical models of Parkinson’s disease in which the triad of classic features of progressive motor dysfunction, nigrostriatal neurodegeneration and \(\alpha\)-synucleinopathy is robustly expressed. However, as with the direct intracerebral rotenone model, this approach was not without its limitations as rats implanted with rotenone-filled osmotic minipumps developed motor dysfunction on both sides of their bodies and began to lose
weight steadily after 2 weeks. Thus, in terms of our aim to develop and characterise a novel multi-hit model, we have shown that combining AAV-\(\alpha\)-synuclein-induced \(\alpha\)-synuclein overexpression with continuous subcutaneous infusion of rotenone can model the main features of the human disease but, because the pesticide is administered systemically, it is associated with bilateral dysfunction and rotenone-induced loss of body weight.

For the first main study, rotenone (1.0 mg kg\(^{-1}\)) was administered by twice-weekly intraperitoneal injection for 13 weeks directly following unilateral intranigral AAV-\(\alpha\)-synuclein administration. In previous studies in which rotenone was administered by intraperitoneal injection, doses in the range of 1.0–5.0 mg kg\(^{-1}\) were administered for up to 60 days (Alam et al., 2002; Bashkatova et al., 2004; Biehlmaier et al., 2007; Greenman et al., 1993). As these studies were associated with loss of body weight and a high rate of mortality, we used a lower non-toxic dose for chronic administration. This study shows that this dose and injection regime (1.0 mg kg\(^{-1}\) i.p. twice-weekly for 13 weeks) did not have any impact on \(\alpha\)-synuclein expression, nigrostriatal integrity or motor function. As expected, injection of AAV-\(\alpha\)-synuclein into the substantia nigra led to widespread expression of the \(\alpha\)-synuclein protein throughout the entire nigrostriatal pathway as well as loss of both nigrostriatal cell bodies and terminals in line with previous reports (Azeredo da Silveira et al., 2009; Gorbatyuk et al., 2008; Kirik et al., 2002; Koprich et al., 2011; Ulusoy et al., 2010; Yamada et al., 2004a). Previous studies have suggested that anterograde transport from the substantia nigra to the axon terminals is responsible for the widespread expression of the AAV transgene product in the striatum (Bjorklund et al., 2000).
The intracellular accumulation of α-synuclein caused significant loss of both nigrostriatal cell bodies and terminals at the time point examined, and led to significant impairments in motor ability on the contralateral side. Interestingly, although rats infused with AAV-α-synuclein exhibited pronounced neglect of contralateral space in the Corridor Test and an impaired ability to make adjusting steps in the Stepping Test, they retained the capacity to make vibrissae-elicited forelimb placings in the Whisker Test. This is intriguing as it indicates that the neurological mechanisms underlying performance of the Corridor and Stepping Tests are compromised by intracellular accumulation of α-synuclein in the nigrostriatal pathway, whereas those underlying the Whisker Test are unaffected. The Whisker Test has previously been shown to be reliant on an intact nigrostriatal pathway as performance of this task is almost completely obliterated after neurotoxic lesions of this system (Grealish et al., 2008). However, it appears that rats with high nigrostriatal load of α-synuclein but only partial lesions retain the ability to perform this task as well as control rats. It is also possible that the Whisker Test relies on other circuits. The barrel circuit in rats relies on both dopaminergic and serotonergic inputs which may remain functional with reduced dopamine levels (Waterhouse et al., 1996). Although Kirik et al. (2002) have previously shown that this model is associated with forelimb stepping impairments, these impairments only manifested after pretreatment of the rats with the tyrosine hydroxylase inhibitor α-methyl-para-tyrosine. Thus, this data confirms and extends previous behavioural data from this model and, for the first time, also demonstrates that it can induce impairments in the Corridor Test. However, the most important finding from the present study was that low-dose systemic rotenone (1.0 mg kg\(^{-1}\) i.p. twice-weekly for 13 weeks) did not have any impact on AAV-α-synuclein-induced α-synucleinopathy, nigrostriatal degeneration or motor impairments. The most obvious explanation for this is that the dose and administration regime used for
rotenone was too low to have any detrimental impact on the nigrostriatal pathway. Although it is clear from previous literature that alternative intraperitoneal administration regimes could provide a more neurotoxic effect (Alam et al., 2004b; Alam et al., 2002; Cannon et al., 2009), it has yet to be systematically established if this could be achieved without a detrimental effect on the rats’ health.

For the subsequent study, we took an alternative approach to developing a multi-hit model and assessed the impact that the pesticide could have on Parkinsonism when administered to rats with a high background of α-synuclein expression. Thus, in this study, the pesticide was administered by continuous subcutaneous infusion (2.5 mg kg$^{-1}$ day$^{-1}$) for 4 weeks from weeks 13-17 following unilateral intranigral AAV-α-synuclein administration. Continuous subcutaneous infusion using osmotic minipumps has been used previously as an approach to modelling Parkinson’s disease. In these studies, doses in the range of 1.5 – 5.0 mg kg$^{-1}$ day$^{-1}$ were administered for up to 60 days (Ferrante et al., 1997; Fleming et al., 2004; Milusheva et al., 2005; Panov et al., 2005; Sherer et al., 2003c). As the studies using higher doses and/or longer durations were associated with loss of body weight and a high rate of mortality, we again used a relatively low dose for chronic administration.

In this study, the rotenone dose and infusion regime selected (2.5 mg kg$^{-1}$ day$^{-1}$ s.c. for 4 weeks) caused significant and progressive loss of body weight from weeks 2-4 after minipump implantation. Despite this, all rats survived until the natural study endpoint by which point they were 87±6% of their pre-implantation weight. Nevertheless, the progressive weight loss is a significant disadvantage of this approach and limits the use of this model for long-term preclinical drug discovery research. The 2.5 mg kg$^{-1}$ day$^{-1}$ dose was selected in an attempt to avoid a repeat of the previous study in which rotenone had
no impact whatsoever on the rats. With hindsight perhaps, we should have selected 2 mg kg$^{-1}$ day$^{-1}$ dose as the pilot study showed that subcutaneous infusion of this dose for 4 weeks did not impact on the rat’s body weight (in this single animal at least). However, since the sensitivity of experimental animals to rotenone is variable (Hoglinger et al., 2006; Richter et al., 2007; Schmidt & Alam, 2006; Uversky, 2004; Zhu et al., 2004) there is no guarantee that administration of this dose to a wider set of rats would not have had detrimental effects. In contrast to rotenone, the AAV-α-synuclein virus has no impact on the rats’ body weight and nor did it significantly exacerbate the rotenone-induced loss of weight.

With regard to the impact of systemic rotenone on Parkinsonism, in its own right, rotenone (2.5 mg kg$^{-1}$ day$^{-1}$ s.c. for 4 weeks) caused significant bilateral motor dysfunction without a statistically significant effect on nigrostriatal integrity. Although not statistically significant, there was a tendency for rotenone to reduce the density of tyrosine hydroxylase immunopositive terminals. Thus, this may have been sufficient to produce the deficits observed. Although this level of behavioural impairment would not normally be expected with such a small loss of terminals, rotenone differs from selective catecholamine neurotoxins like 6-hydroxydopamine in that it is not selective. Therefore, it is possible that the small loss of nigrostriatal terminals combined with loss of other neurons may account for the substantial bilateral motor dysfunction observed. However, a more probable explanation for the behavioural results observed is that the toxic effects of rotenone rendered the rats unable to perform the tasks. This effect was most obvious in the Corridor Test where a large proportion of the rats made little attempt to move at all, with retrievals of less than 5 pellets being common towards the end of the study period. This bilateral effect was also observed in the Stepping and Whisker Tests although the
severity was not at the level of the Corridor Test, possibly due to the fact that during the tests the animal was held in place as the experimenter initiated movement. Closer scrutiny of the data shows that the rats had begun to lose weight before the onset of bilateral rotenone induced impairments, indicating that peripheral toxicity may have been responsible for the behavioural results observed and not the level of nigrostriatal degeneration recorded.

Despite the limitations of bilateral motor dysfunction and body weight induced by systemic rotenone, this study did reveal some important genetic and environmental interactions. The most important finding of this study was the additive effect of systemic rotenone and unilateral intranigral AAV-α-synuclein on ipsilateral nigrostriatal integrity and contralateral motor function. Thus, combining the two risk factors resulted in exacerbation of nigrostriatal terminal loss on the ipsilateral side and progressive motor dysfunction in the Corridor, Stepping and Whisker Tests, on the contralateral side only. Crucially, this additive effect was not observed on the ipsilateral side. At the point of minipump implantation, rats in the Combined group had been exposed to α-synuclein expression for 13 weeks. We have shown in the previous study that by 12 weeks post AAV-α-synuclein infusion, significant nigrostriatal degeneration has occurred and in this study the subsequent systemic administration of the Complex I inhibitor appears to have led to an exacerbation of the AAV-α-synuclein induced degeneration. Dopaminergic neurons already in a state of distress may have been more sensitive to the rotenone induced effects leading to more pronounced effects ipsilateral but not contralateral to the side of AAV-α-synuclein infusion. Clearly, rats with high load of α-synuclein were more sensitive to impact of the rotenone as this was not observed on the ipsilateral side. This is
intriguing as it shows combined impact of genetic and environmental risk factors that are directly relevant to human Parkinson’s disease.

