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NON-MOTOR SYMPTOMS IN THE AAV-α-SYNUCLEIN RAT MODEL OF PARKINSON'S DISEASE: EXERCISE AS A THERAPEUTIC INTERVENTION

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Declaration

All work presented in this thesis is original and entirely my own. The work was carried out under the supervision of Prof. Aideen Sullivan and Dr. Yvonne Nolan between October 2013 and June 2017 in the Department of Anatomy and Neuroscience, University College Cork, Ireland. This dissertation has not been submitted in whole or in part for any other degree, diploma or qualification at any other University.

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Chapter 1

1.0 Abstract

Parkinson's disease (PD) is no longer primarily classified as a motor disorder due to the emergence of a number of non-motor symptoms (NMS) of the disease. These NMS are highly prevalent and greatly affect the quality of life of patients with PD. Thus, an animal model that replicates these symptoms is greatly needed to enhance the translational impact of preclinical research. The AAV-α-synuclein rat model is the only animal model to date that has been shown to robustly and consistently reproduce the primary neuropathological and behavioural features of PD. However, there has been little research on the ability of the model to replicate NMS of the disease. Moreover, this model is most commonly employed unilaterally, which can confound cognitive testing due to contralateral functional compensation. Thus, the aim of this thesis was to use an AAV2/6 viral vector overexpressing human wild-type α-synuclein to characterise NMS of PD, exploring behavioural phenotypes of both unilaterally- and bilaterally-administered α-synuclein. Furthermore, it set out to explore whether voluntary exercise could ameliorate motor and NMS in this PD model, including if exercise could protect against hippocampal-associated cognitive deficits by modulating adult hippocampal neurogenesis.

We demonstrated that unilateral and bilateral administration of AAV-α-synuclein induced distinct patterns of nigrostriatal degeneration and associated motor dysfunction. Overexpression of AAV-α-synuclein was used to model NMS associated with PD, including deficits in hippocampal-associated tasks. This was coupled with α-synuclein-positive immunostaining in the dentate gyrus of the hippocampus, confirming the

propagation of the protein throughout distinct regions of the brain. Bilateral intranigral administration of AAV-α-synuclein was found to induce motor dysfunction and a significant loss of nigral dopaminergic neurons, neither of which were rescued by voluntary running. Overexpression of α-synuclein also resulted in significant impairment on a neurogenesis-dependent pattern separation task, as well as anxiety-like behaviours on both the open field and the elevated plus maze. Voluntary running improved performance on the pattern separation task only. This was substantiated by an effect on hippocampal neurogenesis levels in the dorsal, and not ventral, dentate gyrus, suggesting that the functional effects on pattern separation were mediated by increasing neurogenesis.

Chapter 2

2.0 Abbreviations

AAV – adeno-associated virus

ACC – Anterior cingulate cortex

ADAGIO - (Attenuation of disease progression with Azilect given once-daily)

ADL – Activities of daily living

AHN – Adult hippocampal neurogenesis

ALS/PDC - Amyotrophic lateral sclerosis-parkinsonism dementia complex

BBB – Blood brain barrier

BDNF – Brain-derived neurotrophic factor

BMP – Bone morphogenetic protein

BrdU - 3 5-Bromo-2'-deoxyuridine

BSSG - β-sitosterol β-d-glucoside

CD - Charles river (sd)

CED – Convection-enhanced delivery

CGI – Clinical Global Impression

CHO – Chinese Hamster Ovary

CNS – Central nervous system

COMT - Catechol-O methyl transferase

COX – Cyclo-oxygenase

CRT - Choice reaction time

CRTT – Choice reaction time task

CS – Conditioned stimulus

CSF – Cerebrospinal fluid

CTA – Conditioned taste aversion

DA – Dopamine

DBS – Deep brain stimulation

DCX – Doublecortin

DG – Dentate gyrus

DHA - Docosahexaenoic acid

dlPFC – Dorso-lateral prefrontal cortex

E – Embryonic day

ENS – Enteric nervous system

EPM - Elevated plus maze

FGF – Fibroblast growth factor

FST - Forced swim test

G – Gram

GCL – Granule cell layer

GDF5 – Growth/differentiation factor 5

GDNF – Glial cell-line derived neurotrophic factor

GFAP - Glial fibrillary acidic protein

GFP – Green fluorescent protein

GREFEX - Groupe de Réflexion sur l'Evaluation des Fonctions Exécutives

HMG-CoA - 3-hydroxy-3-methylglutaryl coenzyme A

ICV – Intracerebroventricular

IFN – Interferon

IL – Interleukin

iNOS - inducible nitric oxide synthase

L-dopa – levo-dopa

LE - Long Evans

LH - Lister Hooded

LPS – Lipopolysaccharide

M - Molar

MCI – Mild cognitive impairment

Ml - Millilitre

MFB – Medial forebrain bundle

Mg - Milligram

MMSE – Mini-mental state examination

MPP^+ - 1-methyl-4-phenylpyridine

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSA – Multiple system atrophy

MWM - Morris water maze

NAC - Non-amyloid-β component

NDS – Normal donkey serum

NGS – Normal goat serum

NICE - National Institute for Clinical Health and Excellence

NMDA – N-methyl-D-aspartate

NMS – Non-motor symptoms

NOR - Novel object recognition

NPC – Neural progenitor cell

NTF – Neurotrophic factor

NRTN – Neurturin

OD - Olfactory discrimination

OF – Open field

OFC – Orbitofrontal cortex

OL – Object location

OR - Object recognition

PD - Parkinson's disease

PDD – Parkinson’s disease dementia

PD MCI – Parkinson’s disease with mild cognitive impairment

PDQ - Parkinson’s disease questionnaire

PD SURG – PD surgical trial

PFC – Prefrontal cortex

PFF – Pre-formed fibril

PLK – Polo-like kinase

PPAR - Peroxisome proliferator-activated receptors

PRET PD - Progressive resistance exercise trial in PD

RANTES - regulated on activation, normal T-cell expressed and secreted

RBD – Rapid eye movement sleep behaviour disorder

REM – Rapid eye movement

RL – Reversal learning

RTT - Reaction time task

ROS – Reactive oxygen species

SA - Spontaneous alternations

SD – Sprague Dawley

SGZ – Subgranular zone

SIH – Stress-induced hypothermia

SN – substantia nigra

SNCA – α-synuclein gene

SPT - Sucrose preference test

SNpc – substantia nigra pars compacta

SR - Social recognition

SRTT - Serial reaction time task

SSRI – Selective serotonin reuptake inhibitors

SVZ – Subventricular zone

TCA – Tricyclic antidepressants

TGFβ - transforming growth factor β

TH – tyrosine hydroxylase

TLR – Toll-like receptor

TNF-α – Tumour necrosis factor α

µl – Microlitre

UPDRS – Unified Parkinson's disease rating scale

US – Unconditioned stimulus

Vg – Viral genomes

Vl – Ventrolateral

VM – Ventral mesencephalon

VMAT – Vesicular monoamine transporter

VTA - Ventral tegmental area

Wt – wild-type

6-OHDA – 6-hydroxydopamine

Chapter 3

3.0 General Introduction

Parkinson's disease (PD) was first reported by James Parkinson in "An essay on the shaking palsy" in 1817 (Parkinson, 1817). In it, he described the shaking palsy as an "involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured", and goes on to detail several patients who report a slow but progressive disorder primarily characterised by motor dysfunction including hand and arm tremors, akinesia and gait problems. A small number of cases also refer to non-motor symptoms such as constipation and speech disruption. Since that initial report, now 200 years ago, our knowledge and understanding of PD has been greatly advanced by modern science. PD is now described as a degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) region of the brain, and the concomitant loss of the neurotransmitter dopamine (DA) along the nigrostriatal pathway leads to the primary motor symptoms associated with PD, namely bradykinesia, tremor, rigidity and postural instability. As the disease progresses, secondary symptoms manifest themselves as psychiatric and cognitive dysfunction, broadly termed "non-motor symptoms" (NMS), including depression, dementia and sleep disturbances (reviewed by Cooney & Stacy 2016; Todorova et al. 2014). PD is now the second most common neurodegenerative disease in the world (Feng et al., 2010). Due to an ageing global population, the incidence of PD is on the rise (Savica et al., 2016), and is estimated to double in prevalence by 2030 (Dorsey et al., 2007). This increases not only the financial and economic impact of the disease

(Martinez-Martín et al. 2015; reviewed by Rodriguez-Blazquez et al. 2015) but also caregiver burden (Corallo et al., 2016; Grün et al., 2016) and thus underlines the importance for continued research into new therapies.

3.1 Current treatment of motor symptoms of Parkinson's disease

3.1.1 Pharmacological therapy

Management of the motor symptoms of PD employs various mechanisms to replace the lost DA in the nigro-striatal system. Recommended first line therapy involves treatment with either DA agonists such as pramipexole, rotigotine or ropinirole which act directly at dopamine receptors, or with the amino acid dopamine precursor levo-dopa (L-dopa) (National Institute for Clinical Health and Excellence 2006; reviewed by Kakkar & Dahiya 2015) . L-dopa can be rapidly and extensively converted to dopamine in the periphery, so to prevent this it is administered with dopa-decarboxylase inhibitors such as carbidopa or benserazide, or may be combined with catechol-O methyl transferase (COMT) inhibitors such as entacapone or tolcapone. This not only limits side-effects but also ensures that maximal amounts of free L-dopa are available to cross the blood brain barrier (BBB) and reach effective concentrations in the brain. One of the main issues surrounding L-dopa treatment is the eventual emergence of dyskinesias and fluctuations in response leading to erratic control of motor symptoms, which can become

increasingly problematic for patients (reviewed by Salat & Tolosa 2013). Other serious problems associated with prolonged L-dopa therapy include the development of compulsive disorders and neuropsychiatric symptoms (Voon *et al.*, 2009; Ahlskog, 2011). Duodopa, a new gel infusion of L-dopa and carbidopa in combination which can be administered directly into the gastro-intestinal system, has been shown to significantly reduce motor fluctuations without increasing dyskinesias (Fernandez et al., 2015; Wirdefeldt et al., 2016). However, this preparation is currently only licensed for severe, refractory PD (Healthcare and Products Regulatory Agency, Summary of Product Characteristics) and the administration involves the insertion of a permanent tube via percutaneous endoscopic gastrostomy, which can be invasive and confer additional risks onto the patient. National Institute for Clinical Health and Excellence (NICE) guidance also outlines the use of Amantadine, an N-methyl-D-aspartate (NMDA) receptor antagonist, and β-blockers for symptomatic control of motor symptoms but it does not recommend these agents as first line therapy (National Institute for Clinical Health and Excellence, 2006)

3.1.2 Surgical therapy

As the efficacy of L-dopa treatment wanes in some patients, it generally becomes necessary to use additional therapies to better control motor symptoms. These include surgery such as deep brain stimulation (DBS). DBS involves the implantation of an electrode into a suitable brain area, usually the subthalamic nucleus or globus pallidus, in an attempt to regulate altered

dopaminergic activity that is inherent to PD. Early results from the PD SURG trial (PD surgical trial) (Williams et al., 2010) indicate that patients who received both DBS and current best medical therapy did significantly better in activities of daily living scores and improvements on a PD questionnaire (PDQ) scale, a measure of pain disability, than patients who received medical therapy alone. However, this coincided with an increased number of adverse events that were mostly surgery-related (Williams et al., 2010). There is a nine-year follow-up planned for this trial and thus long-term efficacy will be addressed in the coming years. There are also reports of DBS alleviating some of the non-motor symptoms of PD such as depression or anxiety (reviewed by Kim et al. 2015), although the exact mechanisms of these effects remain unknown.

3.1.3 Neurotrophic factors as therapy in PD

Novel therapeutic strategies in Parkinson's disease are mainly focused on neuroprotection of the existing dopaminergic neurons. Neurotrophic factors (NTFs) are known to play a crucial role in the development and maintenance of neuronal subtypes, including dopaminergic neurons, and so have been extensively researched as a potential therapy for PD (reviewed by Sullivan & Toulouse 2011). NTFs from the TGFβ superfamily are the most characterised in this respect (reviewed by Hegarty et al. 2014), and include glial cell-line derived neurotrophic factor (GDNF), neurturin (NRTN) and growth/differentiation factor 5 (GDF5). Due to promising results from both

in vitro and *in vivo* studies that showed neuroprotective effects (reviewed by Bartus et al., 2007 and Sullivan and Toulouse, 2011), clinical trials in humans have been carried out using both GDNF and NRTN. However, issues surrounding the poor dissemination of GDNF (Nutt et al., 2003) and NRTN (Bartus et al., 2015; Marks et al., 2010) throughout the brain, and the development of anti-GDNF antibodies (Lang et al., 2006; Tatarewicz et al., 2007) have limited progress.

A unique method of adeno-associated viral (AAV) vector-mediated overexpression of GDNF has been shown to achieve, through multiple injections and aided by convection-enhanced delivery (CED), the distribution necessary to ensure successful and widespread GDNF expression in the brain of non-human primates (Richardson et al., 2011). This protocol is currently being implemented in a phase 1 clinical trial in patients with advanced stage PD in the US (NIH trial NCT01621581).

3.1.4 Cell-based therapies

Initial proof-of-concept work pioneered chiefly by Anders Bjorklund's group in Lund, Sweden in the 1980s proved that harvesting dopaminergic neurons from the developing VM of embryonic rodents and subsequently transplanting them into the adult rodent striatum post-neurotoxic lesion resulted not only in cell survival and integration, but in restoration of dopaminergic neuronal activity and alleviation of motor deficits (Bjorklund and Steveni, 1979; Brundin et al., 1987, 1986; Perlow et al., 1979). The

results from the first human studies were initially encouraging, with significant improvements in motor symptoms, dopamine synthesis and storage as well as decreases in the amount of l-dopa medication required by patients (Lindvall et al., 1990, 1989; Sawle et al., 1992; Wenning et al., 1997; Widner et al., 1992). This led to two large placebo-controlled trials in the US, which unfortunately both failed to reach significance in their primary endpoints (Freed et al., 2001; Olanow et al., 2003), Interestingly, longer-term follow up of these patients reported a lasting improvement in UPDRS scores at 2 and 4 years post-grafting (Ma et al., 2010), suggesting a delayed yet sustained effect. Moreover, very long term data recently published supports this theory, with Kefalopoulou *et al* reporting that two patients, 15- and 18 years-post transplant, have remained without dopaminergic medication for the last 10 years and display only mild Parkinsonian symptoms (Kefalopoulou et al., 2014). *Post-mortem* analysis of another graft patient, 24 years post-transplant, confirmed the long-term viability of the transplant (Li et al., 2016). However, due to problems such as the development of graft-induced dyskinesias and ethical issues surrounding the source of foetal tissue for the transplantations, progress in foetal tissue transplantation has largely been halted. Instead, focus has been shifted to developing and optimising protocols for the use of stem cell based therapies, and readying these therapies for clinical trials, which are the main focuses of the newly formed GForce-PD consortium (www.gforce-pd.com).

Although there are a variety of treatment options currently available to patients with PD, the therapies are only aimed at slowing the progression of

the disease and none are without caveats. In order to develop robust and effective new therapeutic options, more representative models of the disease must be used during the screening process to increase the translational impact of new drugs.

3.2 Non-motor symptoms of PD

Although it was initially thought that PD was primarily a motor disorder, in recent years there has been widespread recognition of a broad range of NMS that are associated with this disease. In fact, there have been calls to reclassify PD as a syndrome rather than a disease to underline the importance of recognising PD as a multifaceted condition with a wide variety of symptoms that affect both the central and peripheral nervous system (reviewed by Titova et al. 2016). Interestingly, it has been shown that some of the NMS associated with PD can in fact precede motor symptoms by a number of years, termed the prodromal stage of the disease (Pont-Sunyer et al., 2015). Constipation has been reported to occur more than 10 years prior to motor symptoms, followed by the presence of mood disturbances, loss of smell and fatigue that all occur between 2-10 years preceding diagnosis (Pont-Sunyer et al., 2015; Postuma et al., 2012). Importantly, it was recently shown that some of these NMS can accurately predict cognitive decline and survival in PD patients (De Lau et al., 2014; Fullard et al., 2016; Shoji et al., 2014), and so a thorough understanding of these NMS is key to

better management and outcomes for PD patients (Visanji and Marras, 2015).

3.2.1 Cognitive impairment and dementia

Cognitive impairment is common in PD patients, and includes deficits in working memory, visuospatial processing, language fluency and verbal learning (Siegert et al. 2008; reviewed by Goldman & Postuma 2014). It is mostly associated with frontal lobe dysfunction from either cortical atrophy (Auning et al., 2014; Green et al., 2002; Pereira et al., 2014; Rektorova et al., 2014) or Lewy body pathology (Kehagia et al., 2012). The prevalence of cognitive impairment ranges widely between studies, between 19 and 36% in separate cohorts of early, untreated PD patients (Aarsland et al., 2009; Elgh et al., 2009; Foltynie et al., 2004; Muslimovic et al., 2005). This variability may be explained in part by differences in tests used to assess cognitive functioning, and underlines the difficulty in quantifying cognitive deficits. Due to the emerging importance of NMS and their role in predicting survival in PD patients, the Movement Disorder Society recently published diagnostic criteria for mild cognitive impairment in PD (PD-MCI) (Litvan et al., 2012), with a view to standardise diagnoses and highlight patients with the potential to progress to PD dementia (PDD). PDD is characterised by a broad dysexecutive syndrome, with profound impairments in visuospatial functioning, memory and attention, as well as the development of neuropsychiatric symptoms such as hallucinations (Hanagasi et al., 2016).

Recent studies employing the new criteria reported a prevalence of PD-MCI in approximately 42.5% of patients (Domellöf et al., 2015; Yarnall et al., 2014). Long-term follow-up data from the initially examined cohorts, (the ParkWest study in Norway (Aarsland et al., 2009) and the CamPaiGN study in the UK (Foltynie et al., 2004)) have shown that up to 46% of PD patients with diagnosed MCI in the initial studies progressed to PDD by 10 years (Williams-Gray et al., 2013). The main factors associated with advancing from PD to PDD are MCI, specifically semantic fluency and inability to copy intersecting pentagon figures, age and severity of motor impairment (Pedersen et al., 2013; Williams-Gray et al., 2013, 2009). Other work has demonstrated a link between the presence of co-morbid neuropsychiatric conditions such as depression or hallucinations and PDD (Wang et al., 2014).

Interestingly, a proportion of patients initially diagnosed with MCI in the ParkWest study (Aarsland et al., 2009) reverted to normal cognition by the 1-year follow up, suggesting more complex underlying mechanisms. This heterogeneous nature of MCI and the differences in PDD progression was addressed by Kehagia and colleagues (2012) in their “dual syndrome hypothesis”, which states that MCI and PDD are in fact two independent, although partially overlapping, syndromes. PD-MCI is characterised by a tremor-dominant motor phenotype with deficits in working memory and executive function reflective of dysfunctional fronto-striatal circuitry that is sensitive to DA therapy. However, PDD is characterised by a primarily akinetic motor phenotype with pronounced gait disturbances and deficits in semantic fluency and visuospatial function reflective of posterior cortical

and temporal lobe dysfunction which may be ameliorated by cholinergic therapy (Kehagia et al., 2012). This theory was further strengthened by a study carried out by Nombela (2014), showing that impairments in tasks assessing visuospatial, executive and memory encoding domains were associated with region-specific deficits in cortical activity (Nombela et al., 2014). Since then, a series of papers have further separated PD cohorts into 5 cognitively differentiated subtypes based on cluster analysis: 1) cognitively intact, 2) no cognitive impairment but slight mental slowness, 3) slightly impaired overall cognitive ability, 4) severe mental slowing and overall cognitive impairment and 5) severe cognitive impairment across all domains (Dujardin et al., 2015, 2013). Collectively, the evidence points to a spectrum of severity in cognitive impairment in PD patients, which has been shown to be far more complex and intricate than initially thought.

3.2.2 Dysexecutive syndrome

Executive function encompasses all of the mental processes required for goal-directed behaviours, including planning, decision making, execution and effective performance, and contributes to the ability of a person to be useful, socially responsible and constructive throughout life (Lezak, 1982). So long as executive functioning remains intact, even people with marked cognitive impairment can still maintain their independence and productivity (Lezak, 1982). Due to its complexity, measuring executive function is difficult and includes a wide variety of tasks measuring outputs such as response

inhibition, attentional shifting, cognitive flexibility, rule detection, strategic planning and concept formation (reviewed by Jurado & Rosselli 2007). To combat this, the Groupe de Réflexion sur l'Evaluation des Fonctions Exécutives (GREFEX) published proposed criteria for diagnosing dysexecutive syndrome which encompassed both behavioural and cognitive domains (Godefroy et al., 2010). These criteria have been recently updated and shown to be highly diagnostically accurate (Roussel et al., 2016). Executive dysfunction has been well characterised in PD patients (Kudlicka et al. 2011; reviewed by Ceravolo et al. 2012), and can affect ADL as simple as getting up, dressing, cooking and general multi-tasking (Koerts et al., 2011). It is thought to be due in part to dysfunctional fronto-striatal circuits that connect the basal ganglia to the frontal cortical regions and originate in the dorsolateral PFC (dlPFC), the orbitofrontal cortex (OFC) and the anterior cingulate cortex (ACC) (Lewis et al., 2012; Zgaljardic et al., 2006). Interestingly, recent work has shown that distinct functional-anatomical networks within the PFC may be responsible for the processing of different forms of executive function, with the dlPFC and ACC largely associated with cognitive control and the OFC more related to decision making (Glascher et al., 2012).

3.2.3 Neuropsychiatric

There are a wide variety of neuropsychiatric disturbances that have been associated with PD, and importantly they have been shown to be associated with disease factors such as motor symptom fluctuations and disease

severity and duration (Sagna et al., 2014). PD patients are more likely to suffer from depression than age-matched controls, with an estimated prevalence of 17% of PD patients suffering from major depressive disorder and 35% suffering from clinically significant depressive symptoms (Reijnders et al., 2008). A wide variety of factors are thought to contribute to an individual's risk of developing depression, including environmental factors (Gallagher and Schrag, 2012), genetic contributions (Collins and Williams-Gray, 2016) and psychological factors such as the ability to cope with a diagnosis of PD (Schrag et al., 2001). Interestingly, Even and Weintraub (2012) recently hypothesised that there are three different subtypes of comorbid depression in PD patients: patients who would experience depression regardless of a PD diagnosis, patients who would be depressed if they were diagnosed with another chronic and debilitating illness, and patients whose depression is specifically linked to the pathophysiology of PD (Even and Weintraub, 2012). This distinction may have important implications for the choice of treatment in these patients. Depressive symptoms associated with PD are thought to be due in part to dysfunctional catecholaminergic and serotonergic neurotransmitter systems (reviewed by Aarsland & Gregoric 2015). *Post mortem* analysis of brain tissue from PD patients has shown widespread deficits in dopaminergic, noradrenergic and serotonergic innervation throughout the basal ganglia, cortical and limbic regions of the brain (Buddhala et al., 2015), as well as decreased DA and noradrenaline transporter binding in the locus coeruleus and throughout the limbic system (Remy et al., 2005; Vriend et al., 2013). Moreover, Lewy body

pathology has been discovered in brainstem areas that are linked to depression including the noradrenergic locus coeruleus (Itoi and Sugimoto, 2010) and serotonergic raphe nuclei (Lowry et al. 2008; reviewed by Gallagher & Schrag 2012).

Anxiety disorders are also highly common in PD, with some studies estimating the prevalence of some form of anxiety at up to 50% (Leentjens et al. 2011; Rutten et al. 2015; Pontone et al. 2009). Anxiety disorders include generalised anxiety and panic disorder, as well as social anxiety (Dissanayaka et al., 2010). Although it was initially thought that anxiety in PD is "reactive" as a result of being diagnosed with a chronic and debilitating degenerative condition, growing evidence now suggests that it is directly related to the pathophysiology of PD (Prediger et al., 2012) and can in fact be present for years preceding the onset of motor symptoms (Bower et al., 2010). Anxiety in PD is frequently associated with depression (Brown et al., 2011), and as such it is likely that some of their underlying mechanisms overlap. Dysfunctional transmission in dopaminergic, serotonergic and noradrenergic systems have all been linked to anxiety in PD (reviewed by Prediger et al. 2012), however the exact workings remain poorly understood.

It is also estimated that apathy is present in up to 36% of PD patients (Pagonabarraga et al., 2015). Although the mechanisms underlying apathy in PD remain unclear (Dujardin and Defebvre, 2012), it is thought that dysfunction in both the dopaminergic system (Czernecki et al., 2008) and

neural circuitry connecting the basal ganglia to the prefrontal cortex (Levy and Czernecki, 2006) may play roles.

3.2.4 Autonomic

Autonomic system deficits, also termed dysautonomia, are well characterised in PD and can affect up to 84% of PD patients (Arnao et al., 2015). These symptoms include orthostatic hypotension (Vichayanrat et al., 2016), weight loss (Umehara et al., 2016), differences in pain perception (Rada et al., 2016) and urinary and sexual dysfunction (Pfeiffer, 2010). Notably, they have been shown to markedly impact upon the quality of life of patients, significantly affecting ADL, emotional well-being, social support and communication and body discomfort (Tomic et al., 2016).

3.2.5 Olfaction

Olfactory disturbances are very common and are estimated to affect up to 70% of PD patients (Hawkes et al., 1997). The olfactory bulb is thought to be the first site of α-synuclein pathology according to the Braak hypothesis (Heiko Braak et al., 2003), and smell disturbances have been shown to be present years preceding motor symptoms in PD patients (Pont-Sunyer et al., 2015; Postuma et al., 2012). Interestingly, olfactory deficits appear to be specific to PD and could be used to diagnostically discriminate between PD and other neurodegenerative conditions (Krismer et al., 2016)

3.2.6 Sleep

Sleep disturbances are very common in patients with PD, and encompass a broad range of symptoms including circadian rhythm disturbances, restless legs syndrome, rapid eye movement (REM) sleep behaviour disorder (RBD), insomnia and excessive daytime sleepiness (reviewed by Chahine et al. 2016; Fifel 2016). RBD is defined as a multifaceted parasomnia characterized by the loss of atonia that normally occurs during REM sleep, and is associated with often violent dream enactment behaviour (Chahine et al., 2016; Schenck and Mahowald, 2002). It has recently been shown to be perhaps the most sensitive and specific prodromal marker for future development of PD (Fereshtenejad et al., 2017). Several longitudinal studies have reported that up to 90% of patients with RBD go on to develop either PD or another form of synucleinopathy (Boeve et al., 2013; Iranzo et al., 2014). Similarly, RBD in PD patients is associated with more severe and debilitating motor and NMS (Chahine et al., 2016), and increased α-synuclein deposition throughout the brain (Postuma et al., 2015). Although the neuropathology of these symptoms is currently poorly understood, any alterations in sleep patterns can have drastic effects on quality of life and management of symptoms (Prakash et al., 2016).

3.2.7 Gastro-intestinal

Constipation is the most frequently reported gastro-intestinal symptom of PD, affecting up to 63% of PD patients (reviewed by Stirpe et al. 2016).

Additionally, other gastro-intestinal disturbances include decreased gastric emptying (gastroparesis), malnutrition, dysphagia and small intestinal bacterial overgrowth (reviewed by Fasano et al. 2015). It has also been shown that PD patients exhibit increased intestinal permeability and inflammation when compared to healthy controls (Clairembault et al., 2015, 2014), and that PD patients have significantly different colonic bacterial composition when compared to controls (Keshavarzian et al., 2015).

Due to the fact that constipation is often present for years preceding the onset of motor symptoms in PD patients, there has been a lot of interest surrounding the role of the gastro-intestinal system in PD. The idea that the initial insult that triggers PD could commence in the gastro-intestinal tract was first posited by Braak and colleagues (2003), who outlined potential neuronal networks that could be used to transport α-synuclein from the enteric to the central nervous system (Braak et al., 2003). Since then, there have been numerous reports that support this theory. The vermiform appendix has been shown to be rich in α-synuclein (Gray et al., 2014), and interestingly an appendectomy may protect against the onset of PD (Mendes et al., 2015). Similarly, α-synuclein positive inclusions have been detected in the gastric mucosa of PD patients (Sánchez-Ferro et al., 2014), and in the gastro-intestinal system up to 8 years prior to the onset of motor symptoms (Hilton et al., 2014). Importantly, Holmqvist (2014) injected α-synuclein into the gastro-intestinal system of rats, near the myenteric plexus, and discovered that the protein was transported *via* the vagus nerve into the dorsal motor nucleus of the brainstem, and so provided the first

experimental evidence of α-synuclein propagation from gut to brain (Holmqvist et al., 2014). Furthermore, a truncal vagotomy has been shown to decrease the risk of PD in human studies, underlining the relevance of the vagal nerve to the pathogenesis of the disease (Svensson et al., 2015).