To our knowledge this is the first report describing the impact of combining AAV-α-synuclein gene transfer and systemic environmental risk factor administration in a rat model of Parkinson’s disease. The results of this study are important for two main reasons. Firstly, this intranigral AAV-α-synuclein and systemic rotenone combination model one is the first Parkinson’s disease models to display robust and progressive impairment of spontaneous motor function with associated nigrostriatal α-synucleinopathy and neurodegeneration. It thus replicates the triad of classic Parkinson’s disease features and could be in an invaluable aid to future drug discovery programmes. It also gives us more insight into the effects of combining two Parkinson’s disease risk factors, namely genetic and environmental insults, on the neuropathology and motor impairments associated with the disease. However, while this route of rotenone exposure is relevant to the human condition, the propensity for overt peripheral toxicity places severe limitations on the model. Over longer term studies, weight loss of the magnitude reported here would not be feasible.

In summary, we have shown that unilateral intranigral administration of AAV-α-synuclein and systemic administration of rotenone to the rat leads to a model of Parkinson’s disease in which there is progressive motor dysfunction with underlying nigrostriatal α-synucleinopathy and neurodegeneration. The sequential exposure to these two known risk factors for Parkinson’s disease resulted in a model with more exaggerated motor impairments and neuropathology than either the genetic or environmental risk factor alone. Thus this model may be useful for examining the interplay between genes
and the environment in Parkinson’s disease, however, the ill-effects associated with systemic rotenone are a major limiting factor. Further studies in this thesis will attempt to overcome this limitation in developing a reliable and robust model of Parkinson’s disease.
Chapter 5: Development and characterisation of a novel Parkinson’s disease model induced by intracerebral infusion of rotenone with intranigral administration of AAV-α-synuclein

5.1 Introduction

The low penetrance of some Parkinson’s disease-associated genetic mutations suggests that a combination of insults is required for substantia nigra neuron death (Sulzer, 2007). Although the mechanism of neurodegeneration is still unclear, many triggers involving different, although possibly convergent, pathways have been proposed (Weidong et al., 2009). It has been suggested that substantia nigra neurons might be particularly prone to the accumulation of mitochondrial mutations during aging, and Parkinson’s disease patients tend to express damaged mitochondria with low Complex I activity in a variety of tissues (Dauer et al., 2003). The widening belief is that the pathogenesis of Parkinson’s disease is due for the most part to complex interactions between genetic and environmental factors leading to mitochondrial dysfunction, oxidative stress, inflammation and excitotoxicity, eventually leading to nigral dopaminergic neuron degeneration (Klein & Schlossmacher, 2007; Schapira, 2008; Weidong et al., 2009).

Because of the complexity of the Parkinson’s disease etiology, a multi-hit paradigm may be the most valid approach for modelling the condition in preclinical animals (Goldman et al., 2006; Gorell et al., 1999; Imaizumi, 1995; Ross et al., 2001; Sato et al., 2011; Svenson et al., 1993; Tanner et al., 2011; Van Den Eeden et al., 2003). This approach, which was the basis for the previous chapter, not only has the potential to recapitulate all of the classic features associated with this condition, but could also contribute to our understanding of disease onset and progression after exposure to a combination of genetic
and environmental insults. In the previous chapter, we demonstrated that subcutaneous infusion of rotenone to rats with a high nigrostriatal load of α-synuclein was capable of modelling the triad of classic features associated with the disease, namely, motor dysfunction, nigrostriatal degeneration and α-synuclein accumulation. However, because systemic rotenone infusion was associated with body weight loss and bilateral motor impairments, this limits the utility of this model for long term drug discovery programmes. In contrast, when rotenone was administered directly into the brain, it did not have any detrimental effect on the welfare or body weight of the rats (Mulcahy et al., 2011a).

As intracerebral administration of rotenone is associated with reliable nigrostriatal degeneration and motor dysfunction but not α-synucleinopathy (Mulcahy et al., 2011), whereas viral transfer of α-synuclein is associated with robust α-synucleinopathy but only variable neurodegeneration and motor impairments (Kirik et al., 2002), we considered that combining these two approaches could be a reliable method for modelling Parkinson’s disease.

Thus, the aim of this chapter was to determine if intracerebral administration of rotenone to rats with virally-induced overexpression of α-synuclein in the nigrostriatal pathway could recapitulate the triad of classic features associated with human Parkinson’s disease.

5.2 Materials and methods

All methods have been described in more detail in Chapter 2.
5.3 Experimental design

This chapter will detail the results from two studies which sought to characterise the Parkinson’s disease-associated deficits evoked by sequential α-synuclein overexpression and intracerebral infusion of rotenone. The first study was completed to determine the effect of intranigral AAV-α-synuclein followed by intrastriatal rotenone, while the second study was completed to determine the effect of intranigral AAV-α-synuclein followed by intranigral rotenone.

5.3.1 Study to determine the effect of intranigral AAV-α-synuclein infusion followed by intrastriatal rotenone infusion

Male Sprague Dawley rats were unilaterally infused with 2 µl of either AAV-α-synuclein or AAV-GFP into the substantia nigra. The viral titers were 3.96×10^{11} drp µl^{-1} for AAV-α-synuclein and 1.07×10^{10} drp µl^{-1} for AAV-GFP. Thirteen weeks following virus infusion, rotenone (0.9 µg at 4 sites, 3.6 µg in total), or vehicle (1:1:18 combination of DMSO, Cremophor® and saline, respectively) was infused at 4 sites along the rostro-caudal axis of the striatum resulting in four groups as detailed in Table 5.1. Behavioural testing was carried out using the Corridor, Stepping and Whisker Tests every 4 weeks up until the point of rotenone infusion and weekly thereafter. Rats were then sacrificed by transcardial fixation 24 weeks following initial AAV infusion (11 weeks following rotenone infusion) and the effect of the combined approach was assessed using quantitative tyrosine hydroxylase and α-synuclein immunohistochemistry.
Table 5.1. Final groups used in this study. Rats were unilaterally infused with AAV-α-synuclein or AAV-GFP into the substantia nigra and 13 weeks later this was followed by intrastriatal infusion of rotenone (3.6 μg) or vehicle (DMSO:Cremophor:saline in 1:1:18 ratio).

<table>
<thead>
<tr>
<th>Group</th>
<th>Intranigral infusion</th>
<th>Intrastriatal infusion</th>
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<tbody>
<tr>
<td>Control</td>
<td>AAV-GFP</td>
<td>Vehicle</td>
<td>5</td>
</tr>
<tr>
<td>Rotenone</td>
<td>AAV-GFP</td>
<td>Rotenone</td>
<td>10</td>
</tr>
<tr>
<td>α-Synuclein</td>
<td>AAV-α-synuclein</td>
<td>Vehicle</td>
<td>9</td>
</tr>
<tr>
<td>Combined</td>
<td>AAV-α-synuclein</td>
<td>Rotenone</td>
<td>9</td>
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</tbody>
</table>

5.3.2 Study to determine the effect of sequential intranigral infusion of AAV-α-synuclein and rotenone

For this study, male Sprague Dawley rats were unilaterally infused with 2 μl of either AAV-α-synuclein or AAV-GFP into the substantia nigra. The viral titers were 3.96×10^{11} drp μl^{-1} for AAV-α-synuclein and 1.07×10^{10} drp μl^{-1} for AAV-GFP. Thirteen weeks following virus infusion, rotenone (0.9 μg) or vehicle (1:1:18 combination of DMSO, Cremophor and saline, respectively) was infused at the same site resulting in four groups as detailed in Table 5.2. Behavioural testing was carried out using the Corridor, Stepping and Whisker Tests every 4 weeks up until the point of rotenone infusion and weekly thereafter. Rats were then sacrificed by transcardial fixation 24 weeks following initial AAV infusion (11 weeks following rotenone infusion) and the effect of the combined approach was assessed using quantitative tyrosine hydroxylase and α-synuclein immunohistochemistry.
<table>
<thead>
<tr>
<th>Group</th>
<th>Intranigral virus</th>
<th>Intranigral rotenone/vehicle</th>
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<tbody>
<tr>
<td>Control</td>
<td>AAV-GFP</td>
<td>Vehicle</td>
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<tr>
<td>Rotenone</td>
<td>AAV-GFP</td>
<td>Rotenone</td>
<td>10</td>
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<tr>
<td>α-Synuclein</td>
<td>AAV-α-synuclein</td>
<td>Vehicle</td>
<td>7</td>
</tr>
<tr>
<td>Combined</td>
<td>AAV-α-synuclein</td>
<td>Rotenone</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 5.2. Final groups used in this study. Rats were unilaterally infused with AAV-α-synuclein or AAV-GFP into the substantia nigra and 13 weeks later this was followed by intrastriatal infusion of rotenone (0.9 µg) or vehicle (DMSO:Cremophor:saline in 1:1:18 ratio).

5.4 Results

5.4.1 Study to determine the effect of intranigral AAV-α-synuclein infusion followed by intrastriatal rotenone infusion

In this study, the impact of unilateral intranigral AAV-α-synuclein followed 13 weeks later by unilateral intrastriatal rotenone on body weight, motor function, nigrostriatal integrity and α-synuclein expression was assessed.

5.4.1.1 Unilateral intranigral infusion of AAV-α-synuclein and/or intrastriatal rotenone is not detrimental to the rats’ general health

Intranigral infusion of AAV-α-synuclein and/or intrastriatal rotenone did not induce mortality in any of the rats. Moreover, neither the single nor combined treatments had any effect on the body weight of the animals (Fig. 5.1, Group, $F_{(3,29)}=1.80$, $P=0.17$, ns) and all infusion groups continued to gain weight at the same rate as the control group (Time, $F_{(26,754)}=593.20$, $P<0.0001$; Group x Time, $F_{(78,754)}=0.38$, $P=0.62$, ns).
Fig. 5.1 Unilateral intranigral AAV-α-synuclein and/or intrastriatal rotenone infusion does not affect the rats’ body weights. Unilateral intranigral administration of AAV-α-synuclein followed 13 weeks later by unilateral intrastriatal infusion of rotenone did not affect the body weight of the rats. The XY plot on the left depicts the data collected over the course of the study, while the bar chart on the right represents collapsed data from the post-rotenone infusion period only. Data are shown as mean ± s.e.m. Dashed lines represent point of infusion surgery.

5.4.1.2 Unilateral intranigral infusion of AAV-α-synuclein and/or intrastriatal rotenone induces contralateral motor dysfunction

Because the development of a motor syndrome on the side of the body opposite to the nigrostriatal neuropathology is one of the key features of any unilateral model of Parkinson’s disease, in this study we assessed the impact of the single or combined Parkinsonian insults on motor function by subjecting the rats to a battery of behavioural tests that assesses different aspects of the motor disorder. In line with our previous studies, unilateral intranigral infusion of AAV-α-synuclein or unilateral intrastriatal infusion of rotenone in their own right induced significant contralateral motor impairments in the tests used (Fig. 5.2-5.4; although note the lack of α-synuclein-induced impairments in the Whisker Test). However, the most important finding of this study is the additive effect of the genetic and environmental insults on motor dysfunction. Thus, in the Corridor Test of contralateral neglect (Fig. 5.2), intranigral infusion of AAV-α-
Chapter 5: Combined AAV-α-synuclein and intracerebral rotenone

synuclein followed 13 weeks later by intrastriatal infusion of rotenone significantly reduced the rats’ ability to retrieve food from the contralateral side of their body when compared to either insult alone (Corridor: Group, $F_{(3,29)}=29.52$, $P<0.0001$; post-hoc Newman Keuls confirmed a significant decrease in the combined group relative to the α-Synuclein and Rotenone groups). In the Stepping Test of forelimb akinesia (Fig. 5.3), combining these two known Parkinson’s disease risk factors significantly reduced the number of contralateral forelimb adjusting steps compared to either factor alone (Forehand: Group, $F_{(3,29)}=28.90$, $P<0.0001$; Backhand: Group, $F_{(3,29)}=32.90$, $P<0.0001$; post-hoc Newman Keuls confirmed a significant decrease in the combined group relative to the α-Synuclein and Rotenone groups). In the Whisker Test of sensorimotor integration (Fig. 5.4), combining AAV-α-synuclein and rotenone also significantly reduced the number of vibrissae-elicited contralateral forelimb placings when compared to either insult alone (Whisker: Group, $F_{(3,29)}=42.43$, $P<0.0001$; post-hoc Newman Keuls confirmed a significant decrease in the combined group relative to the α-Synuclein and Rotenone groups).
**Fig. 5.2** Unilateral intrastriatal rotenone infusion exacerbates AAV-α-synuclein-induced spontaneous contralateral motor dysfunction in the Corridor Test. Animals infused with either AAV-α-synuclein and/or rotenone alone showed an increase in ipsilateral retrieval (A) and a decrease in contralateral retrieval (B) over the study period. Importantly, an additive effect was observed following sequential infusion of both insults. The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-rotenone infusion period only. Dashed lines depict the point of infusion surgery. Data are shown as mean ± s.e.m. ***P<0.001 vs. Control; +++P<0.001 vs. α-synuclein; #P<0.05 vs. Rotenone by one-way ANOVA with post-hoc Newman Keuls.
Fig. 5.3 Unilateral intrastriatal rotenone infusion exacerbates AAV-α-synuclein-induced spontaneous contralateral motor dysfunction in the Stepping Test. Animals infused with AAV-α-synuclein and/or rotenone showed a decline in contralateral forehand (A) and backhand (D) stepping over the study period while ipsilateral performance (A & B) was not affected. Importantly, an additive effect was observed following sequential infusion of both of insults. The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-rotenone infusion period only. Dashed lines depict the point of infusion surgery. Data are shown as mean ± s.e.m. **P<0.01, ***P<0.001 vs. Control; +++P< 0.001 vs. α-synuclein; ##P<0.01 vs. Rotenone by one-way ANOVA with post-hoc Newman Keuls.
Chapter 5: Combined AAV-α-synuclein and intracerebral rotenone

Fig. 5.4 Unilateral intrastriatal rotenone infusion exacerbates AAV-α-synuclein-induced spontaneous contralateral motor dysfunction in the Whisker Test. Animals infused with rotenone alone showed a decrease in contralateral placings (B) over the study period while ipsilateral performance (A) was not affected. Importantly, an additive effect was observed following sequential infusion of both of insults. The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-rotenone infusion period only. Dashed lines depict the point of infusion surgery. Data are shown as mean ± s.e.m. ***P<0.001 vs. Control; +++P< 0.001 vs. α-synuclein, ###P<0.001 vs. Rotenone by one-way ANOVA with post-hoc Newman Keuls.

5.4.1.3 Unilateral intranigral infusion of AAV-α-synuclein and/or intrastriatal rotenone induces nigrostriatal degeneration

Once behavioural testing was completed, rats were sacrificed for quantitative immunohistochemical staining (Week 24 post-AAV infusion and week 13 post-rotenone infusion) to assess the impact of rotenone on nigrostriatal integrity. At the level of the terminals in the striatum and the cell bodies in the substantia nigra, infusion of either AAV-α-synuclein or rotenone alone caused a significant loss of nigrostriatal neurons as revealed by tyrosine hydroxylase immunoreactivity (Fig. 5.5. Striatal: Group,
$F_{(3,29)}=13.48, P<0.0001$; Nigral: Group, $F_{(3,29)}=6.01, P<0.001$. Post-hoc testing confirmed significant neurodegeneration in the Rotenone and α-Synuclein groups relative to the Control group in both regions). As can clearly be seen from the photomicrographs (Fig. 5.5A) and volumetric analysis (Fig. 5.5E), the loss of tyrosine hydroxylase immunopositive terminals from the striatum in the single treatment groups occurred in the absence of any loss of striatal volume. Thus unilateral intranigral AAV-α-synuclein or unilateral intrastriatal rotenone caused pronounced dopaminergic deafferentation of the striatum without affecting the intrinsic striatal cells. In sharp contrast, when the pesticide was administered intrastriatally to rats with a high nigrostriatal load of α-synuclein, this resulted in marked loss of striatal parenchyma (Fig. 5.5A and Fig. 5.5E; Group, $F_{(3,29)}=47.77, P<0.0001$. Post-hoc testing confirmed significant loss in striatal volume in the Combined group relative to all other groups). Because of this loss of striatal tissue, it was not possible to reliably assess whether there was an additive effect of the combined treatments on the density of nigrostriatal terminals innervating this region. Nevertheless, we did perform this analysis on the residual striatal tissue and found that, where there was striatal tissue remaining, the density of tyrosine hydroxylase immunopositivity was significantly reduced in the combined group compared to the single treatment groups (ANOVA outcome as above and post-hoc testing confirmed significant neurodegeneration in the Combined group relative to single treatment groups). There was no additive effect of the two insults on the number of tyrosine hydroxylase immunopositive cell bodies in the substantia nigra (Fig. 5.5B&D).
Fig. 5.5 Unilateral intranigral infusion of AAV-α-synuclein and/or intrastriatal rotenone induces nigrostriatal degeneration. Unilateral intracerebral infusion of AAV-α-synuclein or rotenone caused a loss of nigrostriatal terminals (A & C) and cell bodies (B & D) as indicated by a loss of tyrosine hydroxylase immunoreactivity. Importantly, there was an additive effect of the two insults at the striatal but not the nigral level. However, because combined administration resulted in pronounced loss of striatal tissue (A & E), the effect of combined treatment could only be assessed on the small fraction of striatal tissue remaining. **P<0.01, ***P<0.001 vs. Control group; ++P<0.001, +++P<0.001 vs. α-Synuclein group, #P<0.05, ###P<0.001 vs. Rotenone group by one-way ANOVA with post-hoc Newman Keuls. Data are shown as mean ± s.e.m. TH-ir: tyrosine hydroxylase immunoreactivity. Scale bar: A) = 1 mm; B) = 0.5mm.
5.4.1.4 Impact of unilateral intranigral infusion of AAV-α-synuclein and/or intrastriatal rotenone on α-synuclein expression

As expected, unilateral intranigral infusion of AAV-α-synuclein led to significant expression of α-synuclein in the substantia nigra and striatum (Fig. 5.6. Nigral: Group, $F_{(3,29)}=4.40$, $P<0.05$; Striatal: Group, $F_{(3,29)}=6.79$, $P<0.01$. Post-hoc testing confirmed significant α-synuclein expression in the AAV-α-synuclein group relative to the Control group in both regions). Because subsequent intrastriatal infusion of rotenone caused destruction of the striatal tissue, this precluded a reliable assessment of the impact of the pesticide on α-synuclein expression in this region. Nevertheless, assessment of the density of α-synuclein expression in the remaining tissue indicated that there was no combined effect on the density of striatal α-synuclein expression. This was also the case in the ventral mesencephalon.
Fig. 5.6 Impact of unilateral intranigral infusion of AAV-α-synuclein and/or intrastriatal rotenone on α-synuclein expression. Unilateral administration of AAV-α-synuclein into the rat substantia nigra caused significant expression of α-synuclein in the nigrostriatal pathway at the level of the terminals in the striatum (A), as well as in the cell bodies in the substantia nigra (B). However, there was no evidence that subsequent administration of rotenone affected the density of α-synuclein expression in either region. Data is shown as mean ± s.e.m. **P<0.01 vs. Control by one-way ANOVA with post-hoc Newman Keuls. α-synuclein-ir: α-synuclein immunoreactivity; VM: ventral mesencephalon. Scale bar: A) = 1 mm; B) = 0.5mm

5.4.2 Study to determine the effect of sequential intranigral infusion of AAV-α-synuclein and rotenone.

As the previous study established that infusion of rotenone into the striatum 13 weeks after intranigral infusion of AAV-α-synuclein caused pronounced destruction of the striatal parenchyma, in this study, rotenone was instead infused into the substantia nigra
and the impact of the single and combined treatments on body weight, motor function, nigrostriatal integrity and α-synuclein expression was assessed.