3.3 Current treatment of non-motor symptoms of Parkinson's disease

3.3.1 Cognitive impairment, dementia and dysexecutive syndrome

Due to the fact that cortical cholinergic activity is more severely affected in PDD compared to Alzheimer's disease (Bohnen et al., 2003), it stands to reason that drugs affecting the cholinergic system such as cholinesterase inhibitors would be therapeutically appropriate for treatment of these patients. A large systematic review and meta-analysis carried out in 2015 reported that treatment with cholinesterase inhibitors such as donepezil, galantamine and rivastigmine in PDD can significantly improve cognitive function, ADL and caregiver burden and are generally well-tolerated (Wang et al., 2015). Specifically, rivastigmine is the only currently licensed cholinesterase inhibitor for use in PDD (www.bnf.org) and double-blinded placebo-controlled clinical trials have shown that it is effective in improving general cognitive domains (Schmitt et al. 2010), executive function (Schmitt et al. 2010) and ADL (Olin et al., 2010). Memantine, a NMDA receptor

antagonist, did not significantly improve cognitive function measured by the Mini-Mental State Examination (MMSE) but did increase scores on a Clinical Global Impression (CGI) scale (Wang et al., 2015) and has been shown to improve executive function in the form of attention, episodic memory and goal attainment (Leroi et al., 2014; Wesnes et al., 2015). Despite the prevalence of MCI in PD patients and the link between MCI and progression to PDD, there is relatively little evidence to inform treatment of MCI in PD patients. Interestingly, cholinesterase inhibitors do not seem to be effective in MCI, perhaps further supporting the "dual syndrome hypothesis" of two independent but partially overlapping syndromes affecting different neurotransmitter systems (Kehagia et al., 2012). Galantamine was shown to have no effect on memory, executive function or visuospatial performance in non-demented patients (Grace et al., 2009). Similarly, treatment with rivastigmine resulted in non-significant trends toward improvement of cognitive measures (Mamikonyan et al., 2015). Rasagiline, a monoamine oxidase B inhibitor, has shown promise in alleviating attention and certain executive functions (Hanagasi et al., 2011). Atomoxetine, a selective noradrenaline reuptake inhibitor, has been effective in ameliorating executive and cognitive dysfunction in PD, although only in small numbers of patients (Marsh et al., 2009; Weintraub et al., 2010). A clinical trial investigating the effects of piribedil, a dopamine agonist, on motor symptoms also carried out a smaller sub-study investigating the effects on cognition, and found that piribedil significantly enhanced executive function (Castro-Caldas et al., 2006).

3.3.2 Neuropsychiatric conditions

3.3.2.1 Depression

Although depression is a frequent and debilitating co-morbidity in PD, antidepressant treatment either through behavioural therapy or pharmacological means, has been shown not only to increase quality of life (Menza et al., 2009) but also to enhance executive functioning and working memory (Dobkin et al., 2014). Despite this, a series of reports and a Cochrane review have deemed that there is insufficient evidence either for effectiveness or safety of antidepressants for use in treatment of PD (Chung et al., 2003; Ghazi-noori et al., 2003; Liu et al., 2013; Miyasaki et al., 2006; Price et al., 2011; Skapinakis et al., 2010). Notwithstanding, a recent systematic review and meta-analysis concluded that in fact, there is a significant effect of the selective serotonin reuptake inhibitor (SSRI) class drugs (citalopram, sertraline, fluoxetine and paroxetine) on depression in PD patients (Bomasang-Layno et al., 2015). Furthermore, although all the clinical trials to date do not provide sufficient power to allow broad recommendations, evidence gleaned from smaller trials suggest that multiple medications may be beneficial (reviewed by Cooney & Stacy 2016). Tricyclic antidepressants (TCA) (amitriptyline, nortriptyline) and serotonin-noradrenaline reuptake inhibitors (venlafaxine) have shown some degree of efficacy in alleviating depressive symptoms in PD patients (Liu et al., 2013; Richard et al., 2012). Buproprion, a selective noradrenergic and

dopaminergic uptake inhibitor, has been suggested as a suitable treatment due to its combined neurotransmitter effects (Raskin and Durst, 2010) and has been used to great effect but in very small patient populations (Goetz et al., 1984; Załuska and Dyduch, 2011). Pramipexole, a DA agonist used to treat motor symptoms of PD, has shown antidepressant effects (Barone et al., 2010, 2006; Leentjens et al., 2009). The ADAGIO study (Attenuation of disease progression with Azilect given once-daily) was started in 2008 to assess the role of rasagiline, a monoamine oxidase B inhibitor as adjunct therapy in PD (Olanow et al., 2008). Interestingly, when administered with previously initiated antidepressant therapy in trial participants (either a TCA or an SSRI), rasagiline improved depressive symptoms when compared to placebo (Smith et al., 2015). Importantly, given the proven deficits in several brain neurotransmitter systems in depression and PD, it appears logical that a drug, or a combination of drugs, that target more than one system may be the most applicable in PD patients suffering from these disorders.

3.3.2.2 Anxiety and apathy

To date, there are no guidelines for the treatment of anxiety in PD patients and there have been no clinical trials carried out to address this question (Seppi et al., 2011). Treatment of anxiety in patients with PD is the same as those without (Akbar and Friedman, 2015; Cooney and Stacy, 2016), with more cautious consideration recommended regarding the contribution of side-effects to falls risk in PD patients in particular. The situation is similar

with treatment of apathy in PD, with limited evidence to adequately inform guidelines or recommendations. One small double-blinded placebo-controlled clinical trial found that transdermal rivastigmine, a cholinesterase inhibitor, decreased measures of apathy and increased ADL (Devos et al., 2014) while a second found that piribedil, a DA agonist, reduced apathy and depression scores and improved quality of life (Thobois et al., 2013). Methylphenidate, a stimulant that inhibits DA uptake, has also been shown to have positive effects on PD patients suffering from apathy (Chatterjee and Fahn, 2002; Moreau et al., 2012), though again only in very small patient numbers of patients.

3.3.3 Sleep

In general, clonazepam is the most commonly used therapy for sleep disturbances, particularly RBD where it effective in up to 90% of cases (Olson et al., 2000; Schenck et al., 2013; Sforza et al., 1997). However, as it is a benzodiazepine side effects include excessive daytime sleepiness, which can contribute to fall risk in PD patients (Videnovic, 2017) and as such caution should be used when prescribing (Aurora et al., 2010). Several small studies have also shown that melatonin can be effective in increasing REM sleep, either by itself or as an adjunct therapy (Boeve et al., 2003; Kunz and Bes, 1999; Takeuchi et al., 2001). However, in general there are very limited clinical trials for treatment of sleep disturbances specifically in PD patients (Amara et al., 2017; Chahine et al., 2016).

3.3.4 Autonomic dysfunction

Postural hypotension can generally be managed in PD patients, depending on the severity of symptoms. Non-pharmacological measures include increasing water and salt intake and wearing compression stockings (Wu and Hohler, 2015). Fludrocortisone, a corticosteroid that increases systemic sensitivity to circulating catecholamines is the first-line treatment for orthostatic hypotension (Wu and Hohler, 2015). Droxidopa (L-threo-dihydroxyphenylserine), a synthetic pro-drug that is converted to noradrenaline *in vivo*, has been recently approved for the treatment of hypotension in the US and is currently completing phase 3 clinical trials in Europe (Wu and Hohler, 2015). Results from earlier trials have shown that droxidopa can significantly improve symptoms and standing blood pressure measurements as well as decreasing falls (Kaufmann et al., 2015), which is of particular importance in PD patients (Hauser et al., 2016).

3.3.5 Gastro-intestinal dysfunction

Due to the lack of high quality evidence for treatment of gastro-intestinal symptoms specific to PD patients, treatment of constipation and associated gastro-intestinal dysfunctions are usually the same as in the general population (Knudsen et al., 2016; Reichmann et al., 2016).

3.4 Modelling motor and non-motor symptoms of PD

There are currently several experimental animal models of PD in use that can be broadly divided into groups based on the method of neuronal damage. Dopaminergic neuronal damage can be induced by specific neurotoxins, α-synuclein overexpression or by transgenic models. Although some of these agents can be administered systemically, most of the models require stereotaxic surgery. An advantage of this is that the required solution can be injected into the brain unilaterally, allowing the contralateral hemisphere of the same animal to act as a control in *post mortem* tissue analysis. Motor impairment can be assessed using a series of lateralised tasks including the stepping test (Olsson et al., 1995), the cylinder test (Schallert et al., 2000), the corridor test (Dowd et al., 2005) and apomorphine- or amphetamine-induced rotations (Ungerstedt and Arbuthnott, 1970). However, problems arise when attempting to investigate non-motor dysfunction in a unilateral model, as it is well-known that the contralateral hemisphere can compensate for the damaged side and thus mask any potential deficits. For this reason, bilateral stereotactic injections are best used to analyse cognitive function. Motor function in bilateral models can be examined using tests such as motor co-ordination and sensorimotor integration on the Rotarod apparatus (Rozas et al., 1997), or gross locomotor activity in the open field (reviewed by Sestakova et al. 2013).

For the purposes of this thesis, all the papers discussed below are on studies that used rat models, unless otherwise specified.

3.4.1 The 6-OHDA lesion model

This model of PD is characterised by injection of the selective catecholaminergic neurotoxin 6-OHDA into the midbrain, leading to an immediate and profound degeneration of dopaminergic neurons. It has been shown to be a reliable and consistent model of PD, reproducing some of the main pathological features, namely nigro-striatal dopaminergic degeneration, which in turn manifests behaviourally as motor dysfunction (Ungerstedt, 1968). 6-OHDA can be administered either into the MFB, the striatum or the SN, and administration of the toxin at each of the sites results in different behavioural and neuropathological effects (reviewed by Blandini et al. 2008). It is generally administered with noradrenergic and serotonergic protective agents to ensure that it selectively targets dopaminergic neurons (reviewed by Blandini et al. 2008). Although this model has not been shown to induce development of α-synuclein-positive inclusions in the brain (reviewed by Bové & Perier 2012), it has been used to investigate the NMS of PD (see Table 3.1). Bilateral 6-OHDA administration into the rat SN has been shown to impair spatial working memory in a Morris water maze (MWM) task (Ferro et al., 2005), and has also led to anhedonia and apathy-like behaviour, measured by decreased sucrose consumption (Santiago et al., 2015) in a self-administration operant chamber (Favier et al., 2014) and increased time taken to eat 100 sucrose pellets (Pioli et al., 2008), as well as

increased immobility time in the forced swim test (FST) (Santiago et al., 2015). Bilateral striatal administration of 6-OHDA has been more extensively characterised and has been shown to induce a wide variety of cognitive and emotional impairment, including spatial working memory deficits (Lindner et al. 1999; Tadaiesky et al. 2008; Chen et al. 2014; Matheus et al. 2016; Betancourt et al. 2016), anxiety and anhedonia-like behaviour (Chen et al., 2014, 2011; Kumari et al., 2015; Santiago et al., 2015; Silva et al., 2016), working memory deficits including social and object recognition (Aidi-Knani et al., 2015; Chen et al., 2014; Matheus et al., 2016; Tadaiesky et al., 2008) and executive dysfunction in attentional set-shifting and decision making (Courtière et al., 2011, 2005; Eagle et al., 2015; Tait et al., 2016; Temel et al., 2005).

Publication	Breed and sex	Administration site	Dose	Behavioural tests	Result
Chen (2011)	Adult male SD	Dorsal striatum	2 x 10.5µg	MWM EPM FST Social interaction	- ↓ - ↓
Tadaeisky	Adult male Wistar	Vl area of dorsal striatum	2 x 12µg	MWM SR OD	↓ ↓ -
Lindner	Adult male SD, 3m and 12m	Vl area of dorsal striatum	2 x 12.5µg	MWM	↓
Pioli	Adult Wistar – no sex specified	SNpc or VTA, 1w between left and right injections	2 x 8µg for SNpc 2 x 2µg for VTA	Sucrose consumption SA in Y maze Object exploration	↓ in SN ↓ in VTA -
Ferro	Adult male Wistar	SNpc	2 x 6µg	MWM	↓
Courtiere 2011	Adult male LE	Dorsal striatum	2 x 12µg	RTT	↓
Tait	Adult male LH	Dorsomedial striatum	2 x 8µg	Attentional set-shifting	↓ RL
Eagle	Adult male CD	Dorsal striatum	2 x 5.5µg	Stimulus discrimination	↓
Chen (2014)	Adult male Wistar	Striatum	2 x 12µg	EPM SPT Social interaction OR	↓ ↓ ↓ ↓
Favier	Adult male SD	SNpc	2 x 6µg	Operant sucrose self-administration	↓
Temel	Adult male Lewis	Striatum	2 x 20µg	CRT	↓
Courtiere 2005	Adult male LE	Dorsal striatum	2 x 8µg	RTT	↓
Da Silva	Adult male Wistar	Vl area of dorsal striatum	2 x 12µg	EPM SPT	↓ ↓
Domenger	Adult male Wistar	Neostriatum	2 x 8µg	SRTT	↓
Betancourt	Adult male SD	Lateral caudate	2 x 8µg	Barnes maze	↓

Aidi-Knani	Adult male Wistar	Dorsomedial striatum	2 x 12µg	NOR SR EPM	↓ ↓ ↓
Branchi	Adult male Wistar	Dorsal striatum	2 x 10.5µg	MWM EPM SPT SR FST	- ↑ - ↓ ↓
Kumari	Adult female SD	SNpc	2 x 10.5µg	OD FST	↓ ↓
Matheus (2016a)	Adult male Wistar	Dorsolateral striatum	2 x 10µg	NOR Y maze	↓ ↓
Matheus (2016b)	Adult male Wistar	Dorsolateral striatum	2 x 10µg	SPT FST EPM Social withdrawal Splash test	↓ ↓ - ↓ ↓
Santiago	Adult male Wistar	SN	2 x 6µg	FST SPT	↓ ↓

Table 3.1. Summary of the effects of bilateral 6-OHDA lesion on cognitive and emotional behaviours. *↑ denotes increased or enhanced performance, - denotes no change in performance and ↓ denotes decreased or impaired performance. Abbreviations: SD = Sprague Dawley; MWM = Morris water maze; EPM = elevated plus maze; FST = forced swim test; Vl = ventrolateral; SR = social recognition; OD = olfactory discrimination; SNpc = substantia nigra pars compacta; VTA = ventral tegmental area; SA = spontaneous alternations; LE = Long Evans; RL = reversal learning; RTT = reaction time task; LH = lister hooded; CD = Charles river (sd); SPT = sucrose preference test; OR = object recognition; CRT = choice reaction time; SRTT = serial reaction time task; NOR = novel object recognition*

There have also been some reports of cognitive and emotional deficits after unilateral intracerebral administration of 6-OHDA (see Table 3.2). A unilateral lesion of 6-OHDA into the MFB has been shown to induce anxiety-like behaviour in the elevated plus maze (EPM) and marble burying tasks (Jungnickel et al., 2011; O'Connor et al., 2016) as well as spatial working memory deficits in the radial arm maze (Pérez et al., 2009) and the MWM (Ma et al., 2014). However, similar papers have shown conflicting evidence in both anxiety and cognitive measures (Carvalho et al., 2013), highlighting

the variability of using unilateral lesion models to characterise cognitive deficits.

3.4.2 The rotenone model

Rotenone is a naturally occurring toxin commonly used as an insecticide and pesticide, and has been used in PD research since the 1980s. Interestingly, exposure to pesticides has been linked to increased risk of PD in humans (Bellou et al., 2016). The rotenone animal model gained traction when a paper published by Betarbet and colleagues (2000) proved that systemic administration of rotenone in rats could induce both the pathological and behavioural hallmarks of PD, including hypokinesia and rigidity, as well as the development of α-synuclein-positive aggregates in nigral neurons (Betarbet et al., 2000). Since then, it has also been shown to cause a variety of NMS (see Table 3.3).

Bilateral intra-nigral administration of rotenone in rats has been shown to induce deficits in working memory on object recognition tests (Dos Santos et al., 2013), loss of olfactory function (Rodrigues et al., 2014) and depressive-like behaviour in the FST and sucrose preference test (Santiago et al., 2010). Systemic administration of rotenone *via* chronic intra-

Publication	Breed and sex	Administration site	Dose	Behavioural tests	Result
Lelos	Adult female Lister	MFB	12µg	CRTT	↓
Carvalho	Adult Wistar Han	MFB	12µg	EPM MWM SPT Acoustic startle test	- ↓ ↓ ↓
Jungnickel	Adult female SD	MFB – 2 injections	1 x 9µg 1 x 10.8µg	EPM OF	↓ -
Ma	Adult male SD	MFB – 2 injections	2 x 12µg	MWM	↓
Nezhadi	Adult male Wistar	SN	8µg	MWM NOR OL	↓ ↓ ↓
O'Connor	Adult male SD	MFB	13.5µg	EPM Marble burying	↓ ↓
Perez	Male SD	MFB	8µg	Radial arm maze	↓

Table 3.2. Summary of the effects of unilateral 6-OHDA lesion on cognitive and emotional behaviours in rats. *↑ denotes increased or enhanced performance, - denotes no change in performance and ↓ denotes decreased or impaired performance.* ***Abbreviations****: MFB = medial forebrain bundle; CRTT = choice reaction time task; MWM = Morris water maze; EPM = elevated plus maze; FST = forced swim test; SPT = sucrose preference test; OF = open field; NOR = novel object recognition; OL = object location; SD = Sprague-Dawley*

peritoneal injection or osmotic mini-pump infusion can also cause sleep disturbances (García-García et al., 2005; Lax et al., 2012), gastro-intestinal dysfunction such as delayed gastric transit and emptying (Drolet et al., 2009; Greene et al., 2009), as well as depressive-like behaviour in the FST (Bassani et al., 2014; Zaminelli et al., 2014). Although this model displays some of the primary behavioural and pathological features of PD, it is not without its caveats. High variability and low reproducibility of lesions as well as high mortality rates and systemic toxicity have all limited the use of this model

(Cicchetti et al., 2009; Johnson and Bobrovskaya, 2015). Moreover, attempts to circumnavigate some of these issues using different administration methods such as oral or intranasal delivery in mice and rats have had varied success (Inden et al., 2007; Rojo et al., 2007; Sasajima et al., 2015).

Publication	Breed and sex	Administration site	Dose	Behavioural tests	Result
Bassani	Adult male Wistar	Intraperitoneal	2.5mg/kg for 10d	FST	↓
Dos Santos	Adult male Wistar	Bilateral into SNpc	2 x 12µg	OR	↓
Rodrigues	Adult male Wistar	Bilateral into SNpc	2 x 12µg	OD	↓
Santiago	Adult male Wistar	Bilateral into SNpc	2 x 12µg	FST SPT	↓ ↓
Zaminelli	Adult male Wistar	Intraperitoneal	2.5mg/kg for 10d	FST	↓

Table 3.3. Summary of the effects of rotenone administration on cognitive and emotional behaviours in rats. *↑ denotes increased or enhanced performance, - denotes no change in performance and ↓ denotes decreased or impaired performance.* ***Abbreviations****: SNpc = substantia nigra pars compacta; FST = forced swim test; SPT = sucrose preference test; OR = object recognition;*

3.4.3 The MPTP model

This model utilises the metabolism of MPTP *in vivo* to the neurotoxin MPP^+, which impairs mitochondrial respiration (Nicklas et al., 1985) and causes selective damage to the nigro-striatal system. It thus replicates many of the motor deficits seen in PD (reviewed by Bové & Perier 2012). MPTP itself is not toxic and can cross the BBB, meaning that it can be administered systemically. Some forms of cognitive dysfunction have been characterised using the MPTP model (see Table 3.4). Bilateral intra-nigral administration in rats has been shown to cause working memory deficits in avoidance tasks (Gevaerd et al. 2001; Kumar et al. 2009; Gevaerd et al. 2001b), alternation tasks (Braga et al., 2005) as well as social and object recognition (Hsieh et al. 2012; Huang et al. 2015; Ho et al. 2014). A single intra-nasal dose of MPTP has also been shown to induce depressive-like behaviour in the FST and working memory deficits in social recognition (Castro et al., 2013). Similar to the 6-OHDA model, the MPTP model has not been shown to cause α-synuclein aggregation *in vivo* (Shimoji et al., 2005).

Publication	Breed and sex	Administration site	Dose	Behavioural tests	Result
Braga	Adult male Wistar	Bilateral into SNpc	2 x 0.5µmol	Delayed Y maze alternations	↓
Castro	Adult male Wistar	Intranasal	2 x 1mg	FST SPT MWM SR	↓ - - ↓
Gevaerd (both)	Adult male Wistar	Bilateral into SNpc	2 x 1µmol	Two-way active avoidance task	↓
Ho, Hsieh, Huang	Adult male Wistar	Bilateral into SNpc	2 x 1µmol	T maze OR	↓ ↓
Kumar	Adult male Wistar	Bilateral into SNpc	2 x 100µg	MWM Passive avoidance task	↓ ↓
Miyoshi	Adult male Wistar	Bilateral into SNpc	2 x 0.5µmol	MWM x 3 versions: Spatial working; Spatial reference; Cued	↓ - ↓
Ferro	Adult male Wistar	Bilateral into SNpc	2 x 100µg	MWM	↓

Table 3.4. Summary of the effects of MPTP administration on cognitive and emotional behaviours in rats. *↑ denotes increased or enhanced performance, - denotes no change in performance and ↓ denotes decreased or impaired performance.* ***Abbreviations****: SNpc = substantia nigra pars compacta; MWM = Morris water maze; FST = forced swim test; SR = social recognition; SPT = sucrose preference test; OR = object recognition;*

3.4.4 The BSSG model

A novel and interesting model of PD was recently reported by Van Kampen and colleagues (2015). This model utilises a dietary neurotoxin derived from the cycad seed, which has previously been linked with Guamanian amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS/PDC) (Steele and McGeer, 2008). In the report by Van Kampen *et al*, rats were fed a diet containing β-sitosterol β-d-glucoside (BSSG) for 4 months. The animals first displayed olfactory deficits, followed by locomotor impairment in the form of decreased activity and co-ordination, before finally progressing to cognitive dysfunction measured by working memory deficits in both the radial arm maze and alternations in the T-maze. This was coupled with marked degeneration of TH-positive neurons in the SN and striatal innervation by nigro-striatal neurons. Most interestingly, α-synuclein positive inclusions were observed, present first in the olfactory bulb and then at later time-points throughout the striatum, SN, entorhinal cortex and CA1 and dentate gyrus regions of the hippocampus.

3.4.5 Transgenic models

The vast majority of transgenic rodent models of PD are in mice, and so are outside the scope of the purpose of this thesis (Magen and Chesselet, 2010). However, there are a small number of transgenic rat

lines, fewer still those that have investigated non-motor symptoms of PD. Transgenic overexpression of α-synuclein in Sprague-Dawley (SD) rats has been shown to induce olfactory dysfunction and α-synuclein-positive pathology in the olfactory bulbs (Lelan et al., 2011; Nuber et al., 2013), and also to induce an anxiety-like phenotype (Kohl et al., 2016). Interestingly, the birth of new neurons in the adult hippocampus, known as hippocampal neurogenesis, was severely compromised in these rats, which potentially could affect functioning of the hippocampus and associated learning and memory, although this was not examined from a behavioural perspective in this paper (Kohl et al., 2016).

3.4.6 The α-synuclein model

The α-synuclein model of PD was first described by Kirik *et al* (2002), when an AAV vector was used to overexpress both wild-type and mutated forms of human α-synuclein in the dopaminergic neurons of the rat SN. Unilateral administration of either vector induced neuronal degeneration in the nigra specific to DA neurons and a decrease in striatal DA levels. Animals also developed significant unilateral motor impairment in the paw-reaching test and apomorphine-induced rotations (Kirik et al., 2002). This paper was the first proof-of-concept that α-synuclein overexpression in the midbrain could replicate the basic pathological, neurochemical and behavioural features of the disease, and established the model as a viable representation of PD. Since then, the model has been adapted as various capsid protein serotypes

of AAV have been used in order to achieve better transduction of nigral dopaminergic neurons, as it is the capsid protein and not the core protein that determines the entry method of the virus into the cell. Vectors derived from AAV2, 5, 6 and 9 capsid proteins have all been utilised in rats to varying success (M Decressac et al., 2012; Mulcahy et al., 2013; Shahaduzzaman et al., 2015; Taschenberger et al., 2012). A number of studies have been reported that have used AAV1 core protein-derived vectors (Koprich et al., 2011, 2010). One of the distinct advantages of this model is the progressive nature of the neuronal degeneration and motor dysfunction, which more closely replicates the human condition compared to previous models (reviewed by Volpicelli-Daley et al. 2016). Surprisingly, given the success of the model, relatively little has been characterised with regards to NMS (see Table 3.5). Bilateral intra-nigral administration of wild-type α-synuclein has been shown to induce depressive-like behaviour in the FST and sucrose preference test (SPT) (Caudal et al., 2015), although the same tests previously carried out by another group reported no effect of α-synuclein (Campos et al., 2013). A novel approach to inducing overexpression of wild-type α-synuclein in the adult rat forebrain was carried out by injecting the AAV vector bilaterally into the striatum of postnatal day 2-4 pups (Aldrin-Kirk et al., 2014). This group reported that there were no deficits in spatial learning in the MWM (Aldrin-Kirk et al., 2014). However, administration of wild-type α-synuclein into the ventral tegmental area (VTA) and medial septum/diagonal band of Broca of rats has been shown to induce deficits in spatial working memory in the MWM (Hall et al., 2013). Caution should be

exercised when interpreting this result in the context of PD as administration into this region is not wholly representative of a PD model.

The so-called “third generation” of the α-synuclein model of PD has been developed in very recent years, whereby different forms of α-synuclein such as pre-formed fibrils (Paumier et al., 2015), recombinant monomers, oligomers and PD lysate taken from human brains (Holmqvist et al., 2014; Peelaerts et al., 2015) have all been used to investigate and potentially characterise the differential effects of the α-synuclein structure itself on motor symptoms. To date, no studies have reported on non-motor symptoms using these approaches.

Although the α-synuclein model is perhaps the most accurate representation of the progressive nature of human PD (reviewed by Lindgren et al. 2012), it is not without its caveats. The marked variation in the serotype of viral vectors and the different forms of the α-synuclein protein (wild-type, mutated) that are used by various research groups lead to differing neuronal transduction efficiencies, α-synuclein expression and behavioural and pathological phenotypes, which makes it difficult to compare and extrapolate between the studies that have been published.

Publication	Breed & Sex	Administration site	Virus serotype & protein form	Dose	Behavioural tests	Result
Caudal	Adult female SD	SN	AAV6 Human wt α-synuclein	2.3 x 10^{11}	FST SPT EPM SIH	↓ ↓ - -
Alvarsson	Adult female SD	VTA	AAV6 Human wt α-synuclein	2.3 x 10^{11}	Passive avoidance task EPM FST	↓ - -
Campos	Adult male Wistar	SN	AAV2 Human α-synuclein	11 x 10^{11}	EPM FST MWM SPT	- - - -
Aldrin-Kirk	Neo-natal SD	Str	AAV6 Human wt α-synuclein	3.5 x 10^{9}	MWM	-
Hall	Adult female SD	VTA and MS/DBB	AAV5 Human wt α-synuclein	VTA 5.5 x 10^{10} MS/DBB 1.48 x 10^{11}	MWM	↓

Table 3.5. Summary of the effects of α-synuclein overexpression on cognitive and emotional behaviours in rats. *↑ denotes increased or enhanced performance, - denotes no change in performance and ↓ denotes decreased or impaired performance.* ***Abbreviations****: SD = Sprague Dawley; wt = wild-type; SN = substantia nigra; MWM = Morris water maze; FST = forced swim test; SIH = stress-induced hypothermia; SPT = sucrose preference test; EPM = elevated plus maze; VTA = ventral tegmental area*

3.5 α-synuclein and its role in PD

3.5.1 The SNCA gene and PD

One of the earliest indications of the link between α-synuclein and PD was published by Polymeropolous *et al* (1997), where a genotype analysis was carried out on an Italian family with autosomal dominant inheritance of a PD phenotype. They discovered recombination events in the α-synuclein gene (SNCA) located on chromosome 4, namely an alanine to threonine substitution at position 53 (Ala53Thr, later shortened to A53T), which resulted in a shift from α-helix to β-sheet structure of the α-synuclein protein (Polymeropoulos et al., 1997). This link was further strengthened later that same year with a seminal paper from Spillantini and colleagues (1997), who published the first evidence that aggregated α-synuclein was the primary constituent of Lewy bodies and Lewy neurites. Since then, various other point mutations have been characterised and linked to familial PD, including A30P (Krüger et al., 1998) and E46K (Zarranz et al., 2004), as well as both duplication (Chartier-Harlin et al., 2004) and triplication (Singleton, 2003) of the whole α-synuclein gene. Further mutations that have been discovered in recent years include H50Q (Appel-Cresswell et al., 2013; Proukakis et al., 2013), G51D (Kiely et al., 2013; Lesage et al., 2013) and A53E (Pasanen et al., 2014). Importantly, the various mutations each appear to result in distinct phenotypes of PD, both *in vivo* (reviewed by Kasten & Klein 2013) and experimentally *in vitro* (Sahay et al. 2015; Lu et al. 2015; Ono et al. 2011; Burré et al. 2013).

3.5.2 Structure and function of α-synuclein

α-synuclein is a 140-amino acid protein (14.5kDa) which is encoded by the gene SNCA and is highly conserved in vertebrates (Bisaglia et al., 2009). It belongs to a small family of synuclein proteins which also includes β-synuclein and γ-synuclein (Lavedan, 1998). α-synuclein belongs to a class of intrinsically unstructured proteins owing to its lack of defined structure in its native state (Uversky 2003), and this unique characteristic endows a remarkable conformational plasticity which has been shown to be dependent on the cellular environment (Bai et al., 2016; Lawand et al., 2015). In its native form, α-synuclein exists without any stable tertiary structure (Eliezer et al., 2001). Upon binding to phospholipid cellular membranes, α-synuclein adopts a predominantly α-helical structure (Davidson et al., 1998; Eliezer et al., 2001), however it is the misfolding of the protein into a β-sheet-rich conformation and the subsequent aggregation into insoluble fibrils which form the characteristic Lewy bodies and Lewy neurites that are seen in PD.

Structurally, the native protein is made up of three distinct domains (see Figure 3.1): (1) a positively-charged lipid-binding N-terminal region (2) a central hydrophobic region known as NAC (for non-amyloid-β component of Alzheimer's disease) and (3) an acidic carboxyl terminus that remains unstructured (Eliezer et al. 2001; Stefanis 2012; Jain et al. 2013; Burré et al. 2013). Although it was initially thought that the central NAC region was key for the formation of α-synuclein aggregates (Lawand et al. 2015; Burré et al.