5.4.2.1 Unilateral intranigral infusion of AAV-α-synuclein and/or rotenone is not detrimental to the rats’ general health

Intranigral infusion of AAV-α-synuclein and/or rotenone did not induce mortality in any of the rats. Moreover, neither the single nor combined treatments had any effect on the body weight of the animals (Fig. 5.7; Group, $F_{(3,25)}=1.05$, $P=0.38$, ns) and all infusion groups continued to gain weight at the same rate as the control groups of rats (Time, $F_{(26,650)}=452.56$, $P<0.0001$; Group x Time, $F_{(78,650)}=0.39$, $P=1.00$, ns).

Fig. 5.7 Sequential unilateral intranigral AAV-α-synuclein and/or rotenone infusion does not affect rats’ body weights. Unilateral infusion of AAV-α-synuclein and/or rotenone into the rat substantia nigra did not affect the body weight of the rats. The XY plot on the left depicts the data collected over the course of the study, while the bar chart on the right represents collapsed data from the post-rotenone infusion period only. Data are shown as mean ± s.e.m. Dashed lines represent the point of infusion surgery.
5.4.2.2 Unilateral intranigral infusion of AAV-α-synuclein and/or rotenone induces contralateral motor dysfunction

In line with our previous studies, in its own right, unilateral intranigral infusion of AAV-α-synuclein induced significant contralateral motor impairments in the Corridor and Stepping Tests but not the Whisker Test (Fig. 5.8-5.10). Moreover, we also demonstrate for the first time in this thesis or on the published literature that unilateral intranigral infusion of rotenone is also capable of inducing robust motor dysfunction in all of the tests used (Fig. 5.8-5.10). However, as with the previous study, the most important finding of this study is the additive effect of sequential intranigral administration of the genetic and environmental insults. Thus, in the Corridor Test of contralateral neglect (Fig. 5.8), intranigral infusion of AAV-α-synuclein followed 13 weeks later by infusion of rotenone at the same site significantly reduced the rats’ ability to retrieve food from the contralateral side of their bodies when compared to either insult alone (Corridor: $F(3,25)=14.75, P<0.0001$; post-hoc Newman Keuls confirmed a significant decrease in the combined group relative to the α-Synuclein and Rotenone groups). In the Stepping Test of forelimb akinesia (Fig. 5.9), combining α-synuclein overexpression and rotenone administration significantly reduced the number of contralateral forelimb adjusting steps made when either factor was administered singly (Backhand: Group, $F(3,25)=17.95, P<0.0001$; post-hoc Newman Keuls confirmed a significant decrease in the combined group relative to the α-Synuclein and Rotenone groups). In the Whisker Test of sensorimotor integration (Fig. 5.10) combining AAV-α-synuclein and rotenone did not affect the rotenone-induced impairment in vibrissae-elicited contralateral forelimb placings (Whisker: Group, $F(3,25)=16.29, P<0.0001$; post-hoc Newman Keuls confirmed no significant decrease in the combined group relative to the Rotenone group).
Fig. 5.8 Sequential unilateral intranigral AAV-α-synuclein and rotenone administration induces contralateral motor dysfunction in the Corridor Test. Animals infused with either AAV-α-synuclein and/or rotenone alone showed an increase in ipsilateral retrieval (A) and a decrease in contralateral retrieval (B) over the study period. Importantly, an additive effect on contralateral retrieval was observed following sequential intranigral infusion of both insults. The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-rotenone infusion period only. Dashed lines depict the point of infusion surgery. Data are shown as mean ± s.e.m. **P<0.01, ***P<0.001 vs. Control; ++P< 0.01 vs. α-synuclein; ###P<0.001 vs. Rotenone by one-way ANOVA with post-hoc Newman Keuls.
Fig. 5.9 Sequential unilateral intranigral AAV-α-synuclein and rotenone administration induces contralateral motor dysfunction in the Stepping Test. Animals sequentially infused with AAV-α-synuclein and/or rotenone showed a decline in contralateral forehand (C) and backhand (D) stepping over the study period while ipsilateral performance (A & B) was not affected. Importantly, an additive effect on contralateral backhand stepping was observed following sequential infusion of both insults. The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-rotenone infusion period only. Dashed lines depict the point of infusion surgery. Data are shown as mean ± s.e.m. *P<0.05, **P<0.001 vs. Control; *P<0.05, **P<0.001 vs. α-synuclein; #P<0.05 vs. Rotenone by one-way ANOVA with post-hoc Newman Keuls.
Fig. 5.10 Sequential unilateral intranigral AAV-α-synuclein and rotenone administration induces contralateral motor dysfunction in the Whisker Test. Animals infused with rotenone alone showed a decrease in contralateral placings (B) over the study period while ipsilateral performance (A) was not affected. No additive effect was observed following sequential infusion of both of insults. The XY plots on the left depict the data collected over the course of the study, while the bar charts on the right represent collapsed data from the post-rotenone infusion period only. Dashed lines depict the point of infusion surgery. Data are shown as mean ± s.e.m. **$P<0.01$, ***$P<0.001$ vs. Control; +++$P<0.001$ vs. α-synuclein by one-way ANOVA with post-hoc Newman Keuls.

5.4.2.3 Sequential unilateral intranigral AAV-α-synuclein and rotenone administration leads to a progressive decline in motor function.

One of the most important features of human Parkinson’s disease which has proven difficult to model in laboratory animals is the progressive nature of the movement disorder. In the present study, one of the most significant features of the data is the slow and protracted emergence of the motor dysfunction in the Corridor Test in the Combined group (Fig. 5.8B). Thus, in this test, sequential intranigral injection of AAV-α-synuclein and rotenone induced a progressive decline in contralateral motor function that emerged
over a period of weeks (Group x Time, $F_{(30,250)}=4.62$, $P<0.0001$. Post-hoc testing confirmed a progressive decline in contralateral retrieval over the first 3 weeks after rotenone administration in the Combined group ($P<0.01$ vs. Control by Week 1 after rotenone surgery, $P<0.001$ vs. Control by Week 2 after rotenone surgery, $P<0.0001$ vs. Control by Week 3 after rotenone surgery)).

5.4.2.4 Sequential intranigral infusion of AAV-α-synuclein and/or rotenone induces nigrostriatal degeneration.

Once behavioural testing was completed, rats were sacrificed for quantitative immunohistochemical staining (Week 24 post-AAV infusion and week 13 post-rotenone infusion) to assess the impact of rotenone on nigrostriatal integrity. At the level of the terminals in the striatum and the cell bodies in the substantia nigra, administration of either rotenone or AAV-α-synuclein caused a significant loss of nigrostriatal neurons as revealed by tyrosine hydroxylase immunoreactivity (Fig. 5.11 Striatal: Group, $F_{(3,25)}=15.10$, $P<0.0001$; Nigral: Group, $F_{(3,25)}=16.04$, $P<0.0001$. Post-hoc testing confirmed significant neurodegeneration in the Rotenone and α-Synuclein groups relative to the Control group in both regions). When the genetic and environmental insults were sequentially administered, the loss of tyrosine hydroxylase immunopositive terminals from the striatum was significantly greater than that induced by either insult alone (Fig. 5.11A & C, ANOVA outcome as above and post-hoc testing confirmed significant neurodegeneration in the Combined group relative to the single treatment groups in this region). The same trend was also seen in the nigral region with more pronounced loss of tyrosine hydroxylase immunopositive cell bodies from the substantia nigra of the Combined group when compared with the single treatment groups (Fig. 5.11B & D, ANOVA outcome as above and post-hoc testing confirmed significant neurodegeneration...
in the Combined group relative to the Rotenone group in this region). As in the previous
chapter, AAV-α-synuclein caused swollen dystrophic axon terminals in the striatum
which we assessed in tyrosine hydroxylase immunostained sections (Fig. 5.12). 
Intranigral infusion of rotenone did not induce the formation of dystrophic neurites, nor
did it enhance the number of AAV-α-synuclein induced dystrophic neurites in the
Combined group.

Fig. 5.11 Sequential unilateral intranigral AAV-α-synuclein and rotenone infusion
induces nigrostriatal degeneration. Unilateral administration of AAV-α-synuclein
and/or rotenone into the rat substantia nigra caused significant degeneration of the
nigrostriatal pathway which was evident at the level of the terminals in the striatum (A),
as well as the cell bodies in the substantia nigra (B). Note that the impact of the combined
 genetic and environmental insults was more pronounced than either insult alone. The XY
plots on the left depict the data collected over the course of the study, while the bar charts
on the right represent collapsed data from the post-operative period only. Data is shown
as mean ± s.e.m. **P<0.01, ***P<0.001 vs. Control; ##P<0.01 vs. Rotenone; +P<0.05 vs.
α-Synuclein by one-way ANOVA with post-hoc Newman Keuls. TH-ir: tyrosine
hydroxylase immunoreactivity. Scale bar: A) = 1 mm; B) = 0.5mm.
Fig. 5.12 Sequential unilateral intranigral AAV-\(\alpha\)-synuclein and rotenone infusion does not exacerbate AAV-\(\alpha\)-synuclein induced neuritic dystrophy. Immunohistochemistry for tyrosine hydroxylase revealed significant neuritic dystrophy in the ipsilateral striatum in animals infused with AAV-\(\alpha\)-synuclein. Rotenone administration did not exacerbate this effect when combined with AAV-\(\alpha\)-synuclein infusion. ***\(P<0.001\); vs. control groups by one-way ANOVA with post-hoc Newman Keuls. Data are shown as mean ± s.e.m. Scale bar: 0.1 mm.