2013; Rodriguez et al. 2015), there is also increasing evidence to support the importance of the N-terminus in the aggregation process. The N-terminus has been shown to be critical for α-synuclein membrane interaction and permeabilisation (Gaugler et al., 2012; Lorenzen et al., 2014), and all known clinical mutations for PD are located on the N-terminus (Dehay et al., 2015). A recent study evaluating antibodies against either the N-terminus or central region of α-synuclein found that the N-terminus antibody conferred more protection against dopaminergic neuronal cell loss and against some behavioural deficits compared to the central region antibody (Shahaduzzaman et al., 2015). Interestingly, recent work focusing on the role of the C-terminus has also shown that this region can modulate the aggregation of α-synuclein (Izawa et al., 2012; Izumi et al., 2016; Sahin et al., 2016). Furthermore, a new compound that targets the C-terminus can improve motor deficits and reduce α-synuclein accumulation in several transgenic rodent models of PD (Wrasidlo et al., 2016). Taken together, all this evidence further consolidates the fact that still, relatively little is known about the differential effects and interplay of the structural sub-regions of α-synuclein.

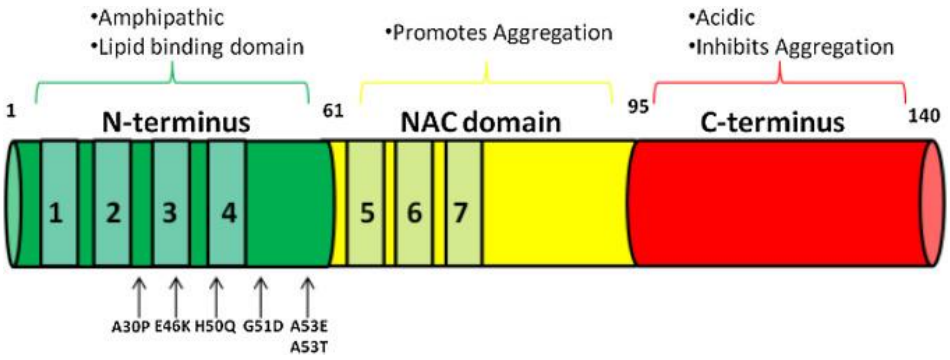

Figure 3.1. Schematic depicting the structure of α-synuclein

The endogenous functions of native α-synuclein are yet to be fully elucidated. Although this protein is found throughout the body (Böttner et al., 2012), it is most highly expressed in the brain. Its localisation at presynaptic nerve terminals and its interaction with membrane proteins suggest that it plays a role in neurotransmitter release (Bendor et al., 2013). Additionally, it has been shown to be involved in facilitating the assembly of the SNARE complex (a family of vesicular fusion proteins) (Burré et al., 2010), vesicular trafficking (Lee et al., 2011; Nemani et al., 2011), neuronal maturation in the dentate gyrus (Winner et al., 2012), DA transmission (reviewed by Butler et al. 2016; Buck et al. 2015) and possibly in transcriptional regulation in the nucleus, though the latter is still highly debated (for review see Wales et al. 2013; Villar-Piqué et al. 2015).

3.5.3 Post-translational modification of α-synuclein

α-synuclein can undergo a wide variety of post-translational modifications, including N-terminal acetylation (Anderson et al. 2006; reviewed by Moriarty et al. 2014), sumoylation (Dorval and Fraser, 2006), ubiquitination (Hasegawa et al., 2002; Nonaka et al., 2005), transglutamination (Junn et al., 2003) and nitration (Barrett & Greenamyre 2015), however it is phosphorylation, specifically at serine residue 129 that has been the most widely studied with respect to PD (for full review on phosphorylation of α-synuclein see Tenreiro et al. 2014; Xu et al. 2015 and Oueslati 2016). First discovered by Fujiwara (2002), s129-phosphorylated α-synuclein is the most abundantly expressed protein in Lewy bodies (Anderson et al., 2006). It is present under normal conditions in the human brain (Muntané et al., 2012), however it is strikingly increased in the brains of patients with PD and other synucleinopathies (Fujiwara et al., 2002). Although increased levels of phospho-s129 have also been reported in animal models of PD, including the 6-OHDA lesion model (Ganapathy et al., 2016), the rAAV-α-synuclein overexpression model (Aldrin-Kirk et al., 2014) and transgenic mouse models (Amschl et al., 2013; Spinelli et al., 2014), the role of phosphorylated α-synuclein in the pathogenesis of PD is poorly understood. Some groups have reported that phospho-s129 potentiates the toxicity of α-synuclein (Gorbatyuk et al., 2008; Sato et al., 2011), and can modulate inclusion formation and α-synuclein fibrillation (reviewed by Tenreiro et al. 2014), while others argue that there are no differences between the effects of wild type and phospho-s129 α-synuclein on nigro-striatal neuronal degeneration

or aggregation kinetics (McFarland et al., 2009; Schreurs et al., 2014). Recent work by Samuel *et al* (2015) and Ma *et al* (2016) suggest that phosphorylated α-synuclein may have different effects depending on whether the wild-type or mutant protein is present. Interestingly, inducing phosphorylation of endogenous α-synuclein through modulation of polo-like kinases (PLKs) does not cause nigral dopaminergic neurodegeneration or accumulation of phosphor-s129 (Buck et al., 2015). Additionally, phosphorylation at serine 87 seems to protect against the aggregation of α-synuclein and associated toxicities (Oueslati et al., 2012; Paleologou et al., 2010), strengthening the argument that the various individual structures of α-synuclein are responsible for its distinct effects.

3.5.4 α-synuclein seeding and the aggregation process

The Braak hypothesis (Braak et al. 2003) describes the pathological staging of PD based on the presence of α-synuclein inclusions in certain regions of the brain, and theorised that α-synuclein proliferates throughout these regions in an ordered and predetermined manner. Starting in the dorsal IX/X motor nucleus (and often in the anterior olfactory nucleus), the disease pathology ascends rostrally in the brainstem and through susceptible brain regions in the midbrain and basal forebrain including subcortical and mesocortical regions and olfactory structures, before reaching the neocortex (Braak et al. 2003). Each stage of the process correlates with increasing severity and type of disease symptoms and clinical manifestations

of the disease. Evidence for the movement of α-synuclein throughout the brain was further strengthened by the discovery of Lewy body pathology within grafted tissue from *post mortem* analysis of patients who had received foetal mesencephalic transplants (Kordower et al. 2008; Kordower et al. 2008b). This also suggests that α-synuclein has the ability to travel between host and graft tissue (Li et al., 2008). This led to the theory that α-synuclein can self-propagate throughout the brain in a "prion-like" manner (reviewed by Sato et al. 2014 and Recasens & Dehay 2014) *via* a seeding mechanism, whereby exogenous α-synuclein is taken up into neurons *via* endocytosis and induces endogenously expressed α-synuclein to form inclusions. These aggregates then cause cell disruption and death, leading to the release of more α-synuclein, which is then available for further neuronal uptake, so continuing the process. There have been several *in vivo* and *in vitro* studies published in support of this theory. Sacino and colleagues (2013) reported that the addition of exogenous pre-formed fibrils (PFFs) to primary mixed neuronal-glial cultures (derived from postnatal day 0 mouse brain) was not sufficient to induce endogenous α-synuclein aggregates. However, overexpression of human wild-type α-synuclein *via* an AAV vector with the extracellular addition of the PFF "seed" resulted in a rapid and marked increase in α-synuclein inclusions primarily formed of endogenous α-synuclein. Interestingly, the morphology of the Lewy body was shown to be primarily dependent on the structure of the seed applied i.e. wild-type or mutant, rather than the type of α-synuclein expressed (Sacino et al., 2013). Furthermore, the addition of α-synuclein PFFs to mouse hippocampal

primary cultures can accelerate the aggregation of monomeric α-synuclein to fibrils (Mahul-Mellier et al., 2015). It has also been shown that injection of human recombinant α-synuclein fibrils into the mouse SN induces accumulation of endogenous α-synuclein up to 3 months after the fibril administration (Masuda-suzukake et al., 2013), as well as propagation of α-synuclein pathology throughout various interconnected brain regions (Masuda-suzukake et al., 2014).

Broadly, the aggregation process (reviewed in depth by Narkiewicz et al. 2014) is the transformation of α-synuclein from its native form to a β-rich fibrillary structure, which has been shown to be the primary component of Lewy bodies (Araki et al., 2015). α-synuclein aggregation begins with the formation of intermediary oligomeric structures through the repeated addition of monomers (Buell et al., 2014). These oligomers then further aggregate, undergoing a series of conformational changes from protofilaments to protofibrils before becoming mature α-synuclein fibrils (Qin et al., 2007). Although it was initially thought that the mature α-synuclein fibrils were cytotoxic, growing evidence suggests that oligomers may also contribute to cell damage and death (reviewed by Forloni et al. 2016 and Roberts & Brown 2015) . Oligomeric α-synuclein has been detected in *post mortem* brain tissue from PD patients (Paleologou et al., 2009), and has been shown *in vitro* to disrupt membranes (Perdersen et al., 2015; van Rooijen et al., 2010) leading to calcium influx (Danzer et al., 2007) and can result in high levels of oxidative stress in neurons (Cremades et al., 2012).

There are a wide variety of factors that can influence the aggregation process, including the lipid composition (Hellstrand et al., 2013; Tsujimura et al., 2015) or pH (Buell et al., 2014) of the cellular environment, the specific isoform of α-synuclein that is present (Bungeroth et al., 2014; Lemkau et al., 2013; Manda et al., 2014; Nielsen et al., 2013), the presence of neurotransmitters (Jain and Bhat, 2014; Outeiro et al., 2009; Pham and Cappai, 2013) and their respective oxidative states (Fischer and Mansfield, 2015; Follmer et al., 2015) and post-translational modifications of the protein (Kang et al., 2013, 2012; Krumova et al., 2011).

Importantly, what is evident is that α-synuclein is an incredibly versatile protein that can dynamically alter its structure in response to various intracellular and extracellular cues. The initial conformation of α-synuclein can drastically impact the final structure of the aggregation process (Bai et al., 2016; Chen et al., 2015). Growing evidence suggests that different structural forms of α-synuclein, or indeed the various post-translational modifications that the protein undergoes, may explain the distinct pathologies seen within the classes of synucleinopathies (Bousset et al., 2013; Peelaerts et al., 2015; Prusiner et al., 2015). Peelaerts and colleagues extensively characterised behavioural and histopathological effects of administration of either α-synuclein ribbons, fibrils or oligomers, and found that each structural moiety elicited a different phenotype (Peelaerts et al., 2015). α-synuclein fibrils proved to be the most neurotoxic, causing progressive motor impairment and cell death, whereas administration of α-synuclein ribbons caused a distinct pattern of phosphorylated α-synuclein

accumulation in oligodendroglial cells, more characteristic of multiple system atrophy (MSA).

3.5.5 Mechanisms of α-synuclein- induced cell death

Various mechanisms of α-synuclein-induced cell death have been reported (reviewed by Gallegos et al. 2015; Waxman & Giasson 2009; Yasuda et al. 2013). These include mitochondrial dysfunction (Parihar et al. 2008; Parihar et al. 2009; Sarafian et al. 2013; Luth et al. 2014; reviewed by Zaltieri et al. 2015), endoplasmic reticulum stress (Colla et al., 2012; Gully et al., 2016), dysregulation of autophagy (reviewed by Wang et al. 2016), lipid membrane disruption (Chaudhary et al., 2014; J. Lee et al., 2012; Mazzulli et al., 2016; Pacheco et al., 2015; Reynolds et al., 2011; Rooijen et al., 2010), pore formation (Tosatto et al., 2012) and calcium influx resulting in abnormal calcium homeostasis (Danzer et al., 2007; Tosatto et al., 2012; Tsigelny et al., 2012), as well as oxidative stress and inhibition of microtubule assembly (Oikawa et al., 2016; Prots et al., 2013). However, there are two conflicting schools of thought on the underlying process of α-synuclein-mediated neuronal death. Although it has been widely accepted that dopaminergic neurodegeneration inherent to PD is caused by the aggregation of α-synuclein in Lewy bodies, a so-called gain-of-toxicity function (Oikawa et al., 2016) more recent work postulates that a loss of endogenous function of pre-synaptic α-synuclein through its sequestration into Lewy bodies could also be involved (reviewed by Benskey 2016). However, evidence from SNCA

knock-out studies does seem to contradict this, as degeneration of dopaminergic cell bodies, fibres or synapses do not develop in α-synuclein null $^{-/-}$ mice (a homozygous knockout model lacking the SNCA gene), nor do these mice display any overt phenotype (Abeliovich et al., 2000). Benskey and colleagues argue that this could be as a result of a developmental coping mechanism from the SNCA gene knock-out (Benskey et al., 2016). Interestingly, SNCA knockdown in adult rats has been shown to reduce striatal DA content (Zharikov et al., 2015) and result in marked TH-positive cell loss with accompanying motor deficits (Gorbatyuk et al., 2010). Additionally, co-expression of endogenous rat α-synuclein with small interfering RNAs (siRNAs) selectively targeting endogenous α-synuclein partially reversed the PD phenotype in these animals (Gorbatyuk et al., 2010). Similarly, knockdown of endogenous α-synuclein in non-human primates also results in degeneration of dopaminergic neurons (Collier et al., 2016).

3.6 Inflammation in PD

Growing evidence supports a role for inflammation in the pathogenesis of PD (reviewed by Lee et al. 2009). Age is the most common risk factor for many neurodegenerative diseases (Ascherio and Schwarzschild, 2016) and is accompanied by a chronic low-grade systemic up-regulation of pro-inflammatory mediators in the absence of overt infection. The term

"inflammaging" has been coined to describe this chronic process (Chung et al. 2009; Franceschi & Campisi 2014). Importantly, dopaminergic neurons in the SN and striatum appear to be particularly vulnerable to inflammatory insult (reviewed by Barnum & Tansey 2010), and so neuroinflammation can significantly contribute to the pathogenesis of PD (Block and Hong, 2007). Of note, head trauma is known to induce an inflammatory reaction in the brain. It has been shown to cause progressive nigro-striatal dopaminergic cell loss in the rat brain (Hutson et al., 2011), and is linked to higher prevalence of PD in humans (Ascherio and Schwarzschild, 2016). Epidemiological evidence for a role of inflammation in PD comes from the fact that the regular use of several anti-inflammatory medications, including the non-steroidal anti-inflammatory drug ibuprofen (Chen et al., 2005; Gao et al., 2011) and the statin (3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors) class of drugs (Gao et al., 2012) decrease the risk of PD. Similarly, peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that play an important role in modulating inflammation (Michalik et al., 2006) and PPAR agonists including pioglitazone and rosiglitazone have been shown to exert neuroprotective effects in animals models of PD (Barbiero et al., 2014; Carta and Simuni, 2014; Falcone et al., 2014).

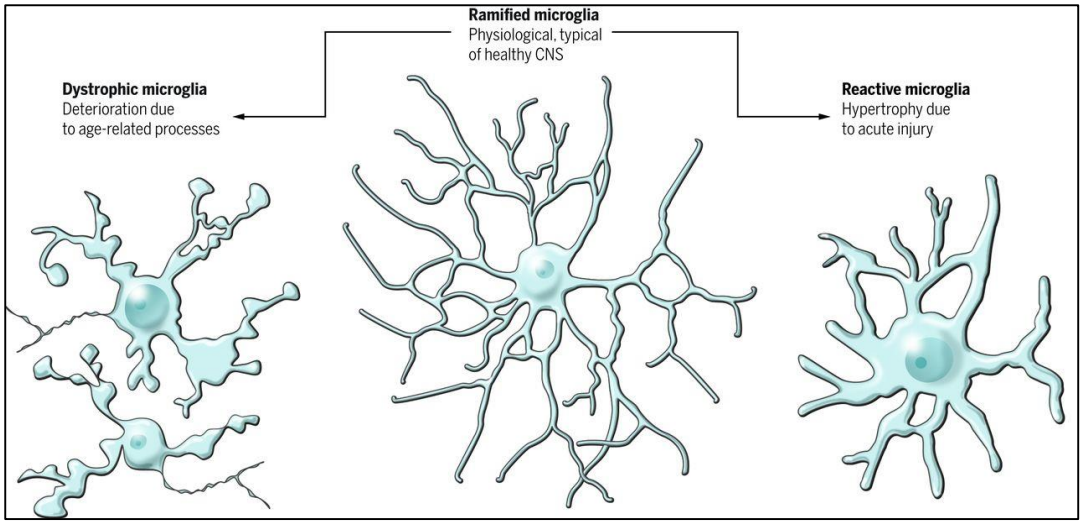

Figure 3.2. Schematic depicting structural and morphological changes in microglia. Taken from Ransohoff (2016).

3.6.1 Inflammation and aging

Microglia are the resident innate immune cells of the brain, and are found in high concentrations in the SN and striatum (Lawson et al., 1990). Their main functions include immune modulation and mediation of innate immune responses (Doorn et al., 2012). They can be activated by a number of stimuli, including stress, head injury, infections, neuronal damage and environmental toxins (reviewed by von Bernhardi et al. 2015), and function to clear the cellular environment *via* phagocytosis of debris from damaged or leaky cells (Nimmerjahn et al., 2005; van Rossum and Hanisch, 2004). In most cases, this reaction is self-limiting. However, in some cases, microglia can remain chronically activated and continually release a myriad of pro-inflammatory cytokines and ROS. This chronic activation leads to aberrant microglial functioning, as they kill otherwise viable cells, particularly neurons (Long-Smith et al., 2009). The resulting cell death triggers further microglial

activation, resulting in a vicious cycle of long-lived and self-propelling inflammation and oxidative stress known as reactive microgliosis.

Interestingly, the process of aging is considered to contribute to neuro-inflammation, and even healthy aged brain tissue displays widespread microglial activation throughout various brain regions (Schuitemaker et al., 2012). However, it remains unclear what drives this increase in upregulated microglia. Aging has been shown to cause changes in brain volume and cortical thinning, as well as neuronal loss and shrinkage (Anderson et al., 1983; Sykova et al., 1998; Terry et al., 1987) and microglia may be reacting to these profound changes in the microenvironment. Alternatively, it has been hypothesised that aging may have a direct impact upon the structure of microglia (Conde and Streit, 2006). Morphological changes have previously been reported in microglia from aged animal and human studies, including cytosolic inclusions, cytoplasmic hypertrophy and other ultrastructural abnormalities collectively termed microglial dystrophy (Vaughan & Peters 1974; Peinado et al. 1998; Streit et al. 2004, see Figure 3.2). Given that the ability of microglia to monitor and respond to pathogens is dependent on their structural complexity, any structural dysfunction could lead to profound functional impairments and subsequently impact on the health of the brain. Microglia in aged mice cause significantly more dopaminergic neuronal death in an MPTP model of PD and are slower to react to acute injury when compared to younger counterparts (Damani et al., 2011; Sawada et al., 2007). Recent work has shown that microglia are differentially susceptible to aging dependent on their phenotype and

location in the brain (Grabert et al., 2016). In addition, several reports have shown that there is a possible shift in cytokine balance with aging, as levels of pro-inflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α in the brain increase (Lukiw, 2004; Ye and Johnson, 1999) and are accompanied by a subsequent decrease in the levels of anti-inflammatory cytokine IL-10 in the brain (Ye and Johnson, 2001). Aging is accompanied by a cumulative increase in environmental toxins, including ROS, in both the body and brain (Chung et al. 2009), and coupled with the increased permeability of the BBB also seen in aging (reviewed by Gorlé et al. 2016), the brain is left particularly vulnerable to inflammatory insult. The shift toward a more pro-inflammatory state and subsequent neuronal damage can lead to prolonged microglial activation and contribute further to reactive microgliosis. Furthermore, there is growing evidence to support the phenomenon of microglial "priming", namely that chronic exposure to inflammation that is consistent with the aging process results in an exaggerated response to a second inflammatory stimulus (reviewed by Hoeijmakers et al. 2016; Perry & Holmes 2014).

Taken together, it is clear that the aging process significantly affects both the structure and function of microglia. However, whether this dysfunction is as a result of chronic over-activation of microglia, or of an inability of microglia to complete normal cellular functions is still highly debated (reviewed by Harry 2013; von Bernhardi et al. 2015). Given that the main predisposing risk factor to developing PD is increasing age (Ascherio and Schwarzschild, 2016),

it stands to reason that the age-related changes in microglial functioning may play a part in the progression of the disease.

3.6.2 Systemic inflammation in PD

Several studies have confirmed the link between systemic inflammation and PD. Increased levels of cytokines TNF-α, IL-2, IL-6, IFN-γ, IL-1β, the chemokine RANTES (regulated on activation, normal T-cell expressed and secreted, also known as CCL-5) and markers of oxidative stress have all been observed in serum of PD patients compared to age-matched control subjects (Andican et al., 2012; Brodacki et al., 2008; Dobbs et al., 1999; Reale et al., 2009; Rentzos et al., 2009, 2007; Stypula et al., 1996). Serum levels of cytokine receptors such as TNF-α receptor 1 are also increased in PD patients (Scalzo et al., 2009). Interestingly, growing evidence suggests that peripheral inflammatory markers may prove useful tools for not only predicting PD (Chen et al., 2008) but also for differentiating between the various subtypes of PD (Constantinescu et al., 2010). It has also been shown that levels of peripheral cytokines and their receptors in PD patients positively correlate with disease progression (measured on the Hoehn-Yahr scale), severity and duration (Reale et al., 2009; Tang et al., 2014) as well as with non-motor symptoms such as depression and fatigue (Lindqvist et al., 2012; Menza et al., 2010; Pereira et al., 2016; Rocha et al., 2014; X.-M. Wang et al., 2016; Williams-Gray et al., 2016).

3.6.3 Central inflammation in PD

The first evidence that linked neuro-inflammation to the progression of PD came from the presence of activated microglia in the SN of *post mortem* brain tissue from PD patients (McGeer et al., 1988). Since then, there has been a myriad of data published supporting this association. Increased levels of cytokines and cytokine receptors have been detected in the SN of PD patients in comparison to controls (Mogi et al., 2000, 2007). Similarly, expression of toll-like receptor (TLR) 2, a potent activator of microglia, is significantly enhanced in the SN and anterior cingulate cortex of PD patients (Doorn et al., 2014; Dzamko et al., 2016). Enzymes associated with inflammation, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), have also been identified *post mortem* in PD brains (Hunot et al., 1996; Knott et al., 2000). Modern imaging studies have shown reactive microgliosis in the SN and striatum (Iannaccone et al., 2013) as well as the occipital, temporal and parietal cortices of PD patients (Gerhard et al., 2006; Terada et al., 2016), highlighting the potential role the inflammatory reaction may play in the pathogenesis of NMS in PD.

3.6.4 Supportive evidence from animal models of PD

Evidence from animal studies further supports the link between neuro-inflammation and PD. Chronic overexpression of the cytokine IL-1β in the SN of rats results in nigral dopaminergic degeneration and motor deficits, as well as glial activation (Ferrari et al., 2006). The MPTP model of PD has been

shown to cause marked microglial activation in the SN in both mice (Czlonkowska et al., 1996) and monkeys (McGeer et al., 2003; Vazquez-Claverie et al., 2009) as well as increases in levels of pro-inflammatory cytokines in the SN of mice (Mandel et al., 2000; Shimoji et al., 2009). Given that the 6-OHDA model by its very nature causes cell death through oxidative stress (reviewed by Barnum & Tansey 2010), it stands to reason that administration of 6-OHDA would also result in a profound inflammatory reaction (reviewed by Cebrian et al. 2015), including enhanced microglial activation and increased levels of pro-inflammatory cytokines (Cicchetti et al., 2002; Marinova-Mutafchieva et al., 2009; Na et al., 2010; Nagatsu and Sawada, 2005). Blockade of TNF-α or the TNF receptor has also been shown to attenuate nigral dopaminergic cell death in a 6-OHDA rat model (McCoy et al., 2006; Mccoy et al., 2008). The rotenone model of PD induces cell death at least in part by the formation of ROS, and so will result in a similar range of neuro-inflammatory effects (Cicchetti et al., 2009). The use of the Gram-negative bacterial endotoxin component LPS as a potent activator of microglia has enabled investigations into the precise contributions of various inflammatory mediators on the pathogenesis of PD. Administration of LPS into the SN activates microglia to release a range of pro-inflammatory cytokines and neurotoxic factors such as ROS (Sharma & Nehru 2015 and reviewed by Liu & Bing 2011). It selectively damages dopaminergic neurons and results in motor deficits similar to those seen in other animal PD models (Hoban et al., 2013). Interestingly, administration of LPS in combination with α-synuclein overexpression results in enhanced dopaminergic

neurodegeneration and motor deficits compared to either LPS or α-synuclein alone (Mulcahy et al., 2013, 2012).

3.6.5 Inflammation and α-synuclein

Overexpression of α-synuclein also results in a robust neuro-inflammatory reaction. Microglia are activated by the presence of α-synuclein (Alvarez-Erviti et al., 2011; Codolo et al., 2013; Wilms et al., 2009), releasing a raft of pro-inflammatory cytokines and ROS that have been shown to contribute to dopaminergic cell death (Wang et al. 2016; Klegeris et al. 2008; Zhang et al. 2007; Theodore et al. 2008; Chung et al. 2009; Sanchez-Guajardo et al. 2010; Cao et al. 2010). Critically, in the context of PD, age-dependent deficits in microglial function have been shown to have deleterious effects on the ability of microglia to protect against α-synuclein-mediated neurotoxicity (Bliederhaeuser et al., 2015). Interestingly, the activation state seems to be dependent on the type of protein present (wild-type, mutant) (Hoenen et al., 2016) and the aggregation state of the protein (Hoffmann et al., 2016). Similarly, microglia isolated from aged animals are unable to efficiently phagocytose α-synuclein oligomers *in vitro* (Bliederhaeuser et al., 2015). Furthermore, previous exposure of microglia to α-synuclein results in a significantly heightened response to TLR stimulation, so-called microglial priming (Roodveldt et al., 2013). A recent paper by Christiansen (2016) showed that vaccination against α-synuclein in mice can modulate the

microglial population to a more anti-inflammatory phenotype (Christiansen et al., 2016).

3.7 Neurogenesis in PD

3.7.1 Neurogenesis

Neurogenesis is defined as the generation of new neurons from neural progenitor cells (NPCs), and it is a key contributor to synaptic plasticity in the adult brain (for reviews see Lee et al. 2012; Regensburger et al. 2014; Sailor et al. 2017). Neurogenesis has been shown to occur, at least in rodents, throughout the lifespan in distinct niches of the brain, namely the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricles (Altman, 1969; Eriksson et al., 1998). Adult neurogenesis has also been shown within the striatum in humans and has been suggested to contribute to the generation of new striatal interneurons (Eriksson *et al.*, 1998; Gage, 2012; Frisén *et al,* 2015). While the functional significance of new adult striatal neurons is yet to be fully understood, it is possible that they contribute to motor and cognitive functions such as behavioural flexibility (Ernst and Frisén, 2015). Some of the newly born neurons become functionally integrated into neural circuitry (Jessberger and Kempermann, 2003; van Praag et al., 2002) , and in the case of adult hippocampal neurogenesis (AHN) they are thought to play a key role in learning and memory processing and emotional regulation (van Praag et

al. 2002; van Praag et al. 2005; Gould et al. 1999; reviewed by Oomen et al. 2015). Specifically, animal studies in which AHN was ablated have revealed that the new-born neurons significantly contribute to spatial memory and pattern separation (Clelland et al. 2009; Raber et al. 2004). Pattern separation is defined as the process by which overlapping or similar representations are transformed into less similar outputs (Sahay et al., 2011), and it is a process that has been shown to be dependent on AHN (Bekinschtein et al., 2014, 2013, 2011; Clelland et al., 2009). Inhibition of AHN, either by pharmacological or transgenic methods, increases anxiety-like behaviours in rodents (Po et al., 2015; Revest et al., 2009). Conversely, enhanced neurogenesis can alleviate anxiety and depressive-like behaviours (Hill et al., 2015), and many anti-depressant medications have been shown to not only increase AHN (Boldrini et al., 2009; Malberg et al., 2000), but to be dependent on AHN for their efficacy (Santarelli et al., 2003).

AHN is sensitive to local and systemic environmental changes. Animal studies in mice and macaques have shown that AHN is enhanced by environmental enrichment (Kempermann et al., 1997), dietary restriction (Lee et al., 2002) and exercise (Farmer et al., 2004; van Praag et al., 1999). Conversely, stress (Gould et al., 1998), peripheral inflammation (reviewed by Chesnokova et al. 2016) and neuroinflammation (Butovsky et al. 2006; reviewed by Sierra et al. 2014; Ryan & Nolan 2016) have all been shown to detrimentally affect AHN. Moreover, blockade of the LPS-induced inflammatory reaction using anti-inflammatory medication (Indomethacin,

a non-steroidal anti-inflammatory drug) can restore AHN (Monje et al., 2003).

Aging also has a profound impact on neurogenesis (reviewed by Lee et al. 2012). Aged animals show significantly decreased levels of NPC proliferation, differentiation and new-born neuron survival (Bondolfi et al. 2004; Kuhn et al. 1996; Heine et al. 2004; reviewed by Drapeau & Abrous 2008). Aging results in widespread microglial activation and upregulation of pro-inflammatory cytokines (H. Chung et al., 2009; Schuitemaker et al., 2012), which can detrimentally affect AHN (Butovsky et al. 2006; reviewed by Sierra et al. 2014; Ryan & Nolan 2016). The functional implications of age-related decreases in AHN, and whether it plays a part in age-related deficits in spatial memory, are still unclear (reviewed by Drapeau & Abrous 2008). Spatial memory impairments in aged animals have been associated with deficits in hippocampal circuitry (Bitencourt et al., 2017; Haberman et al., 2017; Rowe et al., 2007; Yoo et al., 2016). A study by Driscoll and colleagues positively correlated levels of neurogenesis, measured by doublecortin (DCX; a marker of immature neurons) and performance on hippocampal-associated memory tasks in aged animals, including the MWM, a measure of spatial working memory (Driscoll et al., 2006). However, similar studies have not been able to replicate this finding (Bizon and Gallagher, 2003; Merrill et al., 2003).