5.4.2.5 Unilateral intranigral infusion of rotenone does not affect AAV-\(\alpha\)-synuclein-induced \(\alpha\)-synuclein expression

As expected, intranigral injection of AAV-\(\alpha\)-synuclein led to significant expression of \(\alpha\)-synuclein in the substantia nigra and striatum (Fig. 5.13C & D. Nigral: Group, \(F_{(3,25)}=10.05\), \(P<0.001\); Striatal: Group, \(F_{(3,25)}=16.46\), \(P<0.0001\). Post-hoc testing confirmed significant \(\alpha\)-synuclein expression in the \(\alpha\)-Synuclein and Combined groups relative to the Control group in both regions). Qualitative assessment of each \(\alpha\)-synuclein immunostained section revealed that the overall pattern of \(\alpha\)-synuclein immunoreactivity differed between rats but not between groups. For example, in the photomicrographs shown in Fig. 5.13, relative to the AAV-\(\alpha\)-synuclein only rat, in the Combined rat, the striatal \(\alpha\)-synuclein expression extends more ventrally whilst the midbrain \(\alpha\)-synuclein expression is more dense and extends more dorsally and medially. However, subsequent quantitative analysis of both the optical density and area (Fig. 5.13E & F) of
immunostaining confirmed that this was simply due to inherent variability in the AAV transduction and in vivo transgene expression. Thus, sequential administration of the pathogenic virus and rotenone did not have any effect on the level of expression induced by AAV-α-synuclein alone.

Fig. 5.13 Sequential unilateral intranigral AAV-α-synuclein and rotenone infusion did not affect AAV-α-synuclein-induced α-synuclein expression. Unilateral administration of AAV-α-synuclein into the rat substantia nigra caused significant expression of α-synuclein in the nigrostriatal pathway at the level of the terminals in the striatum (A, C, E), as well as in the cell bodies in the substantia nigra (B, D, F). However, there was no evidence that subsequent administration of rotenone affected the level of α-synuclein expression. Data is shown as mean ± s.e.m. **P<0.01, ***P<0.001 vs. Control by one-way ANOVA with post-hoc Newman Keuls. α-synuclein-ir: α-synuclein immunoreactivity; VM: ventral mesencephalon. Scale bar: A) = 1 mm; B) = 0.5 mm.
5.5 Discussion

The overall aim of the experiments described in this chapter was to develop and characterise a novel multi-hit model of Parkinson’s disease by exposing rats to sequential intracerebral infusions of relevant environmental and genetic risk factors. Rats were firstly unilaterally infused with AAV-α-synuclein to the substantia nigra, and this was followed 13 weeks later by infusion of rotenone into the nigrostriatal terminals (striatum) or cell bodies (substantia nigra) on the same side. We found that, although striatal infusion of rotenone to rats with a high nigrostriatal load of α-synuclein caused pronounced motor dysfunction and nigrostriatal degeneration, this approach was also associated with extensive loss of striatal tissue. In contrast, sequential administration of AAV-α-synuclein and rotenone to the same substantia nigra site resulted in a model with α-synuclein expression, nigrostriatal degeneration and motor dysfunction without any overt systemic toxicity. Thus, in terms of our aim to develop and characterise a novel multi-hit model of Parkinson’s disease, we have shown that sequential intranigral infusion of AAV-α-synuclein and rotenone can reliably and robustly model the main features of Parkinson’s disease (Mulcahy et al., 2012).

It has been almost thirty years since the first report of dopaminergic neurotoxicity induced by direct administration of the organic pesticide, rotenone, to the adult rat brain (Heikkila et al., 1985). However, it is only in the past few years that research into this method of modelling Parkinson’s disease has begun in earnest (Klein et al., 2011; Mulcahy et al., 2011; Norazit et al., 2010; Saravanan et al., 2005; Sindhu et al., 2006; Sindhu et al., 2005; Swarnkar et al., 2010; Swarnkar et al., 2011; Xiong et al., 2009). These studies have revealed that direct infusion of rotenone into the substantia nigra (Sindhu et al., 2005; Swarnkar et al., 2010; Swarnkar et al., 2011; Xiong et al., 2009),
medial forebrain bundle (Klein et al., 2011; Norazit et al., 2010; Sindhu et al., 2006; Sindhu et al., 2005) or striatum (Mulcahy et al., 2011; Swarnkar et al., 2010; Swarnkar et al., 2011) can model some of the facets of the condition. The present set of studies provides further evidence that unilateral administration of rotenone into the adult rat striatum or substantia nigra can replicate some of the main features of Parkinson’s disease, namely, a bradykinetic motor syndrome manifest by contralateral neglect, contralateral forelimb akinesia and contralateral sensorimotor dysfunction, as well as nigrostriatal degeneration extending from the cell bodies in the substantia nigra to the terminals in the striatum. Moreover, the Corridor Test revealed that the impairment in contralateral neglect emerged relatively progressively (compared to the other behaviour tests) over three weeks after intranigral administration of the pesticide. However, in line with our previous report (Mulcahy et al., 2011), and in contrast to the effect of systemic administration (Betarbet et al., 2000; Hoglinger et al., 2003; Sherer et al., 2003c; Uversky et al., 2001), neither intrastriatal nor intranigral rotenone administration induced any α-synucleinopathy when administered alone. The reason that systemic, but not intracerebral, rotenone administration can induce α-synucleinopathy is not clear, but it is possible that one or more of the rotenone metabolites might be responsible for this neuropathological feature (Caboni et al., 2004). It is also possible that continuous exposure to the pesticide is required for the accumulation of α-synuclein to occur which could explain the discrepancy between chronic systemic and acute intracerebral models.

As neither intrastriatal nor intranigral infusion of rotenone could induce α-synucleinopathy in its own right, the main aim of this study was to determine if sequentially administering the pesticide to rats with AAV-α-synuclein-induced α-synuclein overexpression could recapitulate the classic features associated with human
Parkinson’s disease. In our first experiment, the pesticide was administered to the nigrostriatal terminals at the level of the striatum 13 weeks after intranigral AAV-α-synuclein administration. Although the behavioural data from this experiment appeared extremely promising with pronounced additive effects on contralateral motor function in the Corridor, Stepping and Whisker Tests, when the rats were sacrificed and their brains examined post mortem, the neuropathological impact of this approach was revealed. Strikingly, intranigral AAV-α-synuclein combined with intrastriatal rotenone led to almost complete destruction of the striatal tissue. This was somewhat surprising as the nigrostriatal dopaminergic neurons and not the intrinsic striatal cells were the intended target of our intranigral AAV virus. Thus, it was puzzling that the striatal cells themselves should have been rendered sensitive to the effect of the pesticide after intranigral AAV-α-synuclein. However, closer perusal of the published literature revealed that intranigral AAV infusion can result in transgene expression in the intrinsic striatal GABAergic neurons (Schneider et al., 2008). Presumably this is due to transduction of the GABAergic striatonigral terminals and transgene expression in the cell bodies after retrograde transport. Whatever the reason for striatal sensitivity to rotenone after intranigral AAV-α-synuclein infusion, this study points to a pronounced and synergistic effect of these genetic and environmental factors on the intrinsic striatal GABAergic neurons. Although the pronounced striatal degeneration nullifies this approach as a model of Parkinson’s disease, it may be useful as a model of multiple systems atrophy as this disease is associated with α-synucleinopathy as well as degeneration of nigrostriatal and striatal projection neurons (Spillantini et al., 1998a; Tu et al., 1998; Wakabayashi & Takahashi, 2006) Moreover, the striking synergistic effect on the striatal cells may have implications for understanding the etiology and pathogenesis of this devastating condition.
Having established that the striatum was too sensitive to the toxic effects of rotenone after intranigral AAV-α-synuclein administration, we then sought to assess the effects of sequential intranigral infusion of the two factors. In this experiment, there was no local toxic effect of AAV-α-synuclein and/or rotenone on the nigral tissue thus enabling us to ascertain the functional impact of this combination on motor function, nigrostriatal degeneration and α-synucleinopathy. When rotenone was infused into the substantia nigra of rats overexpressing α-synuclein, the pesticide did not affect the nigrostriatal α-synuclein load, but did induce motor and neuropathological effects that were more pronounced than either insult alone (Mulcahy et al., 2012). Moreover, one of the most intriguing aspects of this study is the relationship between the extent of neurodegeneration and emergence of motor impairments after treatment with rotenone and/or AAV-α-synuclein. When administered alone, rotenone caused less nigrostriatal degeneration (35% loss of striatal terminals and 30% loss of nigral cell bodies) than AAV-α-synuclein, (48% loss of striatal terminals and 54% loss of nigral cell bodies) however, conversely, the motor deficits induced by rotenone were more pronounced than those induced by AAV-α-synuclein. This indicates that despite being less toxic to the nigrostriatal neurons at a gross anatomical level, rotenone had a more pronounced impact on their functionality. This is not that surprising since rotenone is a highly toxic exogenous inhibitor of mitochondrial respiration whereas α-synuclein is a ubiquitously-expressed physiological protein that is only toxic in high doses (or in mutated forms). Thus, it is likely that even if rotenone does not cause exposed neurons to die, it could impair many adenosine triphosphate-dependent processes that are required for normal neurofunctionality with consequent impact on motor performance. Interestingly, even when AAV-α-synuclein and rotenone were sequentially administered, the resulting cell loss was still relatively modest (~70% loss of striatal terminals and nigral cell bodies)
when compared to 6-hydroxydopamine infusion models. Thus, this combined model could be considered a model of the early symptomatic stage of Parkinson’s disease which is associated with 50-80% neurodegeneration (Becker et al., 2002). Many other widely-used models of early-stage Parkinson’s disease, such as partial lesion models induced by 6-hydroxydopamine, are also associated with similar levels of neurodegeneration and motor dysfunction (Barneoud et al., 1995; Kirik et al., 1998; Lee et al., 1996; Paille et al., 2010). These partial lesion models can easily be modified to produce complete lesion models by adjusting the dose and/or placement of the neurotoxin. Thus, it is tempting to speculate that similar modifications to this sequential intranigral AAV-α-synuclein/rotenone model could also produce more pronounced lesions and motor impairments. However, caution is urged in making such extrapolations as rotenone is highly toxic in higher doses and is known to cause non-specific toxicity with gross anatomical destruction of the neural parenchyma after intracerebral administration (Klein et al., 2011). Another intriguing aspect of the behavioural data generated in this study is that, in the Corridor Test, the AAV-α-synuclein/rotenone-induced neglect of contralateral space emerged in a progressive manner. The reason why impairments in contralateral selection (Corridor Test) but not in contralateral forelimb kinesis (Stepping Test) or contralateral sensorimotor integration (Whisker Test) emerged progressively in the Combined group is not known. Interestingly, we have also observed this phenomenon after nigrostriatal lesions induced by the catecholamine neurotoxin, 6-hydroxydopamine (Grealish et al., 2008) indicating that the Corridor Test, but not the Stepping or Whisker Tests, is capable of revealing the progressive emergence of motor dysfunction as a result of the underlying nigrostriatal lesion development. Although not completed in this study, it will be important in future studies to assess the predictive validity of this novel
Parkinson’s disease model by assessing levodopa-induced restoration of motor function in the various motor tasks.