One way to address the potential link between age-related deficits in AHN and cognitive decline is to delineate the effects of enhancing AHN on

hippocampal-assocated learning and memory tasks (reviewed by Ryan & Nolan 2016). Animal studies have repeatedly demonstrated that exercise is a potent enhancer of AHN, both in young and aged brains (Farmer et al., 2004; Kronenberg et al., 2006; van Praag et al., 2005, 1999). Importantly, exercise has also been shown to improve performance on hippocampal-associated learning and memory tasks, including pattern separation (Creer et al., 2010; Gibbons et al., 2014; Marlatt, 2012; Wu et al., 2015). Although this link is by no means causative, it certainly suggests that exercise-induced cognitive enhancement in neurodegenerative diseases may be dependent on AHN.

3.7.2 Neurogenesis and PD

Although the majority of work surrounding the link between neurogenesis and PD has been focused on neurogenesis in the SVZ (reviewed by Regensburger et al. 2014; Le Grand et al. 2015; Lamm et al. 2014), there are a few studies linking AHN to the pathogenesis of PD in humans (Regensburger et al., 2014). *Post mortem* brain tissue analysis from PD patients revealed decreased NPC proliferation in the SGZ of the hippocampus (Hoglinger et al., 2004). Several studies also support the role of DA in modularing AHN. For example, DA depletion by a 6-OHDA lesion of the nigrostriatal pathway has been shown to impair NPC proliferation in the SGZ of rats (Hoglinger et al., 2004). Similarly, MPTP-treated mice exhibited decreased DA levels in the hippocampus and decreases in the survival of

newly-born neurons in the SGZ (Schlachetzki et al., 2016). The mesocorticolimbic DA pathway and dopaminergic projections to the dorsal hippocampus have also been shown to be of signicant importance in modulating hippocampal-associated learning and memory (Kempadoo et al., 2016; Wisman et al., 2008).

Of particular importance in the context of PD is the growing evidence linking AHN and α-synuclein (reviewed by Le Grand et al. 2015). α-synuclein has been detected in the hippocampus of *post mortem* PD brain tissue (Flores-Cuadrado et al., 2016). A pluripotent stem cell line derived from a PD patient with a SNCA gene triplication exhibited a marked decrease in NPC differentiation when compared to a cell line derived from a healthy control subject (Oliveira et al., 2015). Moreover, animal studies have repeatedly demonstrated that α-synuclein can decrease hippocampal neuronal proliferation and survival both *in vitro* and *in vivo* (Crews et al., 2008; Desplats et al., 2012; Kohl et al., 2016; Marxreiter et al., 2013; Winner et al., 2012, 2004). Given that AHN can be detrimentally affected by α-synuclein pathology, and that it also may contribute to several NMS which occur in PD, it stands to reason that employing neurogenic modulators such as exercise may provide a useful interventional tool for targeting cognitive symptoms in PD.

3.8 Exercise and PD

Although both motor and NMS of PD are primarily managed using pharmaceutical treatments, there is a wealth of evidence to support lifestyle interventions such as aerobic exercise having a role to play in neuroprotection and symptom management for neurodegenerative disorders (reviewed by Paillard et al. 2015 and Inskip et al. 2016). Exercise can be defined as a subset of physical activity that is planned, structured, repetitive, and purposeful in the sense that improvement or maintenance of physical fitness is the objective (Shephard and Balady, 1999). Interestingly, exercise has been shown to not only play a role in attenuating symptoms in patients with existing PD, but vigorous exercise in mid-life has been shown to lead to a reduced incidence of PD in later life (Xu *et al.*, 2010; Ahlskog, 2011).

3.8.1 Systemic effects of exercise

Regular exercise can increase cardiovascular endurance and capacity, enhance muscular tone, increase muscular strength, improve metabolism, and decrease adiposity (reviewed by Bergman 2013; Egan & Zierath 2013; Spielman et al. 2016) and is listed as one of the key recommendations for cardiovascular disease prevention by the National Institute for Clinical Excellence (NICE, www.nice.org.uk). Along with other modifiable lifestyle factors such as diet (for e.g. limited salt and fat intake), epidemiological

evidence supports the fact that exercise can help reduce the risk of cardiovascular disease (Claas and Arnett, 2016), stroke (Niewada and Michel, 2016), diabetes (Schrauwen and van Marken Lichtenbelt, 2016) and cancer (Lemanne et al., 2013). Exercise has been shown to exert systemic anti-inflammatory effects throughout the body, including decreased visceral fat mass and increased production and release of anti-inflammatory cytokines from skeletal muscle (reviewed by Gleeson et al. 2011). Interestingly in the context of inflammation in PD, at least some of these effects appear to mediated *via* TLRs (Zheng et al. 2015; for review see Flynn & McFarlin 2006; Gleeson et al. 2006).

3.8.2 Central effects of exercise

As well as the comprehensive benefits of exercise on systemic health, it can also have profound effects on the CNS including enhanced neuroplasticity, neurorestoration and neuroprotection (Hirsch et al., 2016; Petzinger et al., 2013). Exercise increases grey matter volume (Sehm et al., 2014), cortico-motor excitability (Fisher et al., 2008), striatal DA receptor density and DA levels in studies of patients with PD (Fisher et al., 2013). NTFs such as BDNF are upregulated from the brain after exercise in human studies (Rasmussen et al. 2009; Wagner et al. 2017; reviewed by Dinoff et al. 2016). Exercise profoundly enhances hippocampal neurogenesis (reviewed by Ma et al. 2017) and can improve spatial memory and memory-processing (Vilela et al. 2016; reviewed by Roig et al. 2013). Exercise has been shown to ameliorate

cognitive deficits seen in CNS disorders such as dementia (reviewed by Groot et al. 2016), schizophrenia (reviewed by Vakhrusheva et al. 2016), depression (reviewed by Knöchel et al. 2012), Alzheimer's disease (reviewed by Paillard et al. 2015) and PD (reviewed by Murray et al. 2014; Paillard et al. 2015; Petzinger et al. 2015).

3.8.3 Exercise in PD

The first link between exercise and PD, published over 20 years ago, reported that exercise during adulthood significantly lowered the risk of developing PD in later life (Sasco et al., 1992). This has been confirmed more recently in larger scale epidemiological studies (Shih et al., 2016; Yang et al., 2015). Exercise has also been shown to affect both motor and NMS in patients who have already been diagnosed with PD (reviewed by Cusso et al. 2016 and Dashtipour et al. 2015). A 1h regimen of forced exercise on an exercise bicycle can improve UPDRS scores by up to 50% after a single session (Alberts et al., 2016). The progressive resistance exercise trial in PD study (PRET-PD) was designed to investigate the effects of exercise on both motor and non-motor aspects of PD, and reported that structured exercise programs enhance both physical function (Prodoehl et al., 2015) and cognitive function in the form of attention and working memory (David et al., 2015) in PD patients. Exercise has also been shown to improve various forms of executive function (Altmann et al., 2016; Cruise et al., 2011; Duchesne et al., 2015; Ridgel et al., 2010; Tanaka et al., 2009) as well as

olfaction (Rosenfeldt et al., 2016), mood and language fluency in PD patients (Altmann et al., 2016; Picelli et al., 2016).

3.8.4 Exercise-induced changes in animal models of PD

Animal studies have allowed more detailed research into the mechanisms of exercise-induced neuroplasticity and neuroprotection. In the context of animal models of PD, both the 6-OHDA and MPTP models have been extensively investigated using exercise as an intervention. However, due to the fact that rats are relatively resistant to the effects of MPTP (reviewed by Bové & Perier 2012), all the work carried out to date using this model has been in mice. For the purposes of this thesis, I will limit the discussion to reports of exercise-induced changes in the pathogenesis of PD in studies that used rat models.

Forced exercise in the form of treadmill running (see table 3.6 for further information on type and duration of exercise intervention) either before or after unilateral administration of 6-OHDA into the striatum has been shown to protect against dopaminergic cell death in the SN (Cho et al., 2013; Choe et al., 2012; Real et al., 2013; Yoon et al., 2007), upregulate the expression of NTFs in the striatum (Tajiri et al., 2010) and increase the proliferation of new neurons in the dentate gyrus (Cho et al., 2013). Exercise was found to rescue 6-OHDA-induced motor deficits as measured by rotational behaviour and by the cylinder test (Choe et al., 2012; O'Dell et al., 2007; Real et al.,

2013; Tajiri et al., 2010) and to ameliorate short-term memory deficits in a step-down avoidance task (Cho et al., 2013).

Forced exercise in the form of treadmill running after a unilateral 6-OHDA lesion of the MFB protected against striatal dopaminergic cell degeneration and restored 6-OHDA-induced deficits in striatal DA levels (Poulton and Muir, 2005; Shi et al., 2017; Tillerson et al., 2003). Exposure to either forced or voluntary exercise (in the form of access to a running wheel in the home cage, see Table 3.7 for further information) in the same model rescued motor deficits (Tillerson et al. 2003; Dutra et al. 2012; Hendricks et al. 2012; Howells et al. 2005). It has also been shown to normalise the impaired firing rates of striatal dopaminergic neurons (Shi et al., 2017) and decrease 6-OHDA-induced inflammation in the striatum, as measured by glial fibrillary acidic protein (GFAP) reactivity (Dutra et al., 2012).

Author	Breed and sex	Exercise type and duration	Tests	Effect of exercise on performance
Cho	Adult female SD	Forced treadmill 30m/d for 14d, starting 4wk post-lesion	Step-down avoidance task	↑
Choe	Adult male SD	Forced treadmill 30m twice daily for 16d, starting 5d post-lesion	Rotations	↑
O'Dell	Adult male SD	Forced or voluntary wheel running, starting 2.5wk before lesion and continuing for 4wk	Cylinder Stepping Elevated grid	↑ ↑ ↑
Real	Adult male Wistar	Forced treadmill 3/wk, starting either 4w before lesion for 8 wk or 2d after lesion for 4wk	Rotations	↑
Tajiri	Adult female SD	Forced treadmill, 30m/day, 5d/wk starting 24h post-lesion	Cylinder Rotations	↑ ↑
Yoon	Adult male SD	Forced treadmill for 30m/d starting 24h post-lesion	Rotations	↑

Table 3.6. Effects of exercise on the unilateral striatal lesion of 6-OHDA. *↑ denotes enhanced or recovered behavioural performance. Abbreviations: SD = Sprague Dawley;*

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Interestingly, employing exercise as a neuroprotective rather than neurorestorative intervention has had variable results, with some groups reporting that exercise had no effect on motor symptoms when animals were exposed to either a forced or voluntary exercise regimen prior to a 6-OHDA lesion of the MFB (Landers et al., 2014, 2013). However, it is worth noting that these studies only examined motor behaviours and did not investigate the specific neurobiological effects of exercise. This also serves to highlight the importance of the timing of the exercise regime when

compared to the intervention. To date, no studies have reported on either neuroprotective or neurorestorative effects of exercise using the α-synuclein rat model of PD.

Author	Breed and sex	Exercise type and duration	Tests	Effect of exercise on performance
Poulton	Adult female LE	Forced treadmill 20m twice daily, 6/wk for 1 month. Started either 24h (early) or 1wk (late) post-surgery	Cylinder Stepping Ladder rung walking Rotations	↑ ↑ - ↑ early
Tillerson	Adult male F344	Forced treadmill 15m twice daily for 9d, starting on the day of surgery	Stepping Cylinder Whisker	↑ ↑ ↑
Shi	Adult male SD	Forced treadmill, 5d/wk starting 1d before lesion and continuing for 4wk	Rotations	↑
Dutra	Adult male Wistar	Forced treadmill 5/wk, starting 3d post-surgery for 4wk	Rotations Narrow beam	↑ ↑
Hendricks	Male SD	Voluntary access to running wheel in homecage, starting 1wk pre-surgery	Stepping	↑
Landers (2013)	Adolescent male LE rats	Forced treadmill for 5d/wk or voluntary wheel running for 6wk prior to lesion	Rotations Cylinder Rearings	- - -
Landers (2014)	Adult male LE rats	Forced treadmill for 5d/wk for 4 wks, either before or after lesion	Rotations Cylinder Rearings	- - -
Howells	Adult male LE	Voluntary access to running wheel in homecage, starting 1wk pre-surgery	Rotations	↑

Table 3.7. Effects of exercise on the unilateral MFB lesion of 6-OHDA. *↑ denotes enhanced or recovered behavioural performance, - denotes no change in behaviour. Abbreviations: LE = Long Evans; SD = Sprague Dawley;*

3.9 Summary

To summarise, PD is a multifactorial disorder with a broad range of NMS that are inherent to the disease process, and are thought to be reflective of α-synuclein pathology in internconnected brainr regions. However, to date there is no PD model that accurately and consistently reproduces these important features. Recently, the AAV-α-synuclein rodent model has been shown to recapitulate the progressive nature of PD, however the role of α-synuclein in the development of NMS has yet to be addressed. Thus, the hypothesis for this thesis is:

The propagation of α-synuclein throughout the brain in PD is linked to the presence of NMS

To test this hypothesis, our aims were:

a) To identify whether the AAV-α-synuclein model can replicate the proliferation of α-synuclein through the brain
b) To interrogate the link between the presence of α-synuclein throughout the brain and the pathogenesis of NMS
c) To examine whether a neuroprotective intervention such as exercise could protect against NMS, specifically hippocampal-associated memory tasks
d) To investigate if exercise could protect against α-synuclein-induced cognitive deficits by modulating adult hippocampal neurogenesis

Chapter 4

4.0 Materials and Methods

4.1 Virus preparation

α-synuclein and GFP plasmids were kindly donated by Dr. Eilis Dowd (National University of Ireland, Galway). The viruses were constructed (Vector Biosystems Inc, Philadelphia, USA) as described by Decressac *et al* (2012a). Briefly, AAV2 inverted terminal repeats (ITR) coding for either human wild type human α-synuclein (figure 4.1A) or GFP (figure 4.1B) were packaged using AAV6 capsid proteins to create an AAV2/6 viral vector. Transgene expression was driven by a synapsin-1 promoter and enhanced using a woodchuck hepatitis virus posttranscriptional regulatory factor (WPRE). Viruses were titred by quantitative PCR (qPCR) using the following primers:

Forward 5' tcc ttg tat aaa tcc tgg ttg ctg 3'

Reverse 5' agc tga cag gtg gtg gca at 3'

The final viral titres for AAV2/6-αsyn and AAV2/6-GFP were $5.2x10^{13}$gc/ml and $5.0x10^{13}$gc/ml respectively.

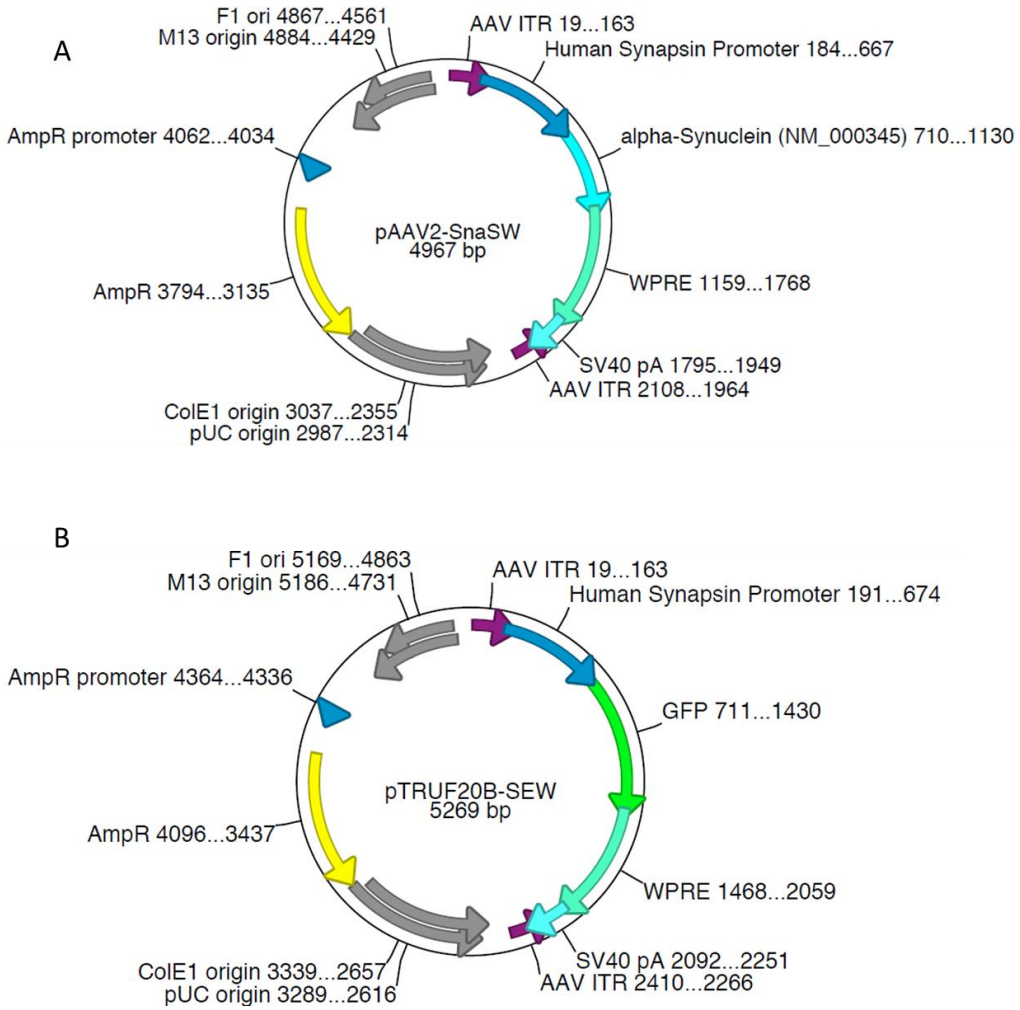

Figure 4.1 AAV2 plasmid encoding (A) human wildtype α-synuclein and (B) GFP control

4.2 Solution preparations

4.2.1 Amphetamine

A 1mg/ml stock solution of amphetamine was prepared by dissolving 30mg D-amphetamine sulphate (Sigma, Ireland) in 30mls sterile water. Animals were injected intraperitoneally (i.p.) with a 2.5mg/kg dose. Amphetamine is imported, stored and used under licence 5/230-1-2013 issued annually by the Healthcare and Products Regulatory Agency (HPRA).

4.2.2 Lithium chloride (LiCl)

A 0.15M solution of lithium chloride (Sigma, Ireland) was prepared by dissolving 3.18g in 500ml of sterile water. Animals received an i.p. dose at 2% of their bodyweight. This dose is based on previously published literature (Inui et al., 2013).

4.2.3 5-Bromo-2'-deoxyuridine (BrdU)

A 10mg/ml stock solution of BrdU was prepared by dissolving 750mg BrdU (Sigma, Ireland) in 75mls sterile water. Animals were injected i.p. with 75mg/kg in 4 doses, each 2h apart.

4.2.4 Phosphate buffered saline (PBS)

A solution containing NaCl (162 mM), Na_2HPO_4 (16.2 mM), $Na_2H_2PO_4$ (3.8 mM) (all Sigma, Ireland) was prepared in dH_2O and adjusted to a pH of 7.2-7.4.

4.2.5 Phosphate buffer solution (PB)

A solution containing $NaH_2PO_4.H_2O$ (0.2M) and $Na_2HPO_4.2H_2O$ (0.2M) was prepared in dH_2O and adjusted to a pH of 7.2-7.4.

4.2.6 Paraformaldehyde (PFA)

PFA (160 g) (Sigma, Ireland) was dissolved in 1500 ml of distilled H_2O (d H_2O) with continuous stirring on a heated stirrer. Once the solution reached 60-65˚C, 1 M NaOH was added drop-wise until the solution cleared. The solution was brought to 2 L with dH_2O, diluted 1:1 with 0.2M PBS to give a final concentration of 4% and adjusted to pH 7.4 before being cooled to 4˚C.

4.3 Animal work

4.3.1 Animal husbandry

Rats were maintained on a 12h:12h light: dark cycle (lights on at 08.00h) under regulated temperature (21±2°C) and humidity (30-50%). Standard rat chow and water were available ad libitum, unless behavioural testing dictated otherwise temporarily. Animals were group- housed 2-3 per cage in either standard housing conditions or cages with access to custom-designed running wheels (Activity Wheel, Tecniplast, UK) unless behavioural testing dictated otherwise temporarily. All experiments were conducted in accordance with the European Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory Authority Ireland (HPRA; AE19130/P010) and approved by the Animal Ethics Committee of University College Cork (Approval number 2013/030).

4.3.2 Stereotaxic surgery

All surgery for the characterisation study was conducted under general anaesthesia induced by an i.p. injection of ketamine and xylazine (80mg/kg and 8mg/kg respectively, Zoetis Ireland Ltd and Vetoquinol Ltd, UK) in sterile 0.9% NaCl. Due to a high mortality rate with injectable anaesthesia and on veterinary advice, all surgery in the running study was conducted under general anaesthesia induced by inhaled isoflurane (Virbac, Ireland).

Animals were placed in a stereotaxic frame (Kopf Instruments) and an incision was made through the skin over the skull and the skull was exposed. Following the location of bregma and the coordinates of the target injection site a drill was used to expose dura. Animals that received a unilateral injection were administered with 3µl (3.1 x 10^8 gc/3µl) of the appropriate solution into the right SN at co-ordinates AP -5.3, ML -2.0 and DV -7.2 relative to bregma. Animals that received bilateral injections were administered with 3 µl (1.5 x 10^8gc/3µl for study 1, 3.1 x 10^9gc/3µl for study 2) of the appropriate solution into each of the left and right SN at co-ordinates AP -5.3, ML ±2.0 and DV -7.2 relative to bregma. All solutions were infused at a rate of 1 µl/min, with an additional two minutes allowed for diffusion before the needle was withdrawn. Following injection, the incision was sutured and the rats were allowed to recover on a heating mat before returning to their home cage. Animals were also administered with the analgesic carprofen (Rimadyl® 5mg/kg, s.c., Zoetis Ireland Ltd) and glucose solution (5% in sterile saline) immediately following the procedure. Animals that received BrdU

injection as a measure of cell survival were administered with 75mg/kg in 4 doses, each 2h apart one week following surgery.

4.3.3 Tissue processing

At the appropriate time-points following surgery, animals to be sacrificed for immunohistochemistry were deeply anaesthetised with sodium pentobarbital (100 mg/kg i.p, Merial Animal Health, UK) and transcardially perfused with 100 ml ice-cold heparinised saline (1000IU/l) (Wockhardt, UK) followed by 150 ml of 4% paraformaldehyde (pH 7.4). The brains were removed and placed into 4% paraformaldehyde overnight for post-fixation prior to equilibration in 25% sucrose for a minimum of 48h. Brains were then frozen and stored at -80°C before being sectioned on a cryostat (Leica, UK). Coronal sections were collected at 40µm thickness in a 1:6 series. For analysis other than immunohistochemistry, animals were decapitated, brains were removed and the required brain regions were dissected out and immediately frozen on dry ice.

4.4 Immunohistochemistry

Endogenous peroxidase activity was quenched by incubating the tissue in a solution of 30% hydrogen peroxide/methanol in distilled water. Non-specific secondary antibody binding was blocked using 3% normal goat serum (NGS) in a solution of phosphate-buffered saline (PBS) with 0.1% Triton-X 100,

adjusted to pH 7.4. Sections were incubated overnight at room temperature with appropriate antibody

(mouse anti-α-synuclein 1:1000, Merck Millipore; mouse anti-TH 1:1000 Merck Millipore) in 1% NGS in PBS with 0.1% Triton-X 100. The sections were then incubated with the appropriate biotinylated secondary antibody (goat anti-mouse Vectastain peroxidase ABC kit, Vector, UK at 1:200 dilution) in PBS for 3 h, followed by incubation in a streptavidin-biotin horseradish peroxidase solution (Vector, UK). Immunoreactivity was visualised by incubating the sections with a 0.5% 3,3-diaminobenzidine tetrachloride (DAB) solution (containing PBS and hydrogen peroxide 30%) for 5 min. Sections were then washed in PBS, mounted on gelatine-coated microscope slides, dehydrated in an ascending series of alcohols (50%, 70% and 100%), cleared in histolene and cover-slipped using DPX mountant (Sigma, UK).

4.5 Immunofluorescence

Non-specific secondary antibody binding was blocked using 3% normal donkey serum (NDS) in a solution of PBS with 0.3% Triton–X100. Tissue sections were incubated overnight (or 48h in the case of doublecortin (DCX)) in the appropriate antibody (see Table 4.1) in 1% NDS in PBS with 0.3% Triton X-100. Sections were then incubated with the appropriate secondary antibodies (see Table 4.2) at 1:200 dilutions in 1% NDS in PBS, and incubated with bisbenzamide (1:5000) for nuclear staining, before being coverslipped (Dako fluorescent mounting media, Dako, UK).

Target	Antibody	Company	Source	Dilution
Dopaminergic neurons	Anti -tyrosine hydroxylase	Merck Millipore, Ireland	Polyclonal rabbit	1:250
Microglia	Anti -Iba1	Wako	Polyclonal rabbit	1:500
α-synuclein	Anti-α-synuclein	Merck Millipore, Ireland	Monoclonal mouse	1:250
GFP	Anti-GFP	Fisher Scientific, Ireland	Polyclonal rabbit	1:250
Immature neurons	Anti-DCX	Santa Cruz, USA	Polyclonal goat	1:100
Neuronal survival	Anti-BrdU	Abcam, UK	Monoclonal rat	1:250
Mature neurons	Anti-NeuN	Merck Millipore, Ireland	Monoclonal mouse	1:100

Table 4.1. Primary antibodies used for immunofluorescence

Secondary antibody	Company	Dilution
AlexaFluor 488-conjugated donkey anti-mouse	Invitrogen, UK	1:500
AlexaFluor 594-conjugated donkey anti-rat	Invitrogen, UK	1:500
AlexaFluor 594-conjugated donkey anti-rabbit	Invitrogen, UK	1:500
AlexaFluor 488-conjugated donkey anti-goat	Invitrogen, UK	1:500
AlexaFluor 488-conjugated donkey anti-rabbit	Invitrogen, UK	1:500

Table 4.2. Secondary antibodies used for immunofluorescence

4.6 Behavioural testing

4.6.1 Motor testing

4.6.1.1 Corridor test

The corridor test of contralateral neglect was carried out as previously described (Dowd et al., 2005). Animals were maintained at 90% of their free-feeding weight for the duration of the test protocol, and were given a small number of food rewards in their cages each day.

On day 1, animals were habituated to the empty apparatus for 5 min. On the next day, food rewards (Coco-pops®) were scattered over the floor and animals were free to explore and collect the food rewards for 5 min. On day 3, animals were first placed into an empty corridor for 5 min before being moved to the second corridor for 5 min, which had food rewards placed into small pots that were located on either side of the corridor. Testing occurred on day 4, and animals were placed into an empty corridor for 5 min of habituation, before being moved to the "test" corridor where each pot contained one food reward. The test concluded when 5 min had elapsed or 20 rewards had been taken. Data were expressed as the number of contralateral retrievals as a percentage of the total retrievals made.

4.6.1.2 Cylinder test

The cylinder test was carried out as previously described (Schallert et al., 2000). Animals were placed in a clear, glass cylinder and the first 20 forepaw touches were analysed. The cylinder was cleaned with 70% ethanol between

trials. Data were expressed as contralateral touches as a percentage of total touches made.

4.6.1.3 Stepping test

The stepping test of forelimb akinesia was performed as described elsewhere (Olsson et al., 1995). Animals were habituated over a number of days to being held with both hindlimbs restrained. On days 1 and 2, animals were held with both hindlimbs restrained and their backs straight with forepaws resting on the countertop. Once the animals were comfortable, they were gently moved forward and backward along the counter in a "wheelbarrow" type motion. On days 3 and 4 of habituation, both hindlimbs and one forelimb were restrained with the remaining free forelimb resting on the countertop. Each animal was then moved both forward and backward over the countertop while making adjusting steps with the free forelimb, and this was repeated until each animal could be held comfortably and reached a stable baseline performance. On the day of testing, both hindlimbs and one forelimb were gently restrained, and the animal was held with the remaining forelimb placed onto the countertop. The animal was then moved sideways across the countertop at a steady pace (90cm in 5s) and the numbers of adjusting steps taken with the unrestrained forelimb over both directions were counted.

4.6.1.4 Rotarod performance

Animals that had received bilateral injections of viral vectors were tested for motor performance using a protocol on the Rotarod apparatus, as described elsewhere (Rozas et al., 1997). Briefly, 24h before testing, animals were trained on the rotarod until they could remain on the apparatus for 120s without falling. On the day of testing, animals were placed on the rotarod and tested on the length of time they were able to stay on the apparatus at increasing speeds (5-40rpm) for a maximum of 300s was recorded.

In the second animal study, a more comprehensive Rotarod protocol was utilised in order to reduce variability that was seen in the first experiment. The protocol (adapted from Marei et al., 2015), consisted of 3 training sessions, each containing three trials of 120s. On day 1, rats were trained at 5rpm. On day 2, rats were trained in the morning at 10rpm, and in the afternoon at 15rpm. Testing took place on day 3, where rats completed 3 trials of 180s at 15rpm. Latency to fall and frequency of falls in the first 60s were measured for all animals. Rats were allowed at least 3min between trials to combat stress and fatigue.

4.6.1.5 Open Field test

Animals were placed in a white, round arena (90cm in diameter) and recorded for 10 min. Motor activity including velocity, distance travelled and time spent in the border zone (thigmotaxis) were measured using Ethovision

XT software (Noldus Information Technology, Noldus, USA). Apparatus was cleaned with 70% ethanol between each test.

4.6.2 Non-motor testing

4.6.2.1 Olfactory discrimination

The olfactory discrimination protocol was adapted from Tadaiesky *et al* (2008). Briefly, a cage (45cm long x 28cm wide x 20cm high) was divided into two equal compartments with an opening to allow free movement of the animal - one side contained each individual animal's used bedding and the other side contained fresh bedding. The animal was put into the fresh bedding compartment at the start of the trial. The time spent in each compartment was recorded over a period of 5 min. The total number of entries into both compartments was also recorded

4.6.2.2 Spontaneous alternations in the Y maze

Working memory was measured using spontaneous alternations in a Y maze, as described previously (Senechal et al., 2007). The Y maze apparatus was black, and consisted of 3 arms, positioned 120° from each other. Each arm was 10cm wide, 20cm high and 40cm long (outside length). Briefly, the animal was placed in a Y-shaped apparatus for one trial lasting 5 min. The animal was placed in the same arm at the beginning of each trial, and allowed to freely explore for the duration of the trial. A spontaneous alternation was defined as entry into all three arms on consecutive choices.

Data were expressed as the percentage of alternation, which was calculated by:

Number of alternations/ (number of entries-2) *100

4.6.2.3 Conditioned Taste Aversion

The conditioned taste aversion test was carried out as described previously, utilising lithium to induce nausea and malaise in animals to develop a taste aversion (Schramm-Sapyta et al., 2011). Prior to testing, animals were given a continuous 48 h exposure to two bottles in the home cage, each containing either tap water or 0.2% sucrose solution. The starting location of the bottles was counterbalanced, and after 24 h, half of the bottles were randomly switched to avoid development of a location bias.

On day 1 of the protocol, animals were water-deprived for 24 h. They were then allowed 15 min access to a bottle containing water, and the water consumption was measured. Animals were then allowed free access to water for 24 h. The next day (day 3), animals were water-deprived again for 24 h before being allowed 15 min access to a 0.2% sucrose solution. Sucrose consumption was measured and the animals were injected with either 0.9% NaCl solution or 0.15M LiCl (i.p.) at 2% of their bodyweight (Inui et al., 2013) before being allowed free access to drinking water. The next day (day 5), animals were again water-deprived for 24 h. On the final day (day 6), animals were allowed access to two water bottles for 15min, containing either water or sucrose solution and the consumption of each was measured.

The percentage preference for the sugar solution was calculated as follows:

(sucrose solution consumed mls/ (total water and sucrose solution consumed mls) *100

4.6.2.4 Social recognition

Short-term social memory was assessed using the social recognition protocol adapted from Tadaeisky *et al* (2008). Adult rats were individually housed for 3 days prior to testing. On the day of testing, a juvenile rat was put in the cage with an adult rat and behaviour was recorded for 5 min. After a 30 min interval, the same juvenile rat was put back in the cage with the adult rat and behaviour was recorded for 5 min. Adult rats were scored for social interaction that included sniffing, touching and grooming of the juvenile rat. The discrimination ratio was calculated as:

Novel Exploration/ (Novel+Familiar Exploration)

4.6.2.5 Discrete alternations in the Y-maze

Spatial reference memory was assessed using the discrete alternations protocol, which was adapted from Deacon & Rawlins (2006) and carried out in a Y-maze apparatus (dimensions 10cm high, 20cm wide and 40cm long). In order to train the animals to enter the open arm of the maze apparatus and retrieve a food pellet, they were first habituated to the apparatus. On day 1, each cage of animals was placed into the Y-maze for four 3-min

sessions, with all doors open and each arm containing a pot loaded with sugar pellets. On day 2, one arm was closed off and each animal was allowed to enter from the starting arm to the open arm to collect the food reward, with an equal number of left and right entries being completed. When all animals freely entered into the open arm to collect the food pellet (generally within 3 days) they were deemed trained and ready for the full test protocol. On the testing day, after each animal had collected the food pellet from the open arm, they were returned to the starting arm. The animal was turned to face the wall, and the door to the closed arm was lifted. The animal then either chose the “new” arm with a food reward, or the “familiar” arm which they had already entered. Correct arm entries were counted and results were presented as a percentage of all trials completed. The protocol was run again the next day, but to increase cognitive load a 1 min delay was added between the animal retrieving the first food pellet in the open arm and returning the animal back to the start arm with the closed arm lifted. The animal was returned to the home cage for this 1 min interval. The apparatus was cleaned with 70% ethanol between trials.

4.6.2.6 Elevated Plus Maze

Animals were assessed for anxiety-like behaviour using the elevated plus maze (Figure 4.2). Each animal was placed in the centre of the apparatus and allowed to explore for 5 min. Each trial was recorded and the number of arm entries and time spent in open and closed arms were manually scored.

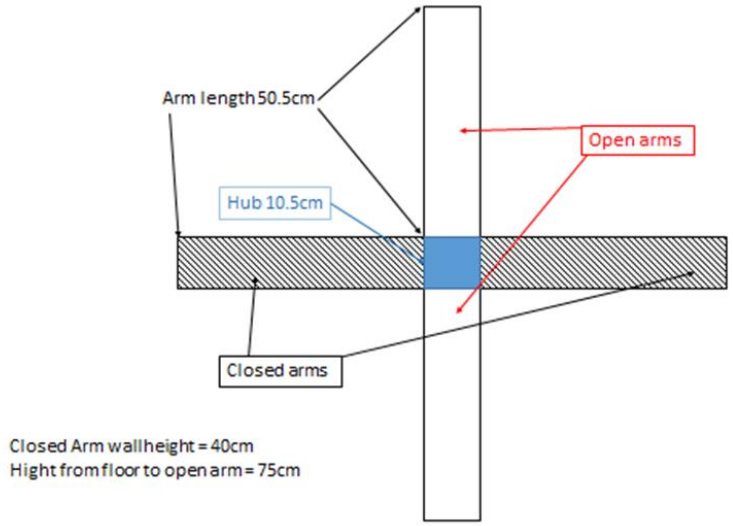

Figure 4.2. Elevated plus maze apparatus and dimensions

4.6.2.7 Modified Spontaneous Location Recognition Task

Pattern separation was assessed using the spontaneous location recognition task, previously described by Bekinschtein et al., (2013). The test was carried out in an open field arena (90cm in diameter), covered with bedding under dim lighting conditions. The testing room had three proximal cues and distal standard furniture. Behaviour was recorded using a camera suspended from the ceiling. All bedding was replaced, and the objects and the arena were cleaned with 70% ethanol between trials to remove odour cues.

Animals were habituated to the empty open field arena for 10 min per day for 5 consecutive days. After this habituation period, the testing protocol began. During the acquisition phase, animals were placed in the arena for 10 min and allowed to explore three identical objects, placed 15cm from the edge of the arena and 30cm from the centre. For the small separation paradigm, two of the objects (A2 and A3) were separated by a 50° angle, and the third (A1) at an equal distance between the two. For the large separation paradigm, the three objects were separated by 120° angles (see figure 4.3). Glass beer bottles (with labels removed) and soda cans were used as objects, and were affixed to the floor with Blu-tac. Twenty-four hours after acquisition, animals were presented with two identical copies of the previously-used objects, one (A4) placed in the same position as A1, and the second (A5) placed halfway between the

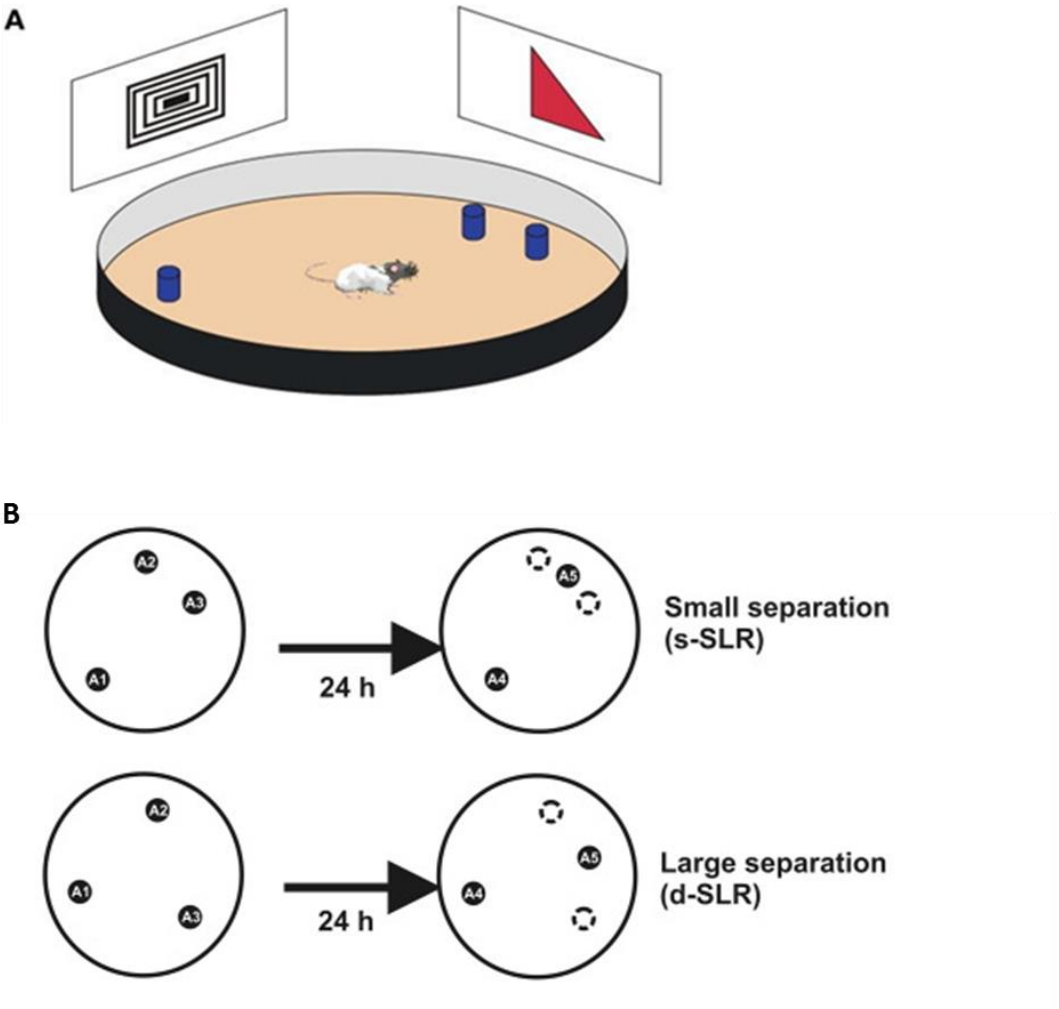

Figure 4.3. Modified spontaneous location recognition task apparatus set-up (A) and object location (B) for small and large separation paradigms. Taken from Bekinschtein et al 2014 and Bekinschtein et al 2013.

acquisition locations of A2 and A3. Animals were allowed to explore the objects for 5 min. Both objects and locations were counterbalanced across all groups. The times each animal spent exploring the object in the novel location (A5) and the familiar location (A4) were recorded and a discrimination ratio was calculated by:

Novel Exploration/ (Novel+Familiar Exploration)

4.7 Imaging and image analysis

Photomicrographs were taken on an Olympus BX53 microscope using Cellsens software package. Confocal photomicrographs were taken on an Olympus FV1000 confocal laser scanning biological microscope using the Olympus FV-10 ASW viewing software package. Images were analysed using ImageJ software (ImageJ v.1.51j8, National Institute of Health, USA).

The fluorescence intensity of TH-positive immunostaining in three coronal sections throughout the striatum was measured using ImageJ software. The mean pixel intensity was analysed in three fields of view per section and was expressed relative to a field through the unstained cortical tissue in each section to control for background fluorescence. Fluorescence intensity was analysed in the right hemisphere of each animal that received a bilateral injection. For animals that received a unilateral injection, the ipsilateral hemisphere was expressed as a percentage of the contralateral hemisphere. For cell quantification in the SN, cells positive for α-synuclein and TH were counted in a 1:6 series. For cell quantification in the dentate gyrus, cells positive for BrdU and NeuN were counted in a 1:6 series, and cells positive for DCX were counted in a 1:12 series. Dorsal co-ordinates for the dentate gyrus were set at -1.8 to -5.2 relative to bregma, and ventral co-ordinates from -5.2 to -6.7 relative to bregma based on previous experience within our group.

4.8 Statistical analysis

All raw data were analysed using the statistical package Statistica (Statistica, US) and graphed using the software GraphPad Prism v5 (GraphPad software, US). Data are expressed as means ± the standard error of the mean (SEM). Statistical analysis was carried out using a one-way analysis of variance (ANOVA) where three or more groups were being compared, repeated measures ANOVA where multiple time-points were being compared or factorial ANOVA where more than one factor was being analysed. This was followed by a *post-hoc* Fisher's LSD test or Dunnett's test if the ANOVA indicated significance. An unpaired Student's t-test was used where only two groups were compared. Differences were considered significant at $p<0.05$.

Chapter 5

5.0 Characterisation of neuropathological, motor and non-motor symptoms in an α-synuclein rat model of Parkinson's disease

5.1 Abstract

The AAV-α-synuclein rat model is the only animal model to date that has been shown to robustly and consistently reproduce the primary neuropathological and behavioural features of PD. However, there has been little research on the ability of the model to replicate NMS of the disease. Moreover, this model is most commonly employed unilaterally, which can confound cognitive testing due to contralateral functional compensation. Thus, the aims of this study were to investigate differences between the effects of unilateral and bilateral administration of AAV-α-synuclein into the rat SN, with regards to both motor and NMS of PD. We found that unilateral and bilateral administration of AAV-α-synuclein induced distinct patterns of nigrostriatal degeneration and associated motor dysfunction. Overexpression of AAV-α-synuclein was used to model NMS associated with PD, including deficits in hippocampal-associated tasks. This was coupled with α-synuclein-positive immunostaining in the dentate gyrus of the hippocampus, confirming the propagation of the protein throughout distinct regions of the brain.

5.2 Introduction

For many years, PD was primarily viewed as a motor disorder, however growing evidence supports the fact that a wide range of NMS are inherent to the disease process. Although there are several experimental animal models of PD currently in use, the recent development of the AAV-α-synuclein overexpression model has been shown to be the most representative of the human disease, including replicating the progressive nature of the model as well as the formation of α-synuclein aggregates. Despite this, relatively little work has focused on the ability of this model to reproduce the NMS of PD, or the link between the propagation of α-synuclein throughout the brain and the presence of NMS. Moreover, the majority of the research to date has employed the model as a hemiparkinsonian model; namely that the viral vector is administered unilaterally, which allows the contralateral hemisphere of the same brain to be used as a control in *post mortem* tissue analyses. Motor impairment is then assessed *in* vivo using well-characterised lateralised tasks including the stepping test (Olsson et al., 1995), the cylinder test (Schallert et al., 2000), the corridor test (Dowd et al., 2005) and apomorphine- or amphetamine-induced rotation tests (Ungerstedt and Arbuthnott, 1970). However, given that motor dysfunction in humans is generally not evident until approximately 30% of dopaminergic neurons in the SN are already damaged (reviewed by Burke and O'Malley, 2013), it is clear that significant compensatory mechanisms exist that allow for normal movement during the early phases of the disease. For example, axonal sprouting after striatal

denervation is well-documented in rodent models of PD and has been linked with behavioural recovery (reviewed by Arkadir et al., 2014; Zeng et al., 2012). Similarly, changes in the functional activation of specific brain regions, characterised by hypermetabolism on imaging studies, are also thought to play a role in compensation (reviewed by Gregory et al., 2017;Kordys et al., 2017). Moreover, recent work confirms not only the presence of cross-hemispheric dopamine projections, but also that these can functionally regulate dopamine release in the contralateral hemisphere (Fox et al., 2016). Taken together, it is evident that a number of compensatory mechanisms exist, and that they can significantly contribute to the pathogenesis of the disease. Additionally, it is well established that difficulties can arise when assessing cognitive deficits in lateralised models, and this is also thought to be due to contralateral compensation.

In light of this, the aims of this chapter are:

a) To identify whether the AAV-α-synuclein model can replicate the proliferation of α-synuclein through the brain
b) To interrogate the link between the presence of α-synuclein throughout the brain and the pathogenesis of NMS
c) To examine neuropathological and behavioural differences between unilaterally- and bilaterally-administered AAV-α-synuclein

To do this, animals received either unilateral or bilateral stereotaxic injections of AAV-α-synuclein or AAV-GFP (control) viral vectors. To replicate

the progressive and long-term nature of the human disease, animals were repeatedly assessed over a period of 40 weeks on a series of motor tasks that measured lateralised and bilateral motor dysfunction, as well a number of cognitive tasks that measured various types of learning and memory processing.

5.3 Experimental design

5.3.1 Animal husbandry

Adult male Sprague Dawley rats (Envigo, UK) were maintained on a 12h:12h light: dark cycle (lights on at 08.00h) under regulated temperature (21±2°C) and humidity (30-50%) and pair-housed. Standard rat chow and water were available *ad libitum*, unless behavioural testing dictated otherwise temporarily. All experiments were conducted in accordance with the European Directive 2010/63/EU, and under an authorisation issued by the Health Products Regulatory Authority Ireland (HPRA, AE19130/P010) and approved by the Animal Ethics Committee of University College Cork (approval number 2013/030).

5.3.2 Stereotaxic surgery

All surgery was conducted under general anaesthesia induced by an i.p. injection of ketamine and xylazine (80mg/kg and 8mg/kg respectively, Zoetis Ireland Ltd and Vetoquinol Ltd, UK) in sterile 0.9% NaCl. Briefly, animals were placed in a stereotaxic frame (Kopf Instruments) and an incision was made

through to the skull. Animals that received a unilateral injection were administered with 3µl (3.1 x 10^8 gc/3µl) of either AAV-α-synuclein or AAV-GFP into the right SN at co-ordinates AP -5.3, ML -2.0 and DV -7.2 relative to bregma. Animals that received bilateral injections were administered with 3 µl (1.5 x 10^8gc/3µl of either AAV-α-synuclein or AAV-GFP into each of the left and right SN at co-ordinates AP -5.3, ML ±2.0 and DV -7.2 relative to bregma. All solutions were infused at a rate of 1 µl/min, with an additional two minutes allowed for diffusion before the needle was withdrawn. Following injection, the incisions were sutured and rats were allowed to recover on a heating mat before returning to their home cages. Animals were administered the analgesic carprofen (Rimadyl® 5mg/kg, s.c., Zoetis Ireland Ltd) and 5% glucose solution immediately following the procedure. An additional cohort of intact control animals did not undergo surgery.

5.3.3 Experimental design

Animals were randomly allocated to one of the following experimental groups as outlined in Table 5.1: AAV-α-synuclein unilateral, AAV-α-synuclein bilateral, AAV-GFP unilateral, AAV-GFP bilateral and intact controls. Motor and cognitive tests were carried out over the time-course of the experiment as outlined in Figure 5.1, and animals were sacrificed at 20 weeks (Cohort 1) or 40 weeks (Cohort 2) post-surgery. Due to significant delays in acquiring ethics approval for this animal work, a pilot study was incorporated into the experimental design. Cohort 1 consisted of smaller groups as they were used

for non-quantitative analysis of transgene expression. Cohort 2 was adequately powered for all behavioural testing and subsequent *post mortem* analysis (n=8-10).

	Number of animals	**Number of animals**
Intact Control	4	8
AAV-GFP bilateral	6	8
AAV-αsyn bilateral	6	10
AAV-GFP unilateral	6	8
AAV-αsyn unilateral	6	10
	Cohort 1	**Cohort 2**
Total	**28**	**44**

Table 5.1 Experimental groups

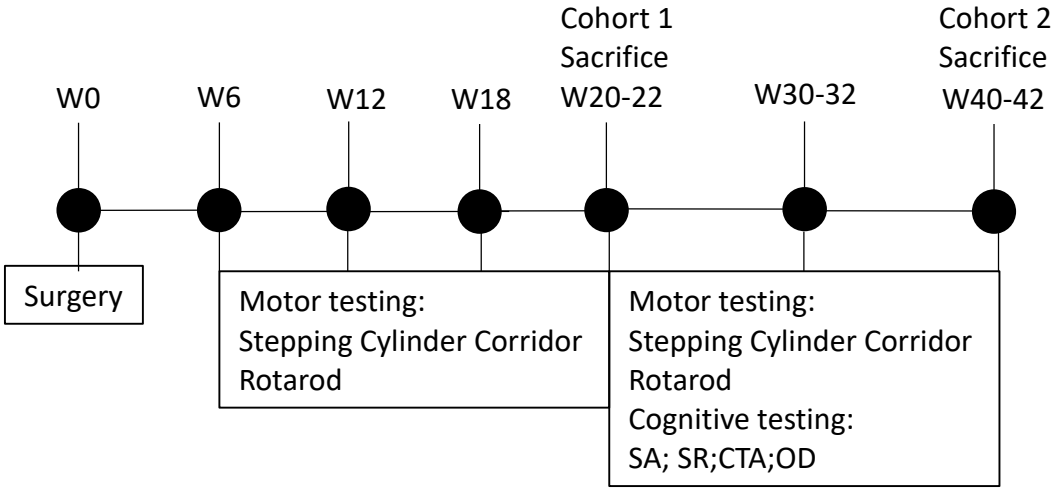

Figure 5.1 Experimental design. Abbreviations: SA = spontaneous alternations; SR = social recognition' CTA = conditioned taste aversion; OD = olfactory discrimination.

5.3.4 Motor testing

Given that lateralised tasks are only appropriate in hemiparkinsonian models, animals that received unilateral injections were assessed for motor dysfunction using the cylinder test, the corridor test, the stepping test and the amphetamine-induced rotation test. Animals that received bilateral injections were assessed for motor deficits on the rotarod. Tests were carried out as described previously (Section 4.6.1). Animals in cohort 1 were tested alongside cohort 2 for motor deficits until they were sacrificed at 20 weeks, and so testing results up to this time-point are cumulative (Cohorts 1 and 2).

5.3.5 Cognitive testing

Cognitive functioning was evaluated using conditioned taste aversion, olfactory discrimination, spontaneous alternations in the Y-maze and social recognition. Animals in cohort 1 were tested (with cohort 2) at the week 20 time-point before they were sacrificed. Results from weeks 30 and 40 post-surgery are those from cohort 2 only. All tests were carried out as described previously (Section 4.6.2).

5.4 Results

5.4.1 Administration of α-synuclein had no effect on body weight

There was no effect of administration of either AAV-α-synuclein or AAV-GFP on the body weight of animals in each group ($F[4,38] = 1.96$, $p = 0.11$; Figure

5.2), although all groups gained weight over the time course of the experiment ($F[4,152] = 141$, $p < 0.001$).

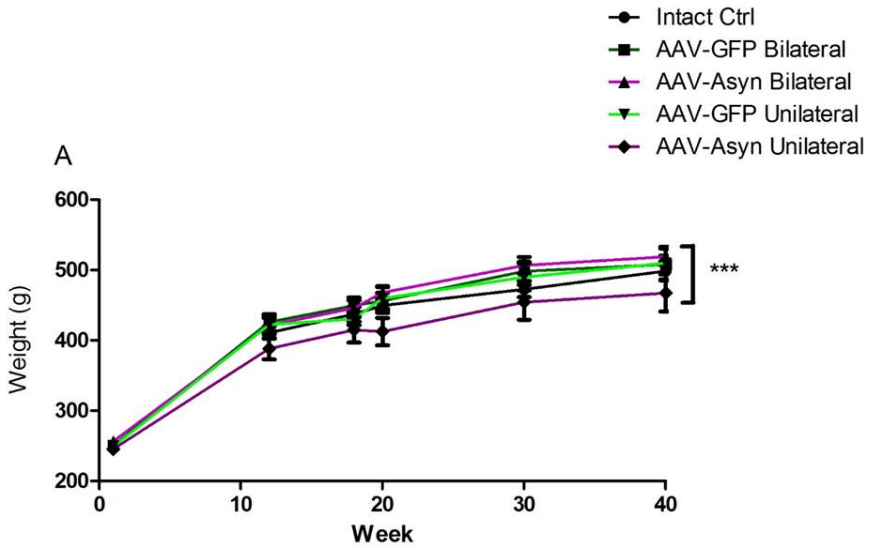

*Figure 5.2. Body weights in each of the treatment groups over the course of the experiment. Data are expressed as mean ± SEM and analysed using repeated measures ANOVA, ***p<0.001 vs week 0.*

Long-term overexpression of unilateral and bilateral AAV-α-synuclein induce distinct patterns of nigrostriatal degeneration

5.4.2 Overexpression of α-synuclein into the SN

Cohort 1 – 20 weeks post-surgery

Cohort 1 was culled at 20 weeks post-surgery to confirm viral transduction and α-synuclein expression. It is important to note that due to low n numbers the following data is not quantitative. Animals that received both unilateral and bilateral injections of AAV-α-synuclein demonstrated considerable α-synuclein expression in the SN (Figure 5.3). In animals injected unilaterally, this was accompanied by an apparent decrease in TH-positive immunostaining in the SN when compared to the intact contralateral hemisphere, although this was not quantified (Figure 5.4).

Cohort 2 – 40 weeks post-surgery

Immunofluorescent analysis of the number of TH+/α-synuclein+ cells in the SN demonstrated that both groups of animals that received AAV-α-synuclein displayed approximately 20% transduction of dopaminergic neurons in the SN (Figure 5.5A). There was no difference in the number of co-localised TH+/α-syn+ neurons between the groups ($t[3] = 0.21$, $p = 0.84$)

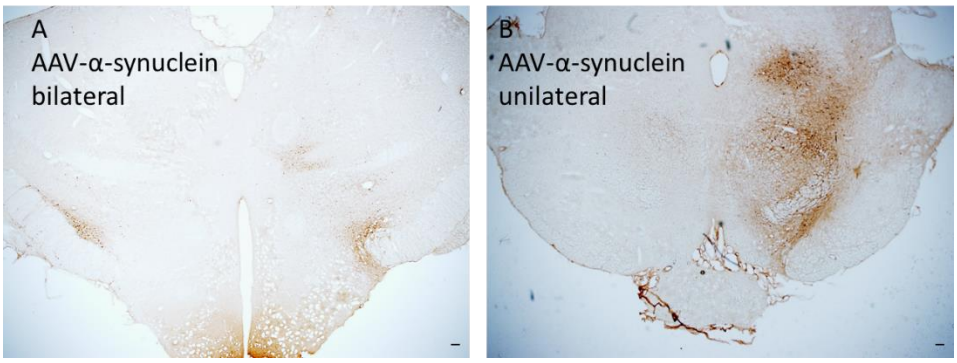

Figure 5.3 Representative photomicrographs of α-synuclein-positive immunostaining in cohort 1 animals administered (A) unilateral and (B) bilateral AAV-α-synuclein. Scale bar represents 10 µm.

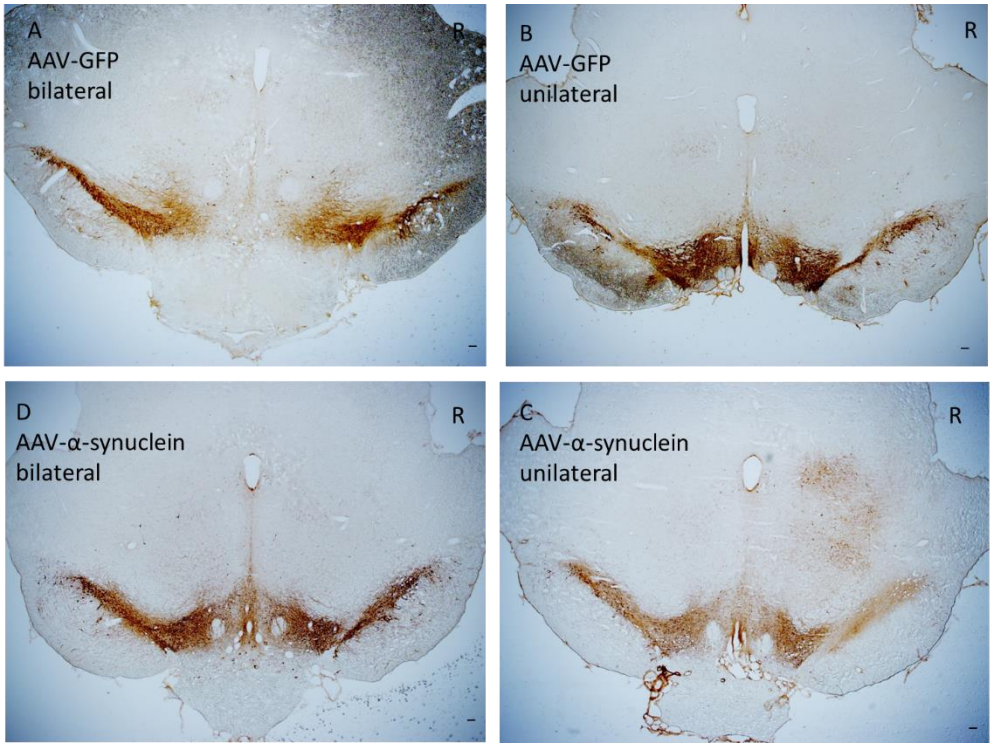

Figure 5.4. Representative photomicrographs of TH-positive immunostaining in cohort 1 animals administered with (A) bilateral AAV-GFP, (B) unilateral AAV-GFP, (C) bilateral AAV-α-synuclein and (D) unilateral AAV-α-synuclein in the SN. Scale bar represents 10 µm, R denotes the right hemisphere.

In animals that received unilateral AAV-GFP and AAV-α-synuclein, the number of TH-positive cells in the SN were expressed as a percentage of those in the intact contralateral hemisphere. There was no significant difference between groups ($F[2,5] = 3.4$, $p = 0.11$). However, *a priori* analysis revealed that overexpression of AAV-α-synuclein resulted in a significant reduction of TH-positive cells when compared to the intact control group ($p = 0.04$; Figure 5.5B). In animals that received bilateral AAV-GFP and AAV-α-synuclein, there was a trend towards a significant reduction in the total number of TH-positive cells when compared to controls ($F[2,7] = 3.77$, $p = 0.07$; Figure 5.5C). *A priori post hoc* analysis revealed that the trend is more likely due to an effect of the α-synuclein overexpression ($p = 0.07$) rather than GFP ($p = 0.12$).