As far as we are aware, there are no published reports of combining AAV-α-synuclein gene transfer and environmental risk factors as an approach to modelling Parkinson’s disease in the rat. Thus, the results of the sequential intranigral AAV-α-synuclein and rotenone study are important for two main reasons. Firstly, this sequential intranigral model is the first Parkinson’s disease model to display robust and progressive impairment of spontaneous motor function with associated nigrostriatal α-synucleinopathy and neurodegeneration, without any overt detriment to the rats (Mulcahy et al., 2012). Thus, it replicates the triad of classic Parkinson’s disease features and could be an invaluable aid to future drug discovery programmes. Secondly, this study also gives some insight into the neuropathological and motor consequences of exposure to two known Parkinson’s disease risk factors, that is, enhanced neurodegeneration and motor disability. Although the molecular mechanisms underlying α-synuclein-induced neurodegeneration remain incompletely understood, abnormal vesicle processing with alterations in protein turnover, mitochondrial function and oxidative stress, is thought to contribute to α-synuclein-induced synaptic dysfunction and death (for review see (Cookson & van der Brug, 2008). Mitochondrial dysfunction and oxidative stress also underlie rotenone-induced neuronal death as this pesticide is a classical inhibitor of Complex I of the mitochondrial electron transport chain (Degli Esposti, 1998). As the motor and neuropathological features were significantly enhanced when rats were sequentially exposed to the genetic and environmental risk factors, this indicates that the processes underlying α-synuclein and rotenone-induced neuronal death are additive in this model system.
In summary, results from this chapter have shown that sequential unilateral intranigral administration of AAV-α-synuclein and rotenone leads to a model of Parkinson’s disease in which there is progressive motor dysfunction with underlying nigrostriatal α-synucleinopathy and neurodegeneration. Sequential exposure to these two known risk factors for Parkinson’s disease resulted in a model with more exaggerated motor impairments and neuropathology than either the genetic or environmental risk factor alone. Thus, this model may be useful for researchers examining the interplay between genes and the environment in Parkinson’s disease, as well as researchers investigating the efficacy of novel anti-Parkinsonian or neuroprotective approaches.
Chapter 6: General discussion

The work presented in this thesis sought to develop and characterise a novel model of Parkinson’s disease by direct intracerebral infusion of the organic pesticide, rotenone, and also by combining rotenone administration with nigrostriatal AAV-mediated α-synuclein overexpression. The main findings from this body of work are: 1) that intrastriatal infusion of rotenone is capable of causing dose-related motor dysfunction and nigrostriatal degeneration without any α-synucleinopathy (Mulcahy et al., 2011), 2) that combining systemic administration of rotenone with intranigral infusion of AAV-α-synuclein can induce the classical features of Parkinson’s disease but is associated with peripheral toxicity, 3) that combining intrastriatal infusion of rotenone with intranigral infusion of AAV-α-synuclein is associated with motor dysfunction, nigrostriatal degeneration and α-synucleinopathy, but is also associated with destruction of the striatal tissue, and 4) that combining intranigral infusion of rotenone with intranigral infusion of AAV-α-synuclein can model the triad of classic features associated with human Parkinson’s disease without any systemic or local toxicity (Mulcahy et al., 2012). Thus, we have shown that sequential intranigral infusion of AAV-α-synuclein and rotenone can reliably and robustly model the main features of this devastating neurodegenerative disease.

Over the course of the studies described in Chapters 3-5, we attempted to model Parkinson’s disease using rotenone and/or AAV-α-synuclein in five different ways. These approaches as well as their associated features are summarised in Table 6.1.
Table 6.1 General overview of the approaches to modelling Parkinson’s disease attempted in this thesis and their associated key features.

The first approach we used (Chapter 3) was infusion of rotenone at four sites along the rostro-caudal axis of the striatum using coordinates established for the Parkinsonian neurotoxin 6-hydroxydopamine (Kirik et al., 1998). In this study, we demonstrated that intrastriatal infusion of the pesticide was capable of causing nigrostriatal degeneration and associated motor impairments in a dose-dependent manner (Mulcahy et al., 2011). This confirms and extends previous reports of the impact of direct intracerebral infusion of rotenone (Antkiewicz-Michaluk et al., 2004; Klein et al., 2011; Norazit et al., 2010; Saravanan et al., 2005; Saravanan et al., 2006; Sindhu et al., 2006; Sindhu et al., 2005; Swarnkar et al., 2010; Swarnkar et al., 2011; Xiong et al., 2009) However, the main limitation of this approach was the lack of α-synucleinopathy meaning that only two of the three classical features were recapitulated. To overcome this hurdle, we incorporated AAV vectors overexpressing normal human α-synuclein in all of the remaining studies.
The next approach we attempted (Chapter 4) was combining systemic administration of rotenone, by twice-weekly intraperitoneal injection or continuous subcutaneous infusion, with intranigral injection of AAV-α-synuclein. Although the regime we used for intraperitoneal administration of rotenone (1.0 mg kg\(^{-1}\) i.p. twice-weekly for 13 weeks after AAV) was low enough to prevent any overt toxicity to the animals, unfortunately it was also too low to induce any Parkinsonian features even in rats with nigrostriatal α-synuclein overexpression. In contrast, when rotenone was administered by continuous subcutaneous infusion (2.5 mg kg\(^{-1}\) day\(^{-1}\) s.c. for 4 weeks from weeks 13-17 after AAV), it did cause enhanced nigrostriatal degeneration and motor impairments in animals with a high nigrostriatal load of α-synuclein. However, these features developed at a cost to the rats’ general health as the animals began to lose weight rapidly after minipump implantation in line with many published reports of rotenone-induced peripheral toxicity (Ferrante et al., 1997; Greene et al., 2009; Lapointe et al., 2004). Thus, in our studies were unable to establish if systemic rotenone could induce Parkinsonism in rats with AAV-mediated α-synuclein overexpression without impacting on their general health. To overcome this, in the final studies, we again administered rotenone by intracerebral rather than by systemic routes.

The final approach we attempted (Chapter 5) was combining intracerebral administration of rotenone, either intrastriatally or intranigrally, with intranigral injection of AAV-α-synuclein. Although combining intrastriatal infusion of the pesticide with intranigral AAV-α-synuclein had an additive effect on motor dysfunction and nigrostriatal degeneration, the two insults together also caused destruction of the striatal parenchyma. In contrast, intranigral infusion of rotenone to animals with a high α-synuclein load led to a progressive decline in motor function which was underpinned by nigrostriatal
degeneration and \( \alpha \)-synuclein accumulation. Thus, this final study shows that sequential intranigral infusion of AAV-\( \alpha \)-synuclein and rotenone can model the triad of classic features associated with human Parkinson’s disease, namely, motor dysfunction, nigrostriatal degeneration and \( \alpha \)-synucleinopathy (Mulcahy et al., 2012).

Due to lack of efficacy (AAV-\( \alpha \)-synuclein with intra-peritoneal rotenone) or overt systemic toxicity (AAV-\( \alpha \)-synuclein with subcutaneous rotenone), the systemic rotenone approaches attempted in this thesis are of limited value as preclinical models of human Parkinson’s disease. Similarly, although the AAV-\( \alpha \)-synuclein with intrastriatal rotenone approach may have relevance to the modelling, etiology and/or pathogenesis of multiple systems atrophy, its striatal toxicity precludes its usefulness as a model of Parkinson’s disease. In contrast, two of the approaches we attempted have generated useful models of the human condition, namely, the four site intrastriatal rotenone model and the dual-hit AAV-\( \alpha \)-synuclein with intrastriatal rotenone model. Thus, both of these models will be discussed further at this stage with specific reference to their advantages and disadvantages over existing models (Table 6.2).
Table 6.2 General overview of the models of Parkinson’s disease developed in this thesis and their associated key features in comparison with more established models.