One-way ANOVA indicated that the extent of TH-positive staining in the striatum was significantly different in groups that received unilateral AAV-GFP and AAV- α-synuclein ($F2,63] = 3.55$, $p = 0.03$; Figure 5.5D). *Post hoc* analysis revealed that this was due to differences between control animals and AAV-α-synuclein ($p = 0.04$). Unilateral administration of AAV-GFP induced a trend towards a significant reduction in TH-positive immunostaining in the striatum ($p = 0.052$). There was a significant difference in the intensity of TH-positive immunostaining in the striatum between intact controls and animals that received either bilateral AAV-GFP and AAV-α-synuclein ($F[2,74] = 7.48$, $p = 0.001$; Figure 5.5E). *Post hoc* analysis revealed a significant decrease in striatal staining intensity in both

AAV-GFP (p = 0.002) and AAV-α-synuclein (p = 0.002) groups compared to intact controls.

5.4.3 Unilateral intra-nigral administration of AAV-α-synuclein has differential effects on contralateral motor function

In the corridor test of contralateral neglect, overexpression of α-synuclein had no effect on the number of contralateral retrievals at any of the time-points examined (Group F[2,17] = 0.36, p = 0.7, Time [4,68] = 1.31, p = 0.27; Figure 5.6A). In the cylinder test of forelimb asymmetry, there were no significant differences in the number of contralateral touches between the groups (F[2,16] = 0.02, p = 0.97; Figure 5.6B), nor did the performance of the groups change over time (F[5,80] = 1.03, p = 0.4). In the stepping test measuring forelimb akinesia, there was no overall difference in the number of adjusting steps taken between the groups (Group F[2,20] = 1.5, p = 0.23; Figure 5.6C). However, the performance of the groups changed significantly over time (F[4,80] = 53.61, p = 0.0001). *A priori post hoc* analysis revealed that α-synuclein animals performed significantly worse than intact controls at week 23 post-surgery (p = 0.04).

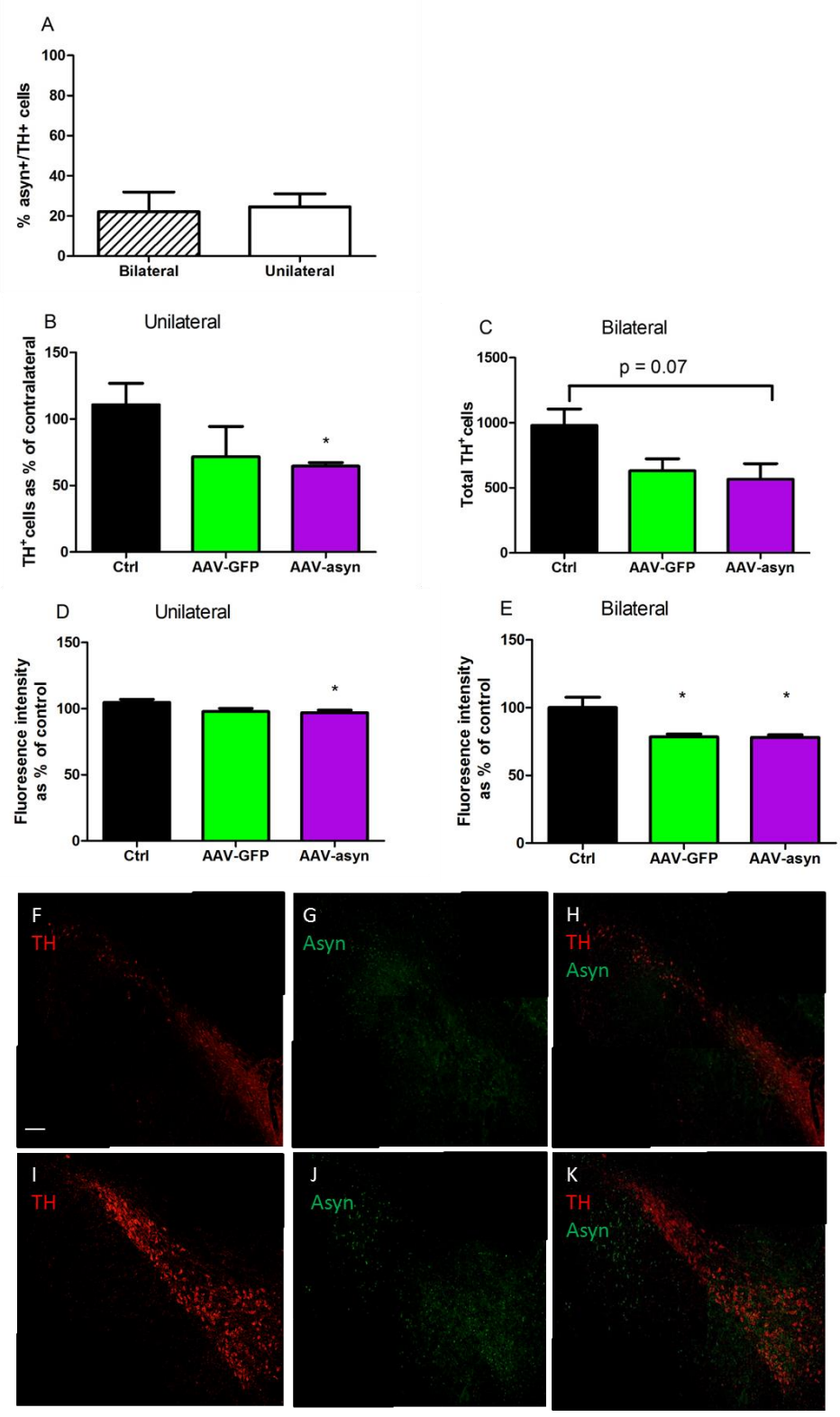

*Figure 5.5 (A) The percentage transduction efficiency of the AAV viral vector animals administered unilateral and bilateral AAV-α-synuclein. TH-positive cell counts in animals administered (B) unilateral and (C) bilateral injections. Fluorescence intensity of TH-positive immunostaining in the striatum in (D) unilateral and (E) bilateral groups. Representative images of TH-positive cells (F, I), α-synuclein-positive cells (G,J) and merged images (H,K) in the SN of groups injected with unilateral (F-H) and bilateral (I-K) AAV-α-synuclein. Scale bar represents 10μm. Data are expressed as mean ± SEM and analysed using one-way ANOVA with post hoc Dunnett's test, *p<0.05 vs intact controls.*

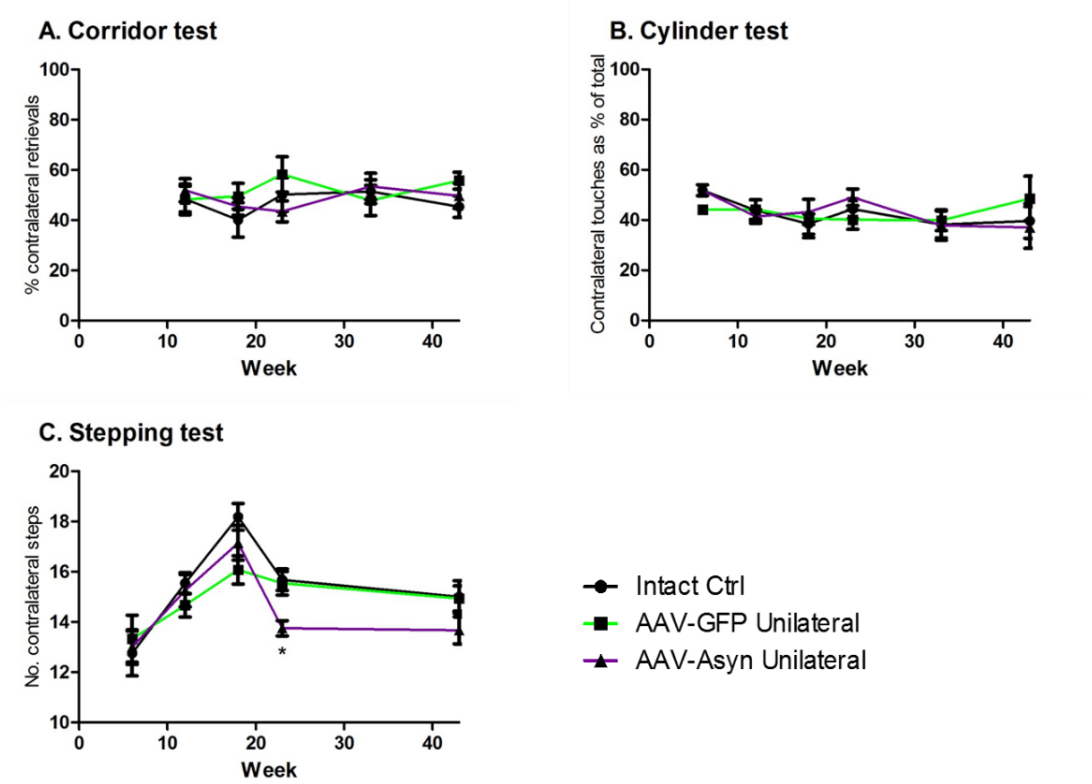

*Figure 5.6 Lateralised motor impairment measured in (A) the corridor test (B) the cylinder test and (C) the stepping test. Data are shown as mean ± SEM and analysed using 2-way repeated measures ANOVA and post hoc Fisher's LSD, *p<0.05 vs intact controls*

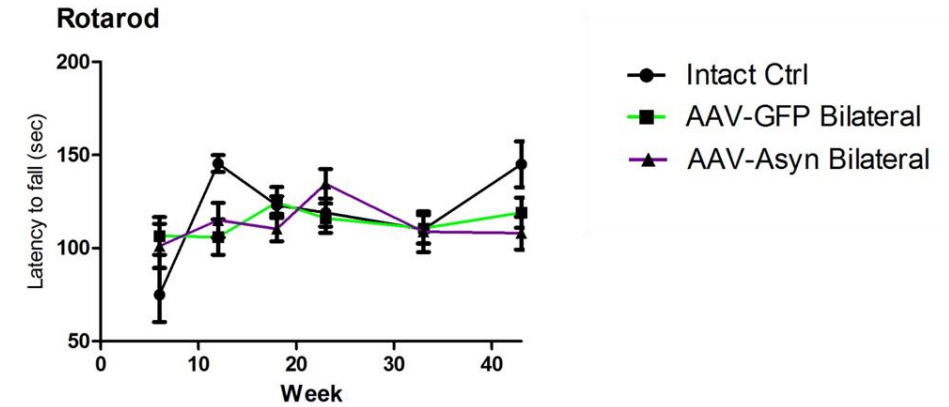

Figure 5.7 Latency to fall measured on the rotarod. Data are shown as mean ± SEM and analysed using 2-way repeated measures ANOVA.

5.4.4 Bilateral intra-nigral administration of AAV- αsynuclein does not affect sensorimotor function

There was no significant difference in the latency to fall off the rotarod between groups that had received bilateral injection of AAV-α-synuclein or AAV-GFP and the intact controls ($F[2,17] = 0.9$, $p = 0.42$; Figure 5.7). All groups performed better on the task over the course of the experiment ($F[5,85] = 9.5$, $p<0.0001$).

5.4.5 Propagation of α-synuclein

The patterns of AAV-mediated α-synuclein were analysed in distinct brain regions for both cohort 1 and cohort 2 (see Table 5.2). Again, it is important to note that due to small group sizes in cohort 1, this data is not quantitative. Specifically, α-synuclein-positive immunostaining was limited to the SN and VTA in cohort 1, which represents 20 weeks of AAV-α-synuclein expression. In cohort 2, 40 weeks post-surgery, α-synuclein-positive immunostaining was detected in the SN and VTA, as well as the dentate gyrus of the hippocampus (Figure 5.8). Interestingly, α-synuclein was expressed in the dentate gyrus of both left and right hemispheres, regardless of whether animals had been injected with AAV-α-synuclein unilaterally or bilaterally.

Brain region	SN	Hippocampus	Amygdala	Cortex	VTA
Cohort 1	+	-	-	-	+
Cohort 2	++	++	-	-	+

Table 5.2 Pattern of α-synuclein expression in distinct brain regions in cohorts 1 and 2. + denotes present and – denotes absent.

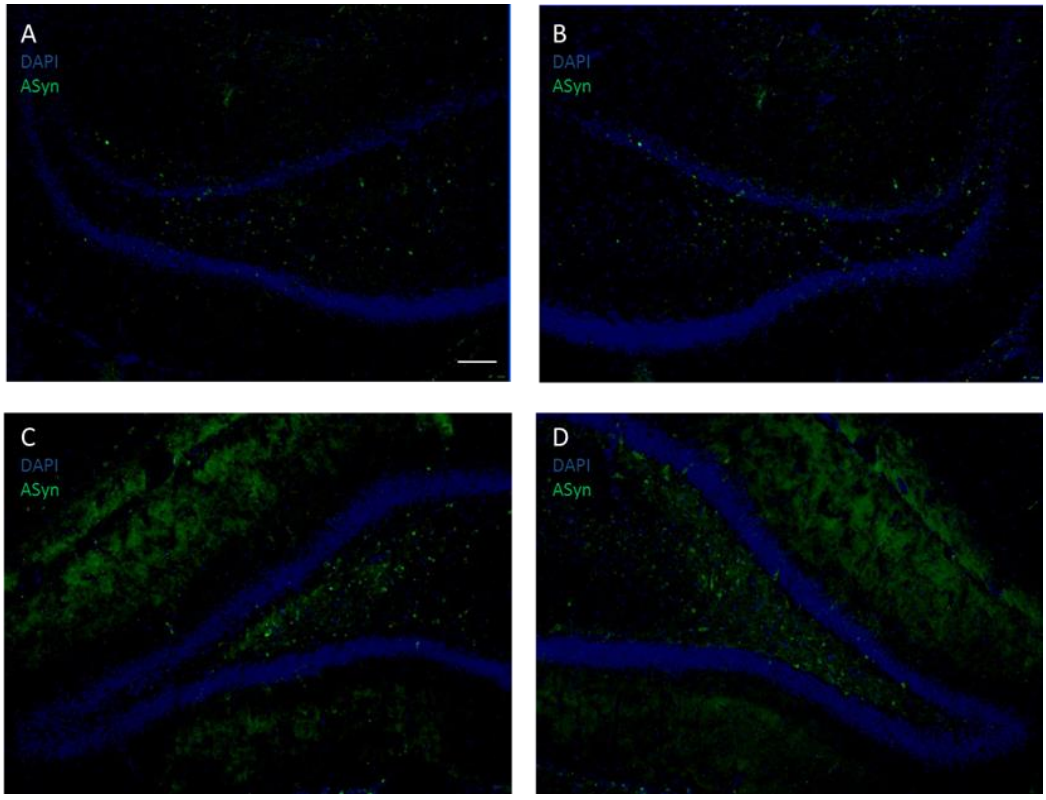

Figure 5.8 Representative photomicrographs of α-synuclein+ immunostaining in (A) the left and (B) the right dentate gyrus of unilateral-lesioned animals and (C) the left and (D) the right dentate gyrus of bilateral-lesioned animals. Scale bar represents 100 µm.

5.4.6 Spontaneous alternations in the Y-maze

There was no significant difference in the percentage of spontaneous alternations between the groups ($F[4,34] = 0.13$, $p = 0.96$; Figure 5.9A). However, the percentage of alternations performed by animals in each group significantly increased at each time-point over the experiment ($F[2,68] = 3.98$, $p = 0.02$). The total number of arm entries were also recorded for this task. There was no difference in the number of entries made by each group ($F[4,38] = 2.1$, $p = 0.08$; Figure 5.9B), although there was a significant decrease in the total number of entries made by all animals over time from week 20 ($F[2,76] = 30.13$, $p<0.0001$).

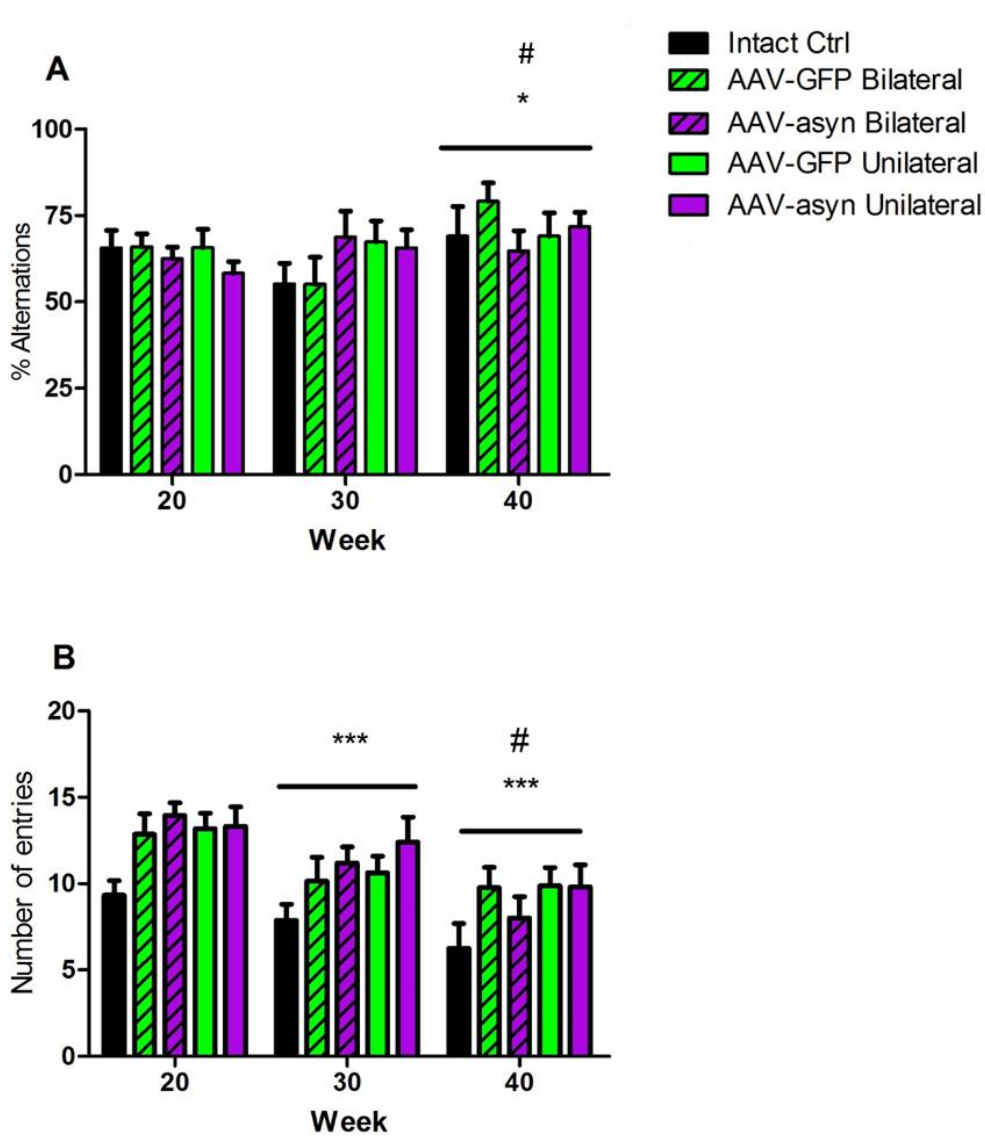

*Figure 5.9 (A) Percentage of alternations in a spontaneous alternations task. (B)The total number of arm entries completed during the task. Data are shown as mean ± SEM and analysed using 2-way repeated measures ANOVA and post hoc Fisher's LSD. ,*p<0.05, ***p<0.001 vs week 20, #p<0.05 vs week 30.*

5.4.7 Conditioned Taste Aversion

Prior to the beginning of this protocol, all animals were given continuous access to water and sucrose solution in the home cage. All groups showed a clear preference for the sucrose solution ($p<0.05$; Figure 5.10A). The difference in preference for sucrose between lithium- and saline-injected animals during the actual test protocol was used as a measure of the degree of aversion induced by lithium. Overexpression of AAV-αsynuclein did not affect an animal's ability to successfully develop a lithium-induced taste aversion, as animals in all groups, with the exception of those injected bilaterally with AAV-GFP, significantly decreased their consumption of sucrose in response to a lithium injection when compared to a saline injection ($p < 0.05$; Figure 5.10B). A one-way ANOVA indicated a significant difference in the percentage of sucrose consumption by animals in saline-injected groups ($F[4,17] = 5.72$, $p = 0.004$), and *post hoc* analysis revealed that animals that received bilateral AAV-GFP consumed significantly less sucrose in the test phase of the paradigm in comparison to intact controls ($p = 0.002$).

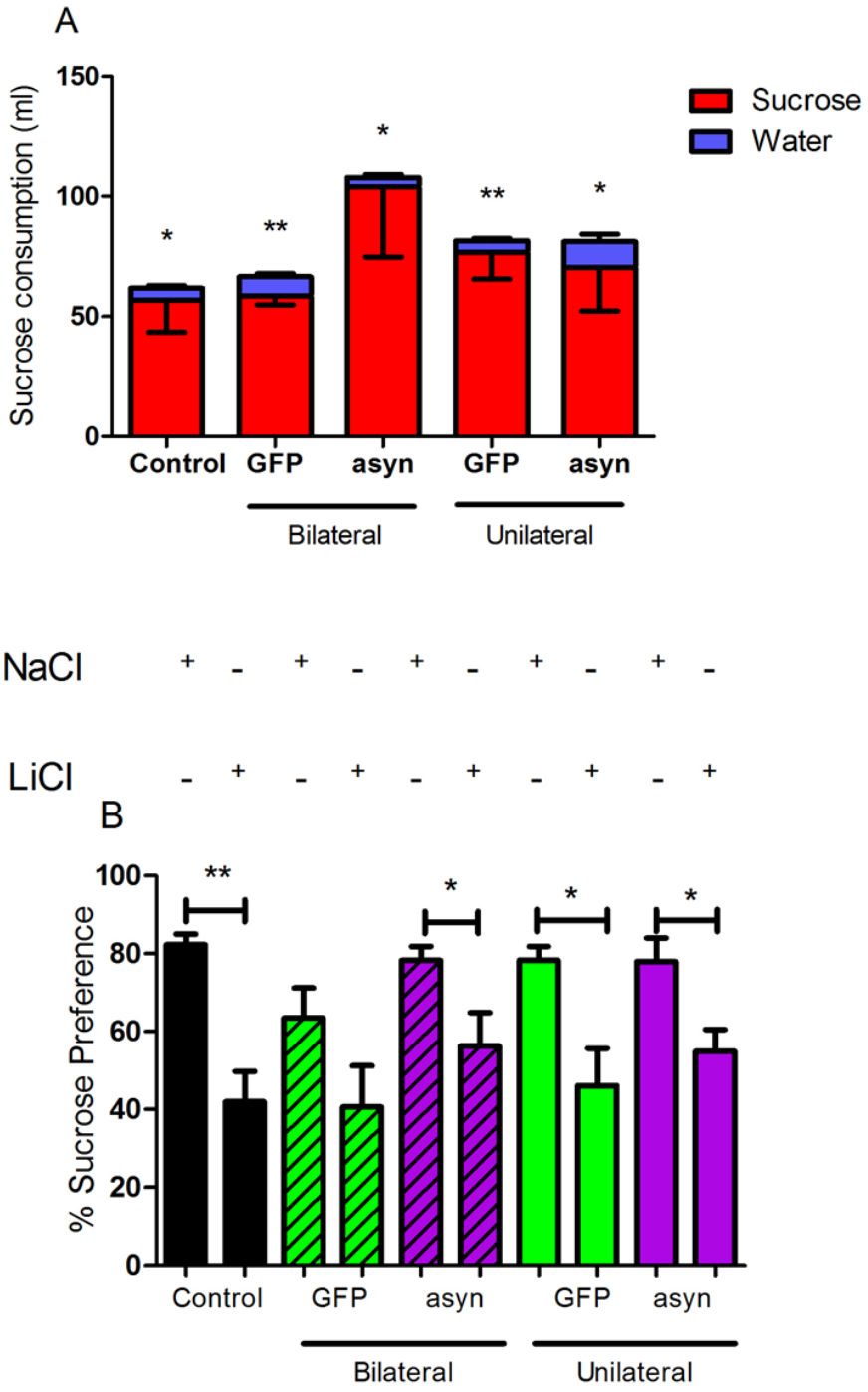

*Figure 5.10 (A) Sucrose and water consumption in all treatment groups. (B) Conditioned taste aversion induced by lithium injected measured in all groups. Data are shown as mean ± SEM and analysed using independent samples t-test. *p<0.05, **p<0.01.*

5.4.8 Olfactory discrimination

In the olfactory discrimination test, there was a significant difference in discrimination between groups regardless of the time-point ($F[4,39] = 4.3$, $p= 0.005$; figure 5.11A). *Post hoc* analysis revealed that the control group consistently spent more time exploring the novel compartment compared to every other group (Control vs AAV-GFP bilateral, AAV-GFP unilateral $p < 0.01$, Control vs AAV-α-synuclein bilateral, AAV-α-synuclein unilateral $p < 0.001$). There was a significant change in the performance of the groups at over each time-point ($F[2,78] = 3.4$, $p = 0.05$), and *post hoc* analysis revealed that all groups spent significantly more time exploring the novel compartment from between weeks 30 and 40 ($p<0.05$). The total number of entries into each compartment was also recorded. There was no difference in the number of entries made between all of the groups ($F[4,35] = 1.1$, $p = 0.37$; Figure 5.11B), however the number of entries decreased significantly over each time-point from week 20 ($F[2,70] = 8.4$, $p = 0.0005$). *Post hoc* analysis revealed that the number of entries made by animals in each group significant decreased from week 20 to week 30 ($p = 0.04$) and week 40 ($p<0.0001$) and again from week 30 to week 40 (0.005).

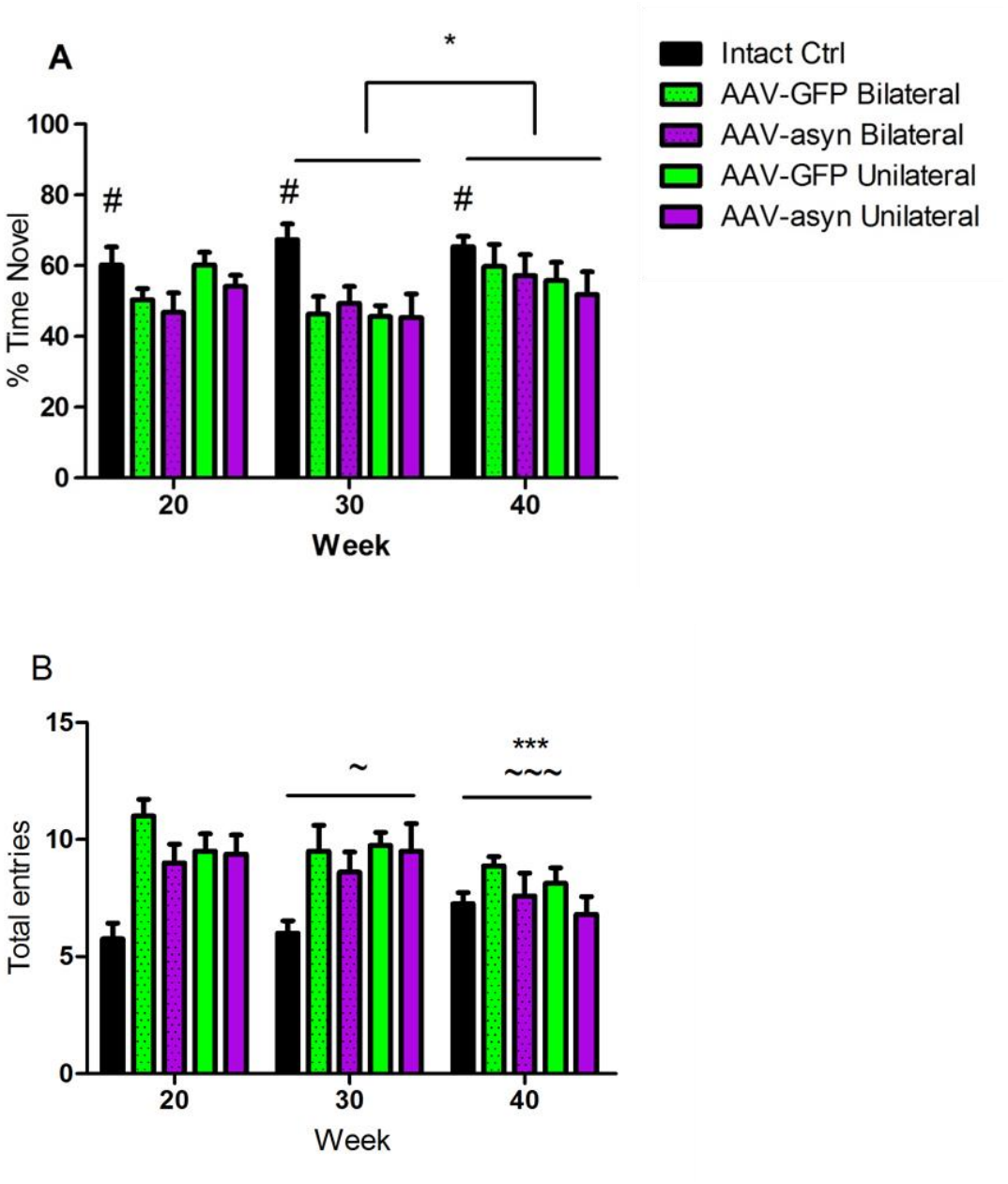

*Figure 5.11 (A)The percentage of time spent in the novel odour compartment in an olfactory discrimination task. (B) The number of entries into each compartment was recorded for all groups. Data are shown as mean ± SEM and analysed using 2-way repeated measures ANOVA and post hoc Fisher's LSD. #p<0.05 vs all other groups, *p<0.05 ***p<0.001 week 30 vs week 40, ~p<0.05 ~~~p<0.001 vs week 20.*

5.4.9 Social recognition

There was a significant difference in the ability of each group of animals to recognise a juvenile rat that they have previously been exposed to ($F[4,39] = 5.6$, $p = 0.001$; Figure 5.12). *Post hoc* analysis revealed that this was due to significant impairment in animals from both AAV-GFP unilateral ($p = 0.01$) and AAV-α-synuclein bilateral groups ($p = 0.004$) when compared to intact controls.