The four site intrastriatal rotenone model compares favourably with established neurotoxic models, such as those induced by 6-hydroxydopamine and MPTP, in so far as it causes nigrostriatal degeneration and motor impairments without any impact on α-synuclein expression. The time frame for the onset of motor dysfunction is also similar to these established models as impairments occur rapidly after lesioning (Blandini et al., 2007; Grealish et al., 2008; Krishnamurthi et al., 2009; Meredith et al., 2008; Rozas et al., 1998). However, the rotenone model confers a distinct advantage over the other models in that rotenone, but not 6-hydroxydopamine and MPTP (for exception see (Langston et al., 1983)), has been shown to be a direct causal agent of Parkinson’s disease and thus this confers enhanced construct validity on the model. Moreover, because injecting the pesticide directly into the brain bypasses the toxicity associated with its systemic administration, this means that the intrastriatal model is associated with fiscal, logistical and ethical benefits when compared with the systemic rotenone models. Thus,
this novel four site intrastriatal rotenone model (Mulcahy et al., 2011) compares favourably with well established and widely-used neurotoxic models of Parkinson’s disease as well as with the systemic rotenone models, and may be of especial interest to researchers investigating the role of environmental agritoxins in the etiology of the human condition.

With regard to the sequential intranigral AAV-α-synuclein and intranigral rotenone model, the key question is whether this dual-hit model confers any advantage over the widely-accepted AAV-α-synuclein model. Clearly previous studies have shown that viral transfer of the human α-synuclein gene into the adult rat substantia nigra can induce α-synuclein expression and aggregation, neuronal dystrophy and degeneration, and associated motor deficits (Cao et al., 2010; Chung et al., 2009; Decressac et al., 2012; Kirik et al., 2003b; Kirik et al., 2002; Koprich et al., 2011; Ulusoy et al., 2010). However, the published literature suggests that this model is highly variable with nigrostriatal degeneration of a magnitude sufficiently high enough to induce motor dysfunction only occurring in a small proportion of the rats (Kirik et al., 2002; Maingay et al., 2005; Ulusoy et al., 2010; Yamada et al., 2004a). Thus it was somewhat surprising to us that, in its own right, AAV-α-synuclein consistently induced nigrostriatal neurodegeneration with significant impairments in contralateral food retrieval (Corridor Test) and forelimb placing (Stepping Test) from ~12 weeks after administration (Chapters 4 & 5 and (Mulcahy et al., 2012)). This begs the question – is there any need for subsequent rotenone administration at all? However, the sequential intranigral AAV-α-synuclein and intranigral rotenone model does confer two main advantages over the existing model. The first of these is that the rotenone administration accelerates the protracted time-course and exacerbates the relatively mild motor dysfunction associated
with the AAV-α-synuclein model. Moreover, because nigrostriatal α-synuclein expression can be detected within 10 days after AAV administration (Lundblad et al., 2012), rotenone could be administered at a much earlier time-point than used in the present study (13 weeks). The rotenone-induced acceleration and exacerbation of the AAV-α-synuclein effect could therefore confer considerable fiscal and logistical advantages over the more established model. The second main advantage of the sequential intranigral AAV-α-synuclein and intranigral rotenone model is that, because it combines an established genetic risk factor and an established environmental risk factor, it has improved construct validity as a model of this neurodegenerative disease which is thought to arise due to interactions between genes and the environment (Gao et al., 2011a; McCulloch et al., 2008; Tanner et al., 2011; Vance et al., 2010). Thus this model may be useful for researchers examining the interplay between genes and the environment in Parkinson’s disease.

**Future validation of these models**

Although we have completed a considerable characterisation of the models generated in this thesis, there are some outstanding questions could form the basis of future work. Firstly, and perhaps most importantly, it will be necessary to establish the time-course, not just for the behavioural impairments, but also for the neuropathological features associated with the different models (i.e. α-synuclein expression, and rotenone/α-synuclein-induced neuronal dystrophy/neurodegeneration). This will require separate cohorts of animals sacrificed at different time points after inducing the lesion(s). It will be important to establish this time-course because human Parkinson’s disease involves a progressive decline in motor function as a result of progressive degeneration of the
nigrostriatal neurons. It will also be important to ascertain the nature of the α-synuclein immunopositive accumulations in the nigrostriatal neurons as the protein accumulates into insoluble aggregates in the human brain (Tanji et al., 2010; Zhou et al., 2011) as well as in other viral gene transfer models (Conway et al., 1998; Kim et al., 2011; Klein et al., 2002; Lo Bianco et al., 2002). Thus it will be important to determine, using proteinase-K digestion, if the α-synuclein expression observed following sequential intranigral AAV-α-synuclein and rotenone infusion is similarly aggregated. One final study which would improve the predictive validity of the models we have generated is to confirm their responsivity to levodopa administration. Since levodopa is the most effective pharmacotherapy for Parkinson’s disease, its administration (with a peripheral DOPA decarboxylase inhibitor) should restore motor function in any relevant model of the disease. The use levodopa treatment in animal models of Parkinson’s disease has been shown to be effective in the most widely used models including the early reserpine, 6-hydroxydopamine and MPTP models (Duty et al., 2011). The further validation of our models by the administration of levodopa, initiated from the point of development of motor dysfunction, similar to the initiation point in the human condition, would strengthen their contribution to the field of Parkinson’s disease research.

The future of Parkinson’s disease modelling

The studies carried out within this thesis have made several contributions to the field of Parkinson’s disease modelling. Most importantly, we have developed a new dual-hit genetic and environmental model that is one of the first models in which the triad of classical features associated with the human condition is reliably and robustly expressed. However, one of the inherent limitations of all of the existing approaches to modelling
human Parkinson’s disease, including the ones described in this thesis, is that they focus on nigrostriatal degeneration and motor dysfunction whereas it is become increasingly apparent that the human condition is much broader. As described earlier, Parkinson’s disease is not just a motor condition and is also associated with a myriad of non-motor symptoms such as dementia, depression, insomnia, and autonomic and gastrointestinal dysfunction (Chaudhuri et al., 2006; Shulman et al., 2002; Ziemssen & Reichmann, 2007). Thus, for a truly relevant model of this disease to be established, these factors will eventually need to be taken into account. In this context, the use of rotenone may have some advantages over other neurotoxins in that it is not selective for dopaminergic neurons. This means that it could also target other systems that play a pivotal role in non-motor features. There have been recent reports of studies carried out concentrating on the non-motor symptoms of Parkinson’s disease include tests of cognition, memory, depression, sleep disturbances and gastrointestinal disorders (McDowell & Chesselet, 2012; Taylor et al., 2009). The adaptation of these to our models may be of significant interest in terms of better understanding the multi-system effects associated with Parkinson’s disease.

Final remarks

Given that Parkinson’s disease is thought to arise as a result of interactions between genes and the environment, modelling the condition using genetic and environmental insults may be a more relevant approach than the single insult models currently in widespread use. Moreover, this body of work has shown that this dual-hit approach is capable of modelling the classical features of human Parkinson’s disease, something that most existing single hit models fail to do. Thus, this model may prove useful in future studies
attempting to develop novel pharmacological, cell or gene therapies for this devastating human condition.


Ferraro, TN, Golden, GT, DeMattei, M, Hare, TA, Fariello, RG (1986) Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on levels of glutathione in the extrapyramidal system of the mouse. *Neuropharmacology* 25(9): 1071-1074.


References


Singer, TP, Ramsay, RR, McKeown, K, Trevor, A, Castagnoli, NE, Jr. (1988) Mechanism of the neurotoxicity of 1-methyl-4-phenylpyridinium (MPP+), the toxic bioactivation product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Toxicology 49(1): 17-23.


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

1.1.1 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 \(-70^\circ C\) 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 day pour 1.5mls of culture into an eppendorf and spin @ 12,000rpm at 4°C for 1min. Store the remainder at 4°C.

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

Appendix 1.1.2: Determining Plasmid concentration by Spectrophotometry

1. Using the nano drop systems, equilibrate and zero machine by 1 ul of water.

2. Place 1ul of AAV plasmid on nanodrop and read at 260: 280nm. Take the 260 reading and record the calculated reading.

3. Place 1ul of P-Truf 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 1.1.3: 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-H$_2$O should be at room temperature.

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

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

Do not freeze CaCl$_2$ - prepare freshly [2.5M]
(3.68g CaCl$_2$ 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-H$_2$O or endotoxin free water for cell biology (calculate the volumes of the other components and add water to a final volume of 52ml)
3. CaCl$_2$ (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, 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, 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$^{\text{Ca+Mg}}$ and centrifuge at 1500 g, 2 min, 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.
4. Add 10 ml of PBS$^{\text{Ca+Mg}}$ on top of the virus suspension.
5. Centrifuge at 1500 g, 18°C, in intervals of 1-3 min until the retained volume is about 250 µl.
6. Repeat steps 4+5.
7. Pipet the concentrated virus solution into a glass. Use 100 µl of PBS$^{\text{Ca+Mg}}$ 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 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 in previously. Allow the pellet of nucleic acid to dry in the air for 10mins.

10. Redissolve 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 2 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 flowthrough.