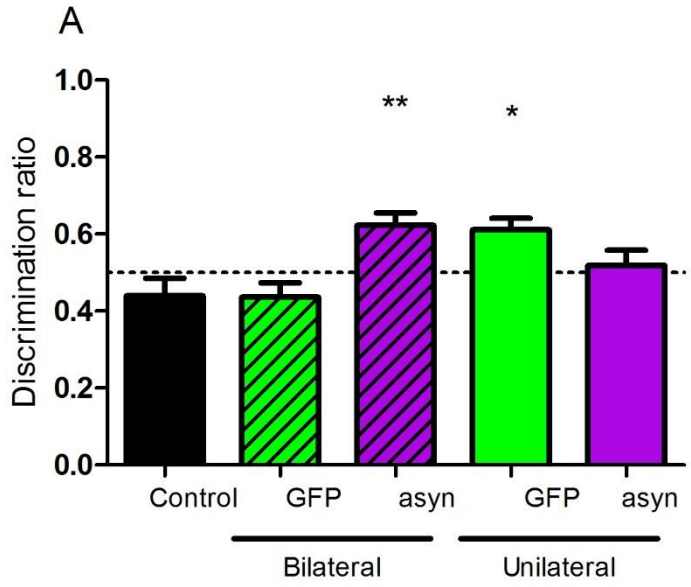

*Figure 5.11 A) Discrimination ratio from a social recognition task. Data are expressed as mean ± SEM and analysed using one-way ANOVA and post hoc Dunnett's test, *p<0.05, **p<0.01 vs intact control group.*

5.5 Discussion

The AAV-α-synuclein model is one of the few animal models of PD that can reproduce the pathological hallmark of the disease; α-synuclein-positive inclusions termed Lewy bodies. Although the model has been well-characterised in terms of neurochemical and neurobiological effects, as well as motor behaviours (Decressac et al., 2012), to date relatively little research has focused on the potential for this model to replicate NMS associated with PD. Moreover, the majority of the studies have employed unilaterally administered AAV-α-synuclein, which enables the contralateral hemisphere to act as a control in *post mortem* analyses. However, given that significant compensatory mechanisms exist, including the recent discovery of cross-hemispheric dopaminergic projections that can modulate dopamine transmission in the contralateral hemisphere (Fox et al., 2016), it is unclear what role these mechanisms may play in unilateral-lesion models. To our knowledge, to date there has been no study published that employed bilateral AAV- α-synuclein as a viable model of PD. Thus, the aims of this study were to employ the AAV-α-synuclein model to compare the neuropathological and behavioural effects induced by both unilateral and bilateral administration into the rat SN. Moreover, we investigated if long-term overexpression of both unilateral and bilateral AAV-α-synuclein could replicate a variety of NMS that are associated with PD.

To date, most of the published work using the AAV-α-synuclein model has also employed an AAV-GFP vector as a control. This doubles as both a control

for surgery and as a control for the expression of the AAV viral vector. However, the majority of these studies are designed to examine the acute effects of α-synuclein overexpression, and as such these experiments are typically no longer than 12-16 weeks in duration (Decressac et al., 2012; Lundblad et al., 2012; Mulcahy et al., 2013). The present study was designed to specifically investigate whether long-term overexpression of α-synuclein leads to propagation of the protein throughout the brain resulting in associated NMS, and so both AAV-α-synuclein and AAV-GFP were overexpressed in the SN for over 40 weeks. In reviewing the data from this chapter, a recurring concern that arose was the behavioural and pathological data from the animals that received the control AAV-GFP viral vector. Unilateral administration of AAV-GFP into the SN led to a marked decrease in the number of TH-positive neurons, while bilateral administration led to reductions in both the number of dopaminergic neurons and the intensity of striatal TH-positive immunostaining, at levels comparable to animals injected with AAV-α-synuclein. Similarly, the performance of animals that received AAV-GFP was worse than animals in the intact control group, both in the social recognition and the conditioned taste aversion tasks. Although the use of GFP as a viral vector control is well-established in *in* vivo studies, a comprehensive overview of the literature demonstrates that, in fact, the effects of AAV-GFP are highly variable. AAV-GFP overexpression has been shown to result in non-significant decreases in dopaminergic neurons in the SN of between 10 and 20% of that of the intact contralateral side (Decressac et al., 2012; Gombash et al., 2013; Gorbatyuk

et al., 2010; Kirik et al., 2003), as well as non-significant reductions in striatal TH-positive fibre density of up to 30% (Taschenberger et al., 2012) and non-significant effects on vesicular monoamine transporter (VMAT, a marker of dopamine transport)-positive neurons in the SN of 20% (Gaugler et al., 2012). Furthermore, overexpression of AAV-GFP has also been shown to increase the number of CD68+ and MHCII+ cells, both indicative of microglial activation (Sanchez-Guajardo et al., 2010). Despite these worrying trends, AAV-α-synuclein groups in these studies are only being compared to AAV-GFP and so any GFP-induced effects are not being adequately controlled for. An elegant study by Koprich and colleagues (2010) compared the AAV-α-synuclein and AAV-GFP vectors to an empty AAV vector, and reported that GFP resulted in a significant 37% reduction in TH-positive neurons in the SN (Koprich et al., 2010). Moreover, a recently published review article addresses concerns surrounding the use of GFP as a control in both *in vitro* and *in vivo* studies, and lists possible mechanisms by which GFP causes cell toxicity and immunogenicity (reviewed by Ansari et al., 2016). These include increased free radical oxygenation, activation of apoptotic pathways and enhanced cell permeability leading to cell death (Ansari et al., 2016). Despite this, the use of AAV-GFP remains the gold-standard for viral vector control in this field of research. However, for the purposes of this chapter, where possible we will directly compare AAV-α-synuclein to intact control animals to negate the possible toxicity of the AAV-GFP groups.

Moreover, based on the findings from this chapter, we planned an additional experiment to delineate both acute and long-term specific AAV-GFP effects

from that of intact control animals as well as animals injected with sterile 0.9% NaCl. Unfortunately, due to problems with the relocation of the animal facility in UCC, animals had to be sacrificed before the expected end of the experiment. Nevertheless, in the subsequent chapter of this thesis, sterile 0.9% NaCl will be used a control instead of AAV-GFP.

Leaving aside the problems surrounding the AAV-GFP vector, in this chapter we show that both unilateral and bilateral administration of AAV-α-synuclein resulted in the transduction of approximately 20% of dopaminergic neurons, and that both paradigms induce distinct patterns of nigrostriatal degeneration, and that the propagation of α-synuclein throughout the brain appears to be at least partially dependent on the duration of expression. Interestingly, there was no difference in the percentage of TH-positive cells transduced with the viral vector between animals that received either unilateral or bilateral injections, despite the difference in doses of vector administered. Unilateral administration of AAV-α-synuclein induced a significant reduction in the number of dopaminergic neurons in the SN and a significant, albeit moderate, decrease in the intensity of TH-positive immunostaining in the striatum. However, bilateral administration of the same vector resulted in a far more robust effect, inducing the loss of approximately 40% of nigral dopaminergic neurons as well as a more profound effect on striatal TH-positive immunostaining. Previously published studies using a similar unilaterally-administered AAV2/6 viral vector reported a reduction of approximately 80% in the number of TH-positive neurons in the SN and 60% decrease in the density of

striatal fibres (Decressac et al., 2012), and although in this study we used the same dose of vector we were unable to replicate the extent of nigrostriatal degeneration previously reported. Decressac and colleagues also demonstrated that a significant portion of the overexpressed α-synuclein was phosphorylated at serine 129 (Decressac et al., 2012). Phosphorylated α-synuclein is abundantly expressed in Lewy bodies (Anderson et al., 2006). Importantly, it is thought to potentiate the toxicity of α-synuclein (Gorbatyuk et al., 2008; Sato et al., 2011) and can modulate the formation of Lewy bodies (reviewed by Tenreiro et al., 2014). It may be that the differences seen between that study and the present study may be explained by alterations in the proportion of α-synuclein that is phosphorylated within the nigrostriatal system. Moreover, the recent discovery of cross-hemispheric dopamine projections that can modulate contralateral dopamine transmission (Fox et al., 2016) may contribute to the moderate reduction seen in striatal TH-positive fibres that we observed. Nevertheless, we have demonstrated that bilateral administration of AAV-α-synuclein reproduces the primary pathological features of PD and is a viable alternative to unilateral animal models of PD.

Due to the complexity of analysing gross motor function, a series of motor tests was carried out to accurately assess and identify any motor impairment across a wide range of parameters. Using this paradigm, we found that unilateral AAV-mediated overexpression of α-synuclein induced significant impairment in the stepping test only, but not in the cylinder test or the corridor test. Previous work investigating behavioural effects of distinct

degrees of lesions of the nigrostriatal system induced by striatal administration of 6-OHDA also found that animals that received partial lesions, induced by a lower dose of 6-OHDA at a single site as opposed to multiple injections, showed minor effects in the stepping test before other motor tests (Kirik et al., 1998). This substantiates our study which only demonstrated deficits in this task. Furthermore, our data is in line with previously a published study that used an AAV2/5 viral vector, which demonstrated that, despite significant nigral dopaminergic neuronal loss and decreased TH-immunoreactivity in the striatum, unilateral intra-nigral administration of AAV-α-synuclein was not sufficient to induce lateralised motor impairment (Naughton et al., 2017). Animals displayed significant motor deficits in the stepping test as well as the cylinder and whisker tests only when administration of AAV2/5- α-synuclein was combined with administration of the organic pesticide rotenone, leading the authors to conclude that the dose of AAV-α-synuclein used was "subclinical" (Naughton et al., 2017). Specifically, in that study animals were administered with 2 x 10^{10} viral genomes (vg) of AAV2/5, and in this study animals were administered with 3.1 x 10^{8} gc of AAV2/6, however due to the different titration methods used across these studies it is difficult to directly compare the doses.

Given that lateralised motor tasks are only appropriate in animals that have received unilateral lesions, in this study animals that were administered with bilateral AAV-GFP or AAV-α-synuclein were assessed for motor dysfunction using the rotarod. Overexpression of α-synuclein had no effect on the

latency to fall off the rotarod apparatus. Moreover, the performance of all groups changed over time. Given that this is an upward trend in duration of time spent on the rotarod, it is likely that the animals became habituated to both the apparatus and the protocol over time, perhaps reflecting intact motor learning in all groups of animals. These animals received the same total dose of viral vector as the groups administered unilaterally, but it was divided equally across both hemispheres so it is likely that overall motor dysfunction could take longer to manifest in comparison. In future studies, a rotarod protocol incorporating more training sessions may allow for more subtle effects of α-synuclein overexpression to be elucidated.

Despite the detection of α-synuclein-positive immunostaining in the dentate gyrus of the hippocampus, the behavioural results from hippocampal-associated tasks were variable. Overexpression of α-synuclein had no effect on the percentage of alternations in a spontaneous alternations task. Moreover, the reduction in the number of entries and enhanced performance by all groups over the course of the experiment suggest that repeated exposure to the apparatus and test paradigm resulted in habituation by the animals. This task is reflective of working memory, in that every response varies in accordance to the arm chosen each time (Sherrick et al., 1979) , and it exploits the animal's natural tendency to explore its environment. However, it is not without its caveats. Deacon and Rawlins (2006) described two main disadvantages to the spontaneous alternations protocol. Firstly, they state that the continuous nature of the task contributes to inter-trial interference and results in moderate alternation

rates typically seen in this paradigm. Secondly, animals with hippocampal damage notoriously adopt side preferences and thus could perform adequately in this task given it's continuous nature (Deacon and Rawlins, 2006). To combat these issues, they suggest that a discrete trial procedure is more suitable for detecting hippocampal damage; this protocol could be adopted in future studies.

The social recognition paradigm employed in this study measures short-term social memory, specifically the ability of an animal to recognise and remember a juvenile rat encountered 30 min previously. Recent evidence points to the involvement of a number of brain regions and neurotransmitter systems in the consolidation and retrieval of social recognition memory, including the dopaminergic systems in the hippocampus and basolateral amygdala (Garrido Zinn et al., 2016; Tanimizu et al., 2017). Moreover, social recognition memory has been shown to be at least partially dependent on adult hippocampal neurogenesis (Pereira-Caixeta et al., 2016). In this study, animals that received AAV-α-synuclein bilaterally spent significantly more time investigating the juvenile rat on the second presentation, indicating that they did not recognise the rat from the first presentation. This is similar to previous work demonstrating that bilateral administration of 6-OHDA could induce deficits in social recognition memory (Tadaiesky et al., 2008). In the present study, animals that received unilateral AAV-α-synuclein did not show significant deficits in social recognition, however the discrimination ratio for this group was higher than controls. In this testing paradigm, it is postulated that animals with intact

social memory capabilities would spend less time exploring the juvenile rat during the second presentation, thus the calculated discrimination ratio would be less than 0.5 (“chance level”, i.e. equal time spent exploring in each presentation). Given that the discrimination ratio for the AAV-α-synuclein unilateral group is higher than this, albeit marginally, it is possible that this group did display some form of social recognition impairment, however the intact contralateral hemisphere compensated for this functional impairment. Interestingly, animals that received unilateral AAV-GFP also showed deficits in social recognition.

Olfactory deficits are a common NMS in patients with PD (Hawkes et al., 1997), and they can often be present years preceding diagnosis (Postuma et al., 2012). Olfactory dysfunction has been demonstrated in animal models of PD such as the 6-OHDA model (Kumari et al., 2015; Tadaiesky et al., 2008) and the MPTP model (Castro et al., 2012), however to date there has been no investigation of olfactory deficits in the AAV-α-synuclein model. In this study, we used a previously published paradigm that involves a rat distinguishing between a novel odour (fresh cage bedding) and a familiar odour (its own used bedding). Although previous work has stated that this protocol relies on an animal showing preference for its own scent as opposed to a novel scent (Tadaiesky et al., 2008), we suggest that actually, the opposite is true. We have previously mentioned that an animal’s natural tendency is to explore a novel environment, a feature that is exploited in many behavioural tasks such as spontaneous alternations in the Y-maze. In light of this, it seems likely that the same is true for this olfactory

discrimination task; an animal can recognise a novel odour over its own familiar odour and thus spend more time in the novel compartment. In this study, we show that control animals spent significantly more time in the novel compartment when compared to all other groups. Moreover, all groups increased the time spent in the novel compartment as the experiment progressed, and this was coupled with decreased number of entries, indicative of habituation to the test protocol. Our data is supported by a recent study employing the rotenone model in adult male rats, which also demonstrated control animals spent significantly more time in the novel compartment compared to familiar (Rodrigues et al., 2014). Although olfactory function is primarily associated with the olfactory bulb, the connections between the olfactory bulb and the hippocampus have been shown to be critical in odour sampling and processing (Chapuis et al., 2013; Gourevitch et al., 2010). Moreover, growing data supports a role for dopamine in olfactory processes, as blocking dopamine receptors in the olfactory bulb has been shown to negatively affect odour discrimination (Escanilla et al., 2009), and more recently a new dopaminergic nigro-olfactory projection has been identified (Höglinger et al., 2015). In this study, it is likely that the combination of α-synuclein pathology in the SN and hippocampus and associated reductions in dopaminergic neurons could be sufficient to induce deficits in olfactory function.

Taken together, it is clear that overexpression of α-synuclein in the SN can lead to propagation of the protein to the hippocampus, and that there it can significantly affect a number of hippocampal-associated behavioural tasks.

However, these effects are variable and so future studies may require more sensitive and specific tests to fully elucidate the subtle effects of α-synuclein expression in the hippocampus. Nevertheless, our data is in line with other published studies that have repeatedly demonstrated the ability of α-synuclein to propagate through interconnected brain regions (Luk et al., 2012; Mason et al., 2016; Paumier et al., 2015; Rey et al., 2013). Furthermore, it is the capacity of α-synuclein to proliferate throughout the brain that has led to PD being considered as a prion-like disorder (reviewed by Brundin et al. 2016), and so any viable animal model of the disease must be able to replicate this.

Conditioned taste aversion is a classic conditioning paradigm and is associated with the basolateral amygdala (Osorio-Gómez et al., 2016), as well as the prefrontal cortex (Gonzalez et al., 2015) and insular cortex (Martinez-Moreno et al., 2016). A conditioned taste aversion is established when the taste of food (conditioned stimulus, CS) is followed by sickness (unconditioned stimulus, US). Generally, animals are quickly able to learn the association between the CS and the US, and the hedonic aspect shifts from positive to negative (reviewed by Yamamoto and Ueji, 2011). In this study, we employed sucrose as the CS and the malaise and nausea induced by lithium administration as the US. Thus, comparing the percentage of sucrose consumption in animals that received a saline (control) injection to animals that received a lithium injection is a measure of the success of the conditioned aversion. Overexpression of α-synuclein had no effect on the ability of animals to display aversive behaviour, as both AAV-α-synuclein

unilateral and bilateral animals consumed significantly less sucrose solution when compared to the corresponding controls. Indeed, α-synuclein-positive immunostaining was not detected in the amygdala, which substantiates the behavioural data. However, animals that received AAV-GFP bilaterally did not display a significant difference in percentage sucrose consumption when compared to their control counterparts, indicating that they did not develop the taste aversion. Although animals in this group showed a clear preference for sucrose during acquisition, and at levels similar to control animals, during the test phase they consumed significantly less sucrose when compared to controls. This may serve to confound the differences in preference percentage and perhaps explain this result.

To conclude, in this study we have shown that unilateral and bilateral administration of AAV-α-synuclein induced distinct patterns of nigrostriatal degeneration in adult male SD rats. Specifically, animals injected with bilateral AAV-α-synuclein demonstrated a more robust combination of nigral dopaminergic neuronal degeneration as well as associated loss of TH-positive fibres in the striatum. Moreover, unilateral overexpression of AAV-α-synuclein induced deficits in the olfactory discrimination task while bilateral overexpression of AAV-α-synuclein induced deficits in both the olfactory discrimination and the social recognition tasks. We show that the AAV-α-synuclein rat model can reproduce the proliferation of α-synuclein throughout the brain, and that it appears that is at least partially dependent on the duration of expression. Moreover, this model can be used to replicate

NMS associated with the disease, although more sensitive behavioural tasks may be required to elucidate the subtle α-synuclein-induced effects.

Chapter 6

6.0 Neuroprotective effects of voluntary running on non-motor symptoms in an α-synuclein rat model of Parkinson's disease

6.1 Abstract

Parkinson's disease (PD) is no longer primarily classified as a motor disorder due to the emergence of a number of non-motor symptoms of the disease, including cognitive dysfunction. These non-motor symptoms are highly prevalent and greatly affect the quality of life of patients with PD, and so therapeutic interventions to alleviate these symptoms are urgently needed. The aim of this study was to investigate the potential neuroprotective effects of voluntary running on cognitive dysfunction in an AAV-α-synuclein rat model. Bilateral intranigral administration of AAV-α-synuclein was found to induce motor dysfunction and a significant loss of nigral dopaminergic neurons, neither of which were rescued by voluntary running. Overexpression of α-synuclein also resulted in significant impairment on a neurogenesis-dependent pattern separation task, as well as anxiety-like behaviours on both the open field and the elevated plus maze. Voluntary running improved performance on the pattern separation task only. This was substantiated by an effect on hippocampal neurogenesis levels in the dorsal, and not ventral, dentate gyrus, suggesting that the functional effects on pattern separation were mediated by increasing neurogenesis.

6.2 Introduction

Recently, due to the increasing awareness of NMS in PD, the disease has begun to be recognised as a multifactorial syndrome rather than simply a motor disorder (reviewed by Titova et al. 2016). In order to increase the translational impact of preclinical research, there is an urgent need for animal models that can reproduce these NMS and so represent a more accurate depiction of the human disease. In chapter 5, we showed that bilateral intranigral administration of AAV-α-synuclein was a robust model of PD, resulting in dopaminergic neuronal degeneration in the SN as well as bilateral propagation of α-synuclein through the brain. More specifically, we demonstrated α-synuclein expression throughout the hippocampus, including in the dentate gyrus. Interestingly, the dentate gyrus is one of only two distinct niches in the brain whereby neurogenesis has been proven to occur throughout the adult lifespan (Altman, 1969; Eriksson et al., 1998). These newly born neurons have been shown to contribute to learning and memory processing, particularly pattern separation (Clelland et al., 2009), as well as emotional regulation (reviewed by Oomen et al. 2014). Inhibition or ablation of neurogenesis in animal models has been shown to increase anxiety and depressive-like behaviour and impair performance in cognitive tasks (reviewed by Ryan & Nolan 2016); conversely enhancing neurogenesis using methods such as environmental enrichment (reviewed by Bekinschtein *et al.*, 2011), dietary restriction (reviewed by Morgan *et al.*,

2017) or aerobic exercise can alleviate these mood disturbances and improve cognitive performance (Creer et al., 2010; Hill et al., 2015).

The first link between exercise and PD was published by Sasco and colleagues (1992), who demonstrated that moderate exercise during adulthood can protect against the risk of developing a PD in later life (Sasco et al., 1992). This has been further strengthened by the recent publication of a large-scale prospective epidemiological study that also confirms the association (Yang et al., 2015). Moreover, exercise has also been shown to play a role in restoring motor function (Collett et al., 2017; Santos et al., 2017) and alleviating NMS in patients already diagnosed with PD (reviewed by Cusso et al. 2016 and Dashtipour et al. 2015).

Given the previous chapters finding that α-synuclein propagates from the SN to the dentate gyrus, the role this region plays in specific cognitive and emotional functioning and it's potential to be modulated with interventions such as exercise, the aims of this chapter are:

a) To investigate the effects of AAV-α-synuclein overexpression on specific hippocampal-associated behaviours, including pattern separation
b) To examine the potential neuroprotective effects of aerobic exercise on α-synuclein-induced cognitive dysfunction
c) To elucidate the mechanisms of exercise-induced neuroprotection, specifically considering the role of adult hippocampal neurogenesis

6.3 Experimental design

6.3.1 Animal husbandry

Adult male Sprague Dawley rats (Envigo, UK) were maintained on a 12h:12h light: dark cycle (lights on at 08.00h) under regulated temperature (21±2°C) and humidity (30-50%) and pair-housed. Standard rat chow and water were available *ad libitum*, unless behavioural testing dictated otherwise temporarily. All experiments were conducted in accordance with the European Directive 2010/63/EU, and under an authorisation issued by the Health Products Regulatory Authority Ireland (HPRA, AE19130/P010) and approved by the Animal Ethics Committee of University College Cork (approval number 2013/030).

Stereotaxic surgery

All surgery was conducted under general anaesthesia induced by inhaled isoflurane. Due to concerns regarding toxicity of long-term expression of AAV-GFP from the previous chapter, in this study sham animals were administered with sterile 0.9% NaCl. Briefly, animals were placed in a stereotaxic frame (Kopf Instruments) and an incision was made through to the skull. Animals were administered with 3 µl of either AAV-α-synuclein (3.1 x 10^9gc/3µl) or 0.9% sterile saline solution (sham) bilaterally into the SN at co-ordinates AP -5.3, ML ±2.0 and DV -7.2 relative to bregma. All solutions were infused at a rate of 1 µl/min, with an additional 2 min allowed for diffusion before the needle was withdrawn. Following injection, the incisions were sutured and rats were allowed to recover on a heating mat before

returning to their home cages. Animals were administered the analgesic carprofen (Rimadyl® 5mg/kg, s.c., Zoetis Ireland Ltd) and 5% glucose solution immediately following the procedure.

6.3.2 Experimental design

One week following surgery, animals were administered with i.p. BrdU (Sigma, Ireland) 75mg/kg in 4 doses, each 2h apart. They were randomly divided into 4 groups; 'sham + sedentary' (n = 7), 'α-synuclein + sedentary' (n = 8), 'sham + running' (n = 6) and 'α-synuclein + running' (n = 8), and were pair-housed in either cages with free access to running wheels ('running' groups) (Activity wheel, Techniplast, UK) or standard housing cages ('sedentary' groups). Running wheels were connected to counters which allowed wheel revolutions to be continuously monitored. Motor and cognitive testing was carried out at selected time-points following surgery based on the previous study (see Figure 6.1 for experimental timeline).

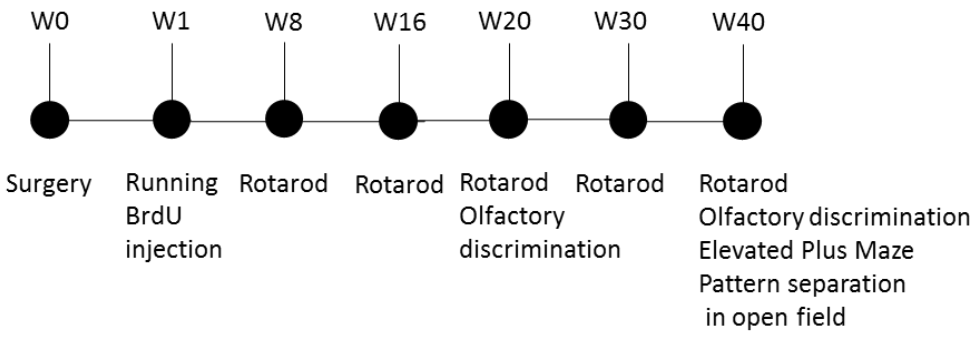

Figure 6.1 Experimental design

6.3.3 Behavioural testing

Behavioural testing was carried out as described previously (Section 4.6.2), and carried out at the time-points detailed in figure 6.1.

6.4 Results

6.4.1 Running distance and body weights

There was no significant difference in the average distance run per month between animals that were administered AAV-α-synuclein or those administered saline ($F [1,5] = 0.03$, $p = 0.86$; Figure 6.2A). Both groups of animals ran significantly more in the initial 10 weeks of the experiment compared to baseline ($F [9,45] = 15.5$, $p < 0.0001$), however this declined at later time-points. Animals in all groups gained weight over the course of the experiment ($F[9,207] = 862$, $p < 0.001$; Figure 6.2B), however animals in the running groups weighed significantly less than their sedentary counterparts ($F[1,23] = 20.97$, $p = 0.00013$). Overexpression of α-synuclein had no effect on weight gain ($F[1,23] = 0.14$, $p = 0.71$).

6.4.2 Overexpression of α-synuclein into SN and associated motor impairment

Both groups of animals that received AAV-α-synuclein displayed extensive transduction of dopaminergic neurons in the SN. There was no difference in

the number of co-localised TH+/α-syn+ neurons between the groups (t (4) = 0.89, p = 0.42; Figure 6.3A). AAV-mediated overexpression of α-synuclein resulted in a significant degeneration in the number of dopaminergic neurons in the SN and this was persistent across each level of the SN (AP -5.2, F [1,3] = 813, p < 0.0001; AP -5.6, F[1,4] = 19.45, p = 0.01; AP -6.0, F[1,4] = 8.6, p = 0.04; Figure 6.3B).

Voluntary running increased the number of dopaminergic neurons, only at the level immediately proximal to the administration site (AP -6.0, F [1,3] = 52.92, p = 0.005). There was a trend towards a significant reduction in TH-positive immunostaining in the striatum in the AAV-α-synuclein groups compared to sham controls (F [1,135] = 3.15, p = 0.07; Figure 6.3C), and *a priori post hoc* analysis revealed this was specific to the sedentary groups (sham sedentary vs α-synuclein sedentary p = 0.06). The degeneration of dopaminergic neurons in the SN caused by α-synuclein overexpression led to a significant impairment in sensorimotor integration on the rotarod (F [1,37] = 5.26 p = 0.02; Figure 6.3D). Voluntary running had no effect on motor function in the same task (F [1,37] = 0.33, p = 0.56).

6.4.3 General locomotor activity

Voluntary running had no impact on the distance travelled (F [1, 23] = 0.57, p=0.45) or the average velocity (F [1, 23] = 0.57, p = 0.45) of the animals in the open field test. Overexpression of α-synuclein resulted in a hyperactive phenotype, with animals travelling a significantly greater distance than

controls (F [1, 23] = 4.4, p=0.045; Figure 6.4A) and at a significantly higher velocity (F [1,23] = 5.2, p = 0.03; Figure 6.4B).