7. **Loadin 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 the 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 recentrifuge for 5 min. Air dry the pellet for 10min and redissolve 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:

- P-truf = 604.3µl
- AAV5 = 2697.94 µl

But for a 1000µg (20 plate) transfection:

- P-truf = 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 labeled 1, for a 20 plate transfection add:
2.5ml CaCl₂
1.2086ml of P-truf
5.39588ml of AAV5
Make the remainder up with water up to 25mls.

3. In a tube labeled 2 add:
25ml of 2X HBS \textbf{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 off 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.

### Preparation of Iodixanol solutions:

<table>
<thead>
<tr>
<th>Percentage Iodixanol</th>
<th>Iodixanol</th>
<th>5M NaCl</th>
<th>5X PBS-MK</th>
<th>H₂O</th>
<th>Phenol Red</th>
</tr>
</thead>
<tbody>
<tr>
<td>15%</td>
<td>12.5ml</td>
<td>10ml</td>
<td>10ml</td>
<td>17.5ml</td>
<td>-</td>
</tr>
<tr>
<td>25%</td>
<td>20.8ml</td>
<td>-</td>
<td>10ml</td>
<td>19.2ml</td>
<td>100µl</td>
</tr>
<tr>
<td>40%</td>
<td>33.3ml</td>
<td>-</td>
<td>10ml</td>
<td>6.7ml</td>
<td>-</td>
</tr>
<tr>
<td>60%</td>
<td>50ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100µl</td>
</tr>
</tbody>
</table>

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)

11.1**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 1.2: Rotenone preparation

Appendix 1.4.1: 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 1.2.2: Rotenone preparation for systemic administration

For systemic intraperitoneal injection, rotenone was dissolved in a 1:1:18 combination of dimethyl sulfoxide (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 solution was injected at 1 ml kg⁻¹.

For systemic administration via Alzet osmotic minipumps, rotenone was dissolved in a 1:1 combination of DMSO and polyethylene glycol (PEG). The following example demonstrates the calculations involved prior to filling the pumps:

**Step 1.** The desired dose to the administered to the study animals was 2.5 mg day⁻¹ kg⁻¹, or 0.105 mg hr⁻¹ kg⁻¹. Average weight of animals = 500 g, therefore, concentration was = 0.053 mg hr⁻¹ rat⁻¹. The concentration of rotenone in the infusion medium (DMSO:PEG) that was necessary to accomplish the desired infusion of 0.053 mg hr⁻¹ was calculated:

Mass flow = volume flow rate x concentration,

or \( k_o = Q \cdot C_d \)

Here \( k_o \) (µg/hr) was the mass delivery rate, \( C_d \) (µg µl⁻¹) represented the concentration of rotenone solution, and \( Q \) (µl hr⁻¹) the pumping rate of the pump. To calculate the concentration of rotenone delivered from the solution used to fill the pump, the total dose administered (per hour) was divided by the pumping rate of the pump.

**Step 2.** The volume flow rate of the 2ML4 pump is 2.5 µl hr⁻¹ (Q), so the required concentration \( (C_d) \) was:

\[
C_d = \frac{0.053 \text{ mg hr}^{-1}}{2.5 \text{ µl hr}^{-1}} = \frac{0.021 \text{ mg µl}^{-1}}{21 \text{ µg µl}^{-1}} = 0.021 \times 10^3 \text{ µg µl}^{-1} = 21 \text{ µg µl}^{-1}
\]
Step 3. The Model 2ML4 pump has a nominal reservoir volume of 2000 µl. In order to achieve the desired concentration of 21 µg µl\(^{-1}\), the amount of rotenone which was dissolved in 2000 µl is:

\[(21 \text{ µg µl}^{-1}) \times 2000 \text{ µl} = 42000 \text{ µg} = 42 \text{ mg}\]

Therefore, in order to infuse rotenone in the rat at 2.5 mg day\(^{-1}\) kg\(^{-1}\) (or 0.053 mg/hr/rat), a solution with a concentration of 42 µg µl\(^{-1}\) of rotenone in solution (DMSO:PEG) was prepared. Excess solution was made for each animal to allow for a safety margin during pump filling.
### Appendix 1.3: General immunohistochemistry protocol

Suitable for 30-40um 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.

<table>
<thead>
<tr>
<th>Step</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quench (Solution 1) below</td>
<td>5 min</td>
</tr>
<tr>
<td>Wash in TBS (Solution 2 below)</td>
<td>3 x 10 min</td>
</tr>
<tr>
<td>Block in 3% serum in TXTBS (Solution 3 below)</td>
<td>2 hours</td>
</tr>
<tr>
<td>Transfer without washing to primary antibody in TXTBS with 1% serum</td>
<td>Overnight at room temp or 2-3 days at 4°C. On a rotating mixer.</td>
</tr>
<tr>
<td>Wash in TBS</td>
<td>3 x 10 min</td>
</tr>
<tr>
<td>Biotinylated secondary antibody in TBS with 1% serum.</td>
<td>3 hours</td>
</tr>
<tr>
<td>Wash in TBS</td>
<td>3 x 10 min</td>
</tr>
<tr>
<td>ABC Kit DAKO (Solution 4 below) in TBS with 1% serum</td>
<td>2 hours</td>
</tr>
<tr>
<td>Note: ABC complex must be made up 30 min in advance</td>
<td></td>
</tr>
<tr>
<td>Wash in TBS</td>
<td>3 x 10 min</td>
</tr>
<tr>
<td>Wash in TNS (Solution 5 below)</td>
<td>2 x 5mins or overnight at 4°C.</td>
</tr>
<tr>
<td>DAB solution (Solution 6 below)</td>
<td>Until light background but dark specific stain. Check microscopically</td>
</tr>
<tr>
<td>Wash in TNS</td>
<td>2 x 5min</td>
</tr>
<tr>
<td>Store at 4 degrees until mounting</td>
<td></td>
</tr>
<tr>
<td>Mount in TNS (with a little TXTNS added) on gelatinised slides and air dry overnight.</td>
<td></td>
</tr>
<tr>
<td>Dehydrate in an ascending series of alcohols.</td>
<td>50% EtOH for 5 min</td>
</tr>
<tr>
<td></td>
<td>70% EtOH for 5 min</td>
</tr>
<tr>
<td></td>
<td>100% EtOH for 5 min</td>
</tr>
<tr>
<td></td>
<td>100% EtOH for 5 min</td>
</tr>
<tr>
<td>Clear in xylene in the fume hood.</td>
<td>1st Xylene for 5 min</td>
</tr>
<tr>
<td>Coverslip using DPX.</td>
<td>2nd Xylene for 5 min</td>
</tr>
</tbody>
</table>
Buffers for perfusions

0.2M Phosphate buffer

**Stock A**
Sodium dihydrogen phosphate monohydrate \((\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O})\) \(\text{MW}=137.99\)

- 1M=137.99g in 1L d H\(_2\)O
- 0.1M=13.799g in 1L d H\(_2\)O
- 0.2M=27.598g in 1L dH\(_2\)O

**Stock B**
Disodium hydrogen phosphate di-hydrate \((\text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O})\) \(\text{MW}=177.99\)

- 1M=177.99g in 1L d H\(_2\)O
- 0.1M=17.799g in 1L d H\(_2\)O
- 0.2M=35.598g in 1L d H\(_2\)O

→ For 100ml PB
9.5ml stock A + 40.5ml stock B + 50ml d H\(_2\)O=100ml PB
→ For 1000ml (1L) PB
95ml stock A + 405ml stock B + 500ml d H\(_2\)O=1000ml PB
→ For 2000ml (2L) PB
190ml stock A + 810ml stock B + 1000 ml d H\(_2\)O=2000ml PB 0.2M
Stock A: 27.598g/L x 0.19L = 5.24362g in 190ml d H\(_2\)O
Stock B: 35.598g/L x 0.81L = 28.83438g in 810ml d H\(_2\)O
Alternatively add both to 1L of d H\(_2\)O and dissolve
Make up to 2L with water

Fixative: 4% Paraformaldehyde (4L)

1. Heat 1.5L of d H\(_2\)O to 60°C
2. Add 160g of paraformaldehyde powder
3. Add a few NaOH pellets in order to dissolve paraformaldehyde
4. Stir until clear
5. Fill to 2L with d H\(_2\)O
6. Stir until clear
7. Add 2L of 0.2M PB to give a final volume of 4L
8. Ph to 7.4 and cool to 4°C

Heparinised Saline

5,000 units of heparin is added per 1L saline
Each small vial of heparin contains 25,000 units per ml
0.2ml i.e. 5,000 units of heparin is added per 1L saline
25% w/v Sucrose Solution (1L)
1. Dissolve 5 PBS tablets in ~500ml d H₂O (Note: 1 PBS tablet per 200ml water)
2. Add 250g of sucrose
3. Stir until dissolved, apply heat if necessary
4. Make up to 1L with d H₂O

0.1% w/v TBS-Azide
1. Weigh out 12g Trizma, 9g NaCl, 1g sodium azide
2. Add to 1L of d H₂O and dissolve
3. Ph to 7.4
## 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°C 20mg in 2 ml aliquot

### DAB working
- DAB stock 2 ml
- TNS (fresh) 40 ml
- Hydrogen peroxide (30%) 12 μl
  This solution may be diluted to 1 in 5 with TNS if the reaction proceeds too quickly.
Slide subbing

Materials

Gelatin (10g/l)
Chromic Potassium Sulphate (500mg/L)
Distilled H2O
Slides (twin frosted)

1. Heat H₂O to +40°C and add gelatine slowly allowing to dissolve before adding more.
2. Add chromic Potassium sulphate
3. Subbing Medium cooled to ~ +35°C
4. Slides placed into slide holders and dipped into subbing medium for ~ 1 min
5. Remove slides and place in clean dry slide box to dry for ~ 1 wk