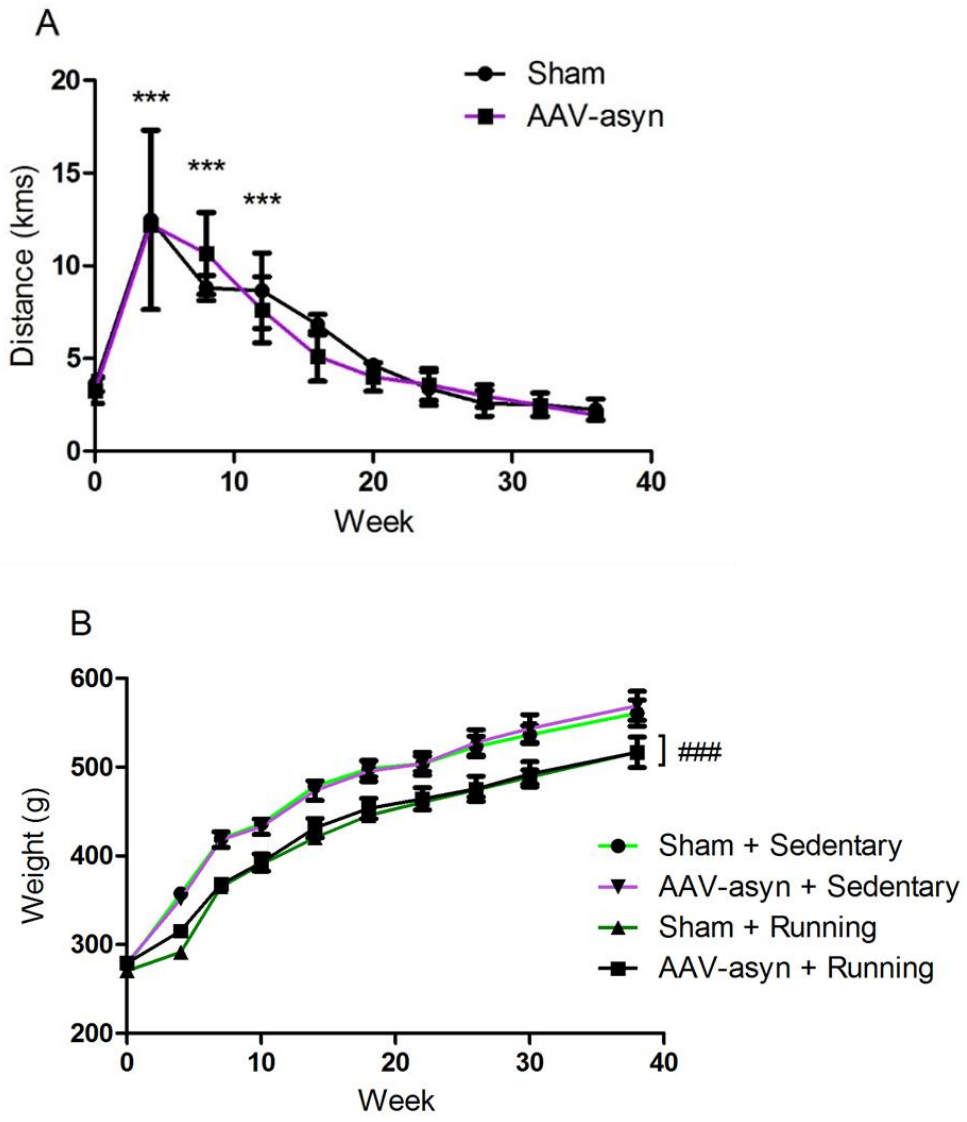

*Figure 6.2. (A) Average running distance per month of sham and AAV-α-synuclein groups. (B) Average weight gain over the course of the experiment. Data are shown as mean ± SEM and analysed using repeated measures ANOVA, ***p<0.001, ###p<0.001.*

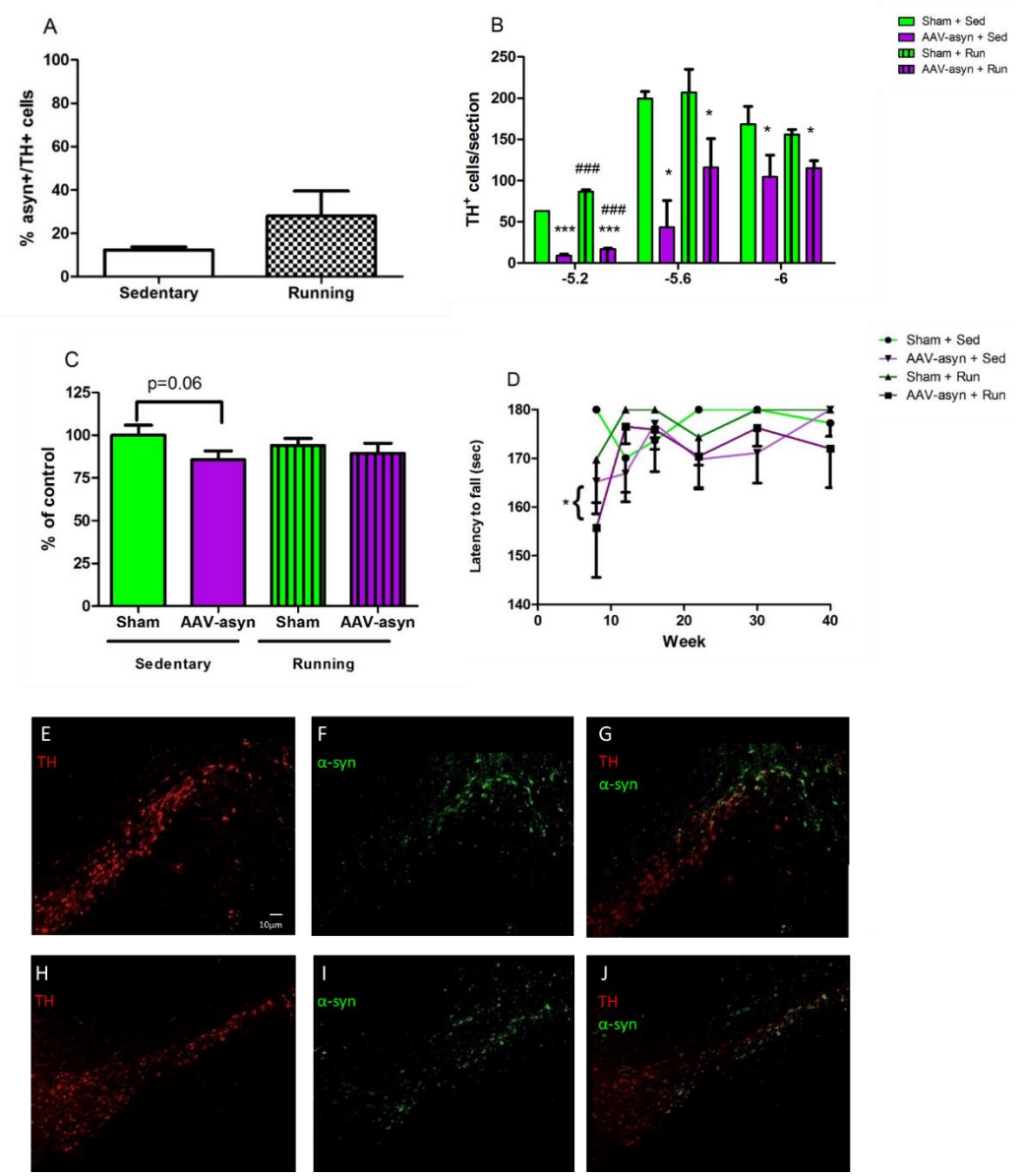

*Figure 6.3 (A) The percentage transduction efficiency of the AAV viral vector in both sedentary and running α-synuclein groups. (B) TH-positive cell counts in the SN and (C) fluorescence intensity of TH-positive immunostaining in the striatum. (D) Motor performance measured on the rotarod. Representative images of (E, H) TH-positive cells in the SN, (F, I) α-synuclein expression in the SN and (G-J) merged images. Scale bar represents 100 µm. Data are expressed as mean ± SEM, *p<0.05, ***p<0.001 vs corresponding sham group, #p<0.05, ###p<0.001 vs corresponding sedentary group.*

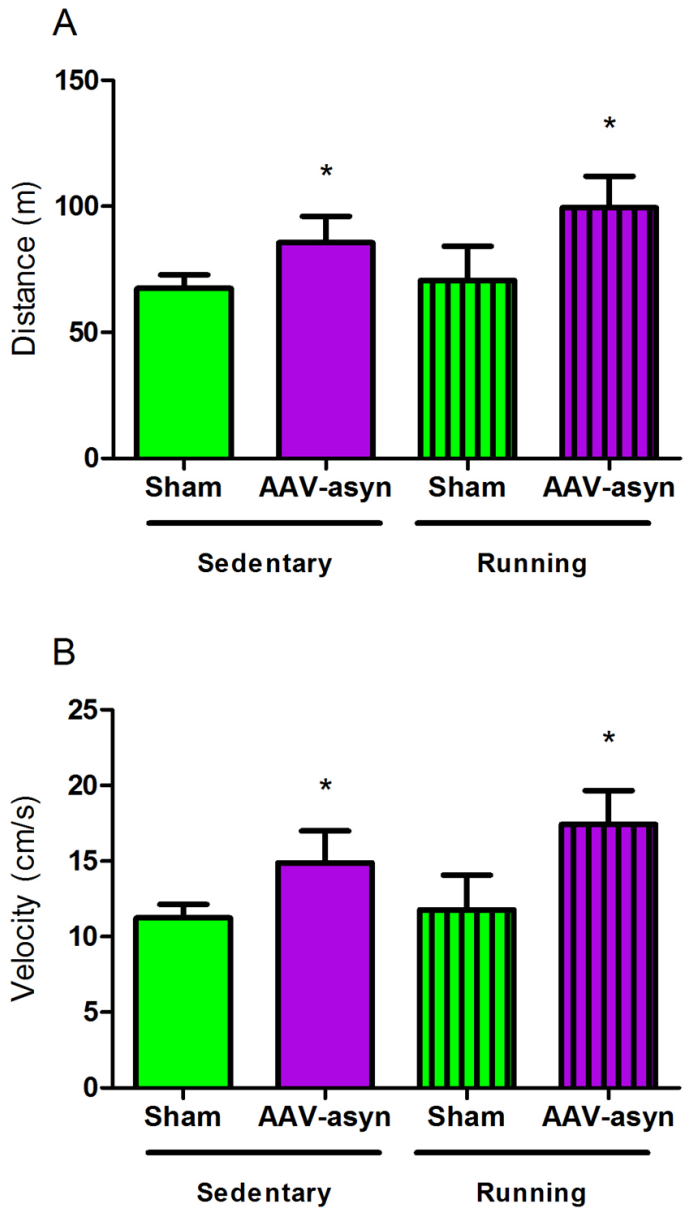

*Figure 6.4 General locomotor activity measured by (A) distance travelled and (B) velocity travelled in the open field. Data are shown as mean ± SEM and analysed using 2-way factorial ANOVA and post hoc Fisher's LSD, *p<0.05 vs corresponding sham group.*

6.4.4 Hippocampal-associated memory tasks

The percentage of alternations in a standard discrete alternation task was not affected by overexpression of α-synuclein ($F [1,22] = 0.86$, $p=0.36$; Figure 6.5A) or by voluntary running ($F [1,22] = 1$, $p= 0.32$). *A priori* testing revealed a trend towards a significant impairment in this task in α-synuclein compared to sedentary groups ($t (11) = 1.92$, $p = 0.08$). To increase the cognitive load of the task, a 1 min delay was inserted into the protocol. However, neither overexpression of α-synuclein ($F [1,22] = 0.46$, $p = 0.5$; Figure 6.5B) or voluntary running ($F [1,22] = 0.73$, $p = 0.4$) had an effect on the percentage of alternations in this paradigm.

In the modified spontaneous location recognition task, there was no effect of α-synuclein ($F [1, 20] = 0.001$, $p =0.99$) or voluntary running ($F [1, 20] = 0.56$, $p = 0.46$) on the ability of the animals to discriminate between the novel and familiar object locations in the large separation paradigm (Figure 6.5C). However, in the small separation paradigm, voluntary running significantly enhanced the discrimination ratio compared to that of the sedentary counterparts ($F [1, 22] = 11.64$, $p=0.002$; Figure 6.5D). There was a trend towards a significant

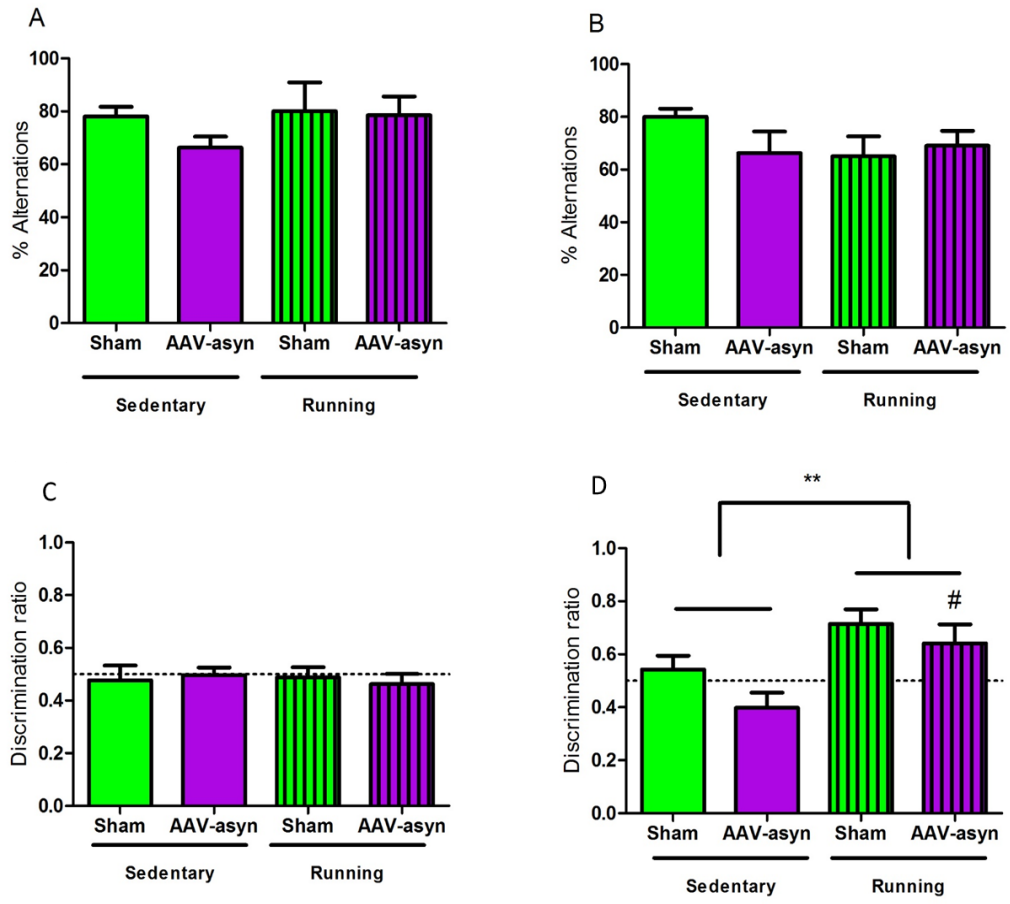

*Figure 6.5 The percentage of alternations in (A) a standard discrete alternations protocol and (B) with a 1 min delay. Modified spontaneous location recognition test in both (C) a large separation paradigm and (D) a small separation paradigm. Data are shown as mean ± SEM and analysed using 2-way factorial ANOVA and post hoc Fisher's LSD, **p<0.01 running vs sedentary counterparts, #p<0.05 vs α-synuclein sedentary.*

impairment in performance of the same task by α-synuclein groups compared to sham groups (F [1, 22] = 3.24, p=0.08), and *a priori post hoc* analysis revealed that voluntary running rescued the partial deficit that was evident in α-synuclein groups (α-synuclein sedentary vs α-synuclein running p = 0.007).

These data indicating a deficit in hippocampal-associated tasks are substantiated by the observation of human α-synuclein expression in the dentate gyrus (Figure 6.6), showing that α-synuclein was transported to this brain area following administration of AAV-α-synuclein into the SN.

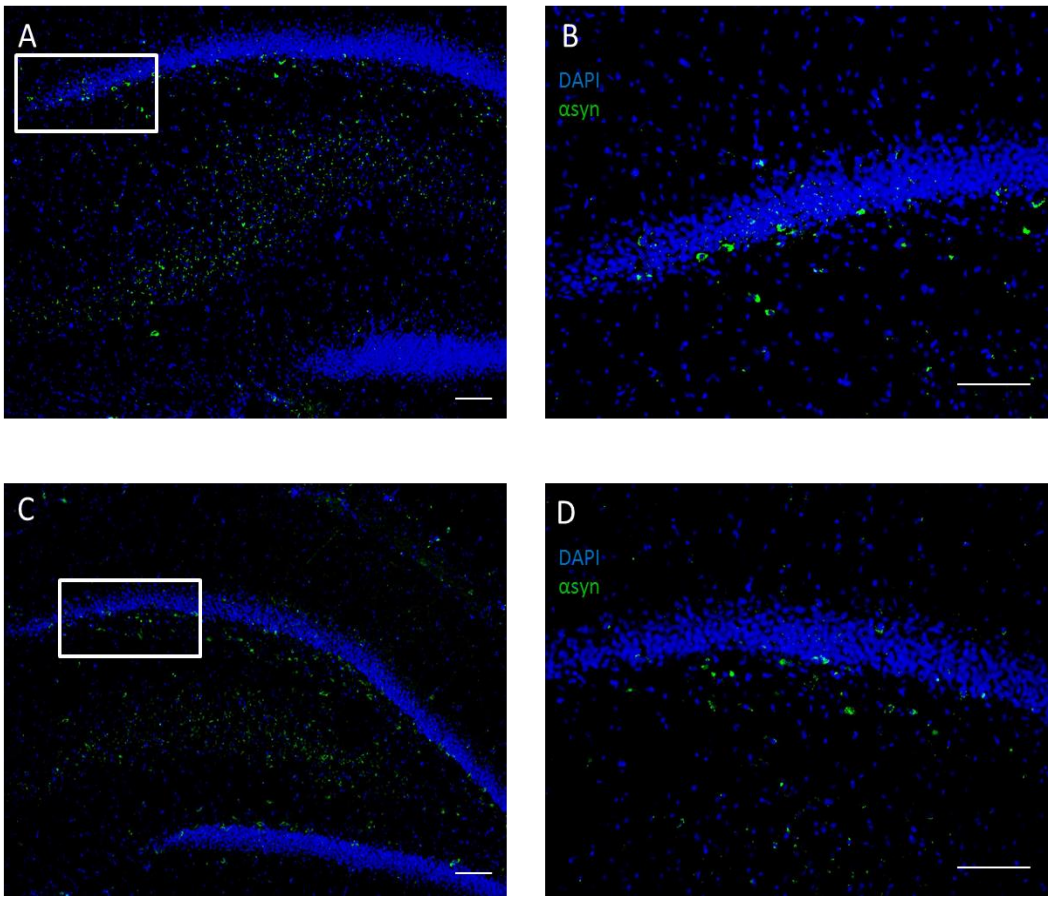

Figure 6.6 Representative photomicrographs of α-synuclein staining in the dentate gyrus of (A, B) AAV-α-synuclein + sedentary and (C, D) AAV-α-synuclein + running groups. Images taken at (A, C) 10x and (B, D) 20x. Scale bar represents 100 µm.

6.4.5 Anxiety-related behaviours

Testing in the elevated plus maze revealed that overexpression of α-synuclein resulted in the animals spending significantly more time in the closed arms of the maze when compared to control animals ($F [1, 23] = 18.95$, $p=0.0002$; Figure 6.7A) Voluntary running also increased the amount of time spent in the closed arms in comparison to sedentary animals ($F [1, 23] = 4.39$, $p=0.04$). Factorial ANOVA showed a significant interaction effect (treatment x running, $F [1, 23] = 9$, $p=0.006$), and *post hoc* analysis revealed a significant difference between the control sedentary and the α-synuclein running groups ($p=0.02$), and a small effect of α-synuclein on the non-running animals (saline sedentary vs α-synuclein sedentary, $p=0.08$). Neither overexpression of α-synuclein ($F [1, 23] = 0.14$, $p=0.7$; Figure 6.7B) or voluntary running ($F [1, 22] = 0.89$, $p=0.35$) had any impact on the total number of closed arm entries per group.

In the open field, overexpression of α-synuclein resulted in a significant increase in thigmotaxis behaviour, as measured by the time the animals spent in the border zone of the open field arena, compared to sham controls ($F [1, 23] = 5.5$, $p =0.027$; Figure 6.7C), Voluntary running did not affect thigmotaxis behaviour (($F [1, 23] = 3.5$, $p= 0.074$).

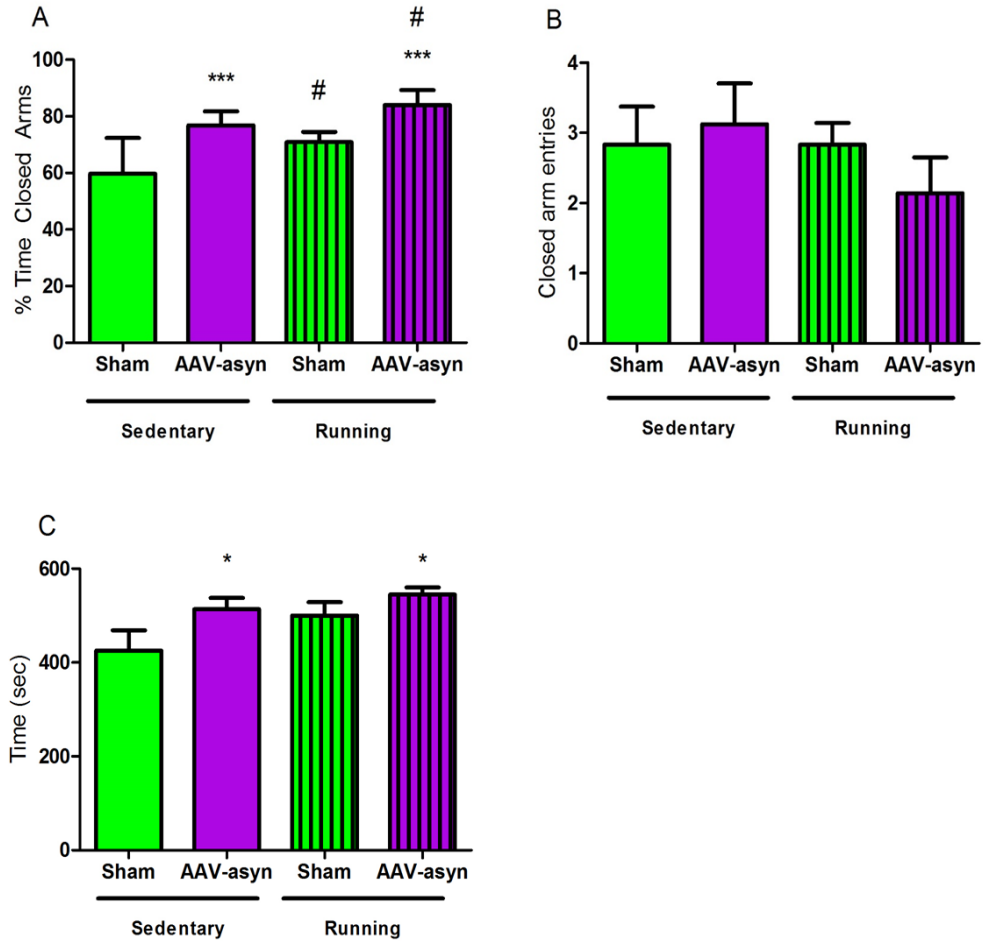

*Figure 6.7 (A) Percentage of time spent in the closed arms of the elevated plus maze and (B) total number of entries into the closed arms. (C) Thigmotaxis behaviour measured by time spent in the border zones of the open field. Data are shown as mean ± SEM and analysed using 2-way factorial ANOVA and post hoc Fisher's LSD, *p<0.05, ***p<0.001 vs corresponding sham controls, #p<0.05 vs sedentary counterparts.*

6.4.6 Olfactory discrimination

Overexpression of α-synuclein did not affect the percentage of time spent by the animals in the novel compartment (F [1, 23] = 1.45, p=0.24; Figure 6.8), nor was there a difference in the animal's performance between time points (F [1, 23] = 0.97, p=0.33). Voluntary running significantly decreased the amount of time spent by the animal in the novel compartment (F [1, 23] = 5.8, p=0.02). Post hoc analysis revealed that this effect was specific to α-synuclein groups (p=0.03), but that there was no effect of running on the control groups.

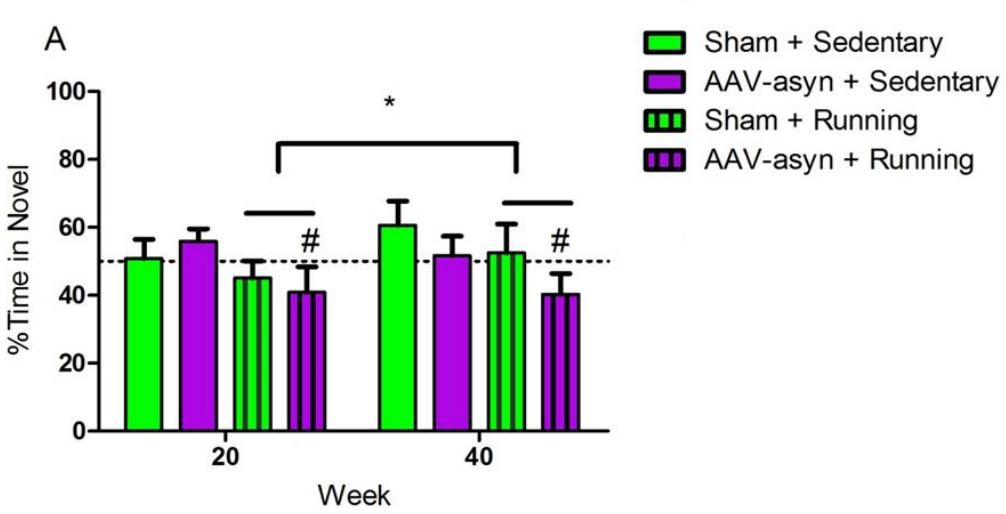

*Figure 6.8 Performance in an olfactory discrimination task. Data are shown as mean ± SEM and analysed using 2-way repeated measures ANOVA and post hoc Fisher's LSD *p<0.05 vs corresponding sedentary controls, #p<0.05 vs α-synuclein sedentary.*

6.4.7 Hippocampal neurogenesis

The total number of DCX-positive cells in the dentate gyrus was significantly decreased by overexpression of α-synuclein ($F [1,12] = 5.12$, $p = 0.043$; Figure 6.9E). Subdivision of dentate gyrus regions revealed that this effect was specific to the dorsal ($F [1,12] = 8.07$, $p = 0.01$) and not found in the ventral region ($F [1,12] = 0.16$, $p = 0.69$). Voluntary running had no effect on the number of DCX-positive cells in the dentate gyrus ($F [1,23] = 0.54$, $p = 0.47$).

Overexpression of α-synuclein in the SN significantly decreased the total number of BrdU-positive neurons in the dorsal dentate gyrus ($F[1,12] = 18.31$, $p = 0.001$; Figure 6.10E) and this effect was persistent in the sub-regions of the dentate gyrus, including the granule cell layer

($F[1,12] = 4.94$, $p = 0.04$) and the subgranular zone ($F[1,12] = 12.38$, $p = 0.004$; Figure 6.10E). Factorial ANOVA showed a significant interaction effect (treatment x running $F[1,12] = 7.1$, $p = 0.02$). *Post hoc* analysis revealed that, although voluntary running alleviated α-synuclein-induced deficits in the number of BrdU-positive cells (α-synuclein sedentary vs α-synuclein running, $p = 0.006$), this effect was specific to the dorsal subgranular zone (α-synuclein sedentary vs α-synuclein running, $p = 0.003$). Neither overexpression of α-synuclein ($F[1,12] = 2.97$, $p = 0.1$) nor voluntary running ($F[1,12] = 1.36$, $p = 0.26$) affected the number of BrdU-positive cells in the ventral dentate gyrus (Figure 6.10F).

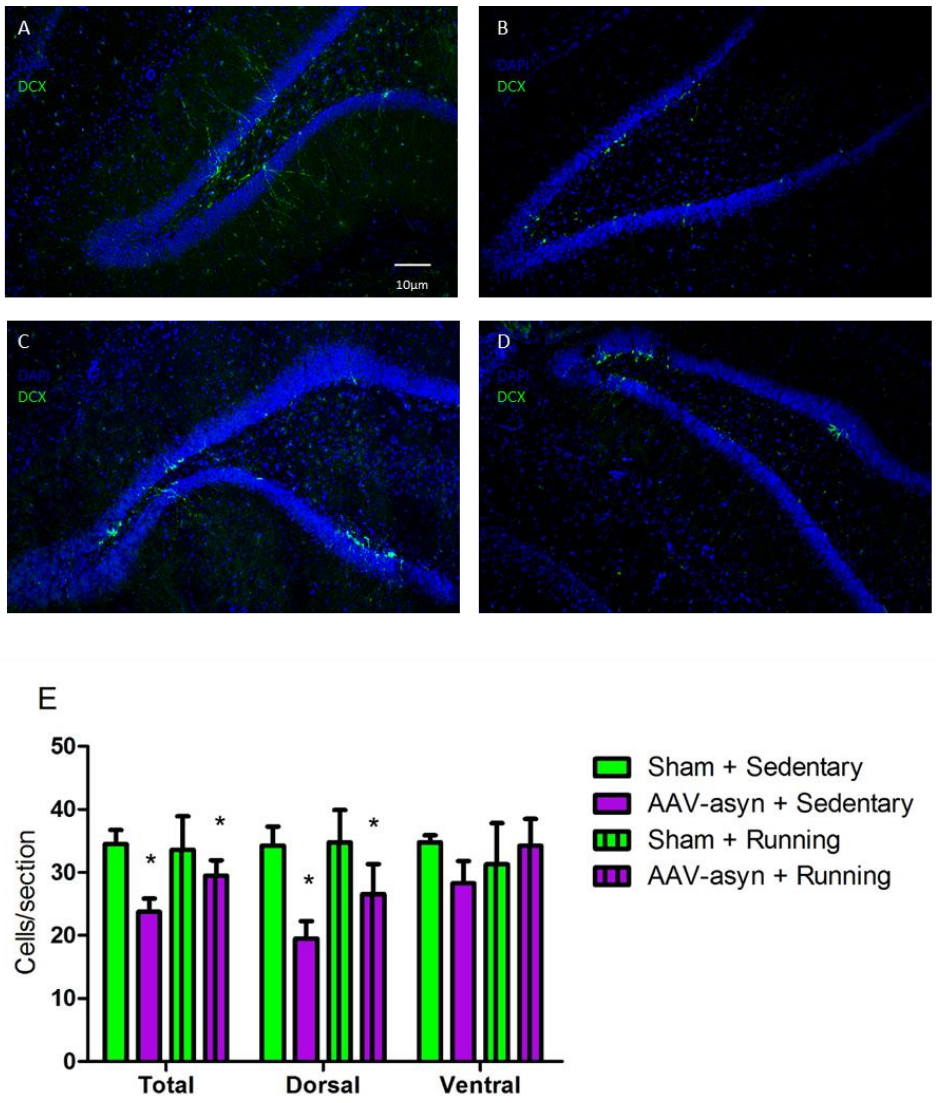

*Figure 6.9 DCX-positive immunostaining in the dentate gyrus of the hippocampus in (A) Sham + sedentary (B) AAV- α-synuclein + sedentary (C) Sham + running and (D) AAV-α-synuclein + running groups. (E) Number of DCX+ cells in the total, dorsal and ventral dentate gyrus. Data are shown as mean ± SEM and analysed using 2-way factorial ANOVA and post hoc Fisher's LSD, *p<0.05 vs corresponding saline control. Scale bar represents 100 µm.*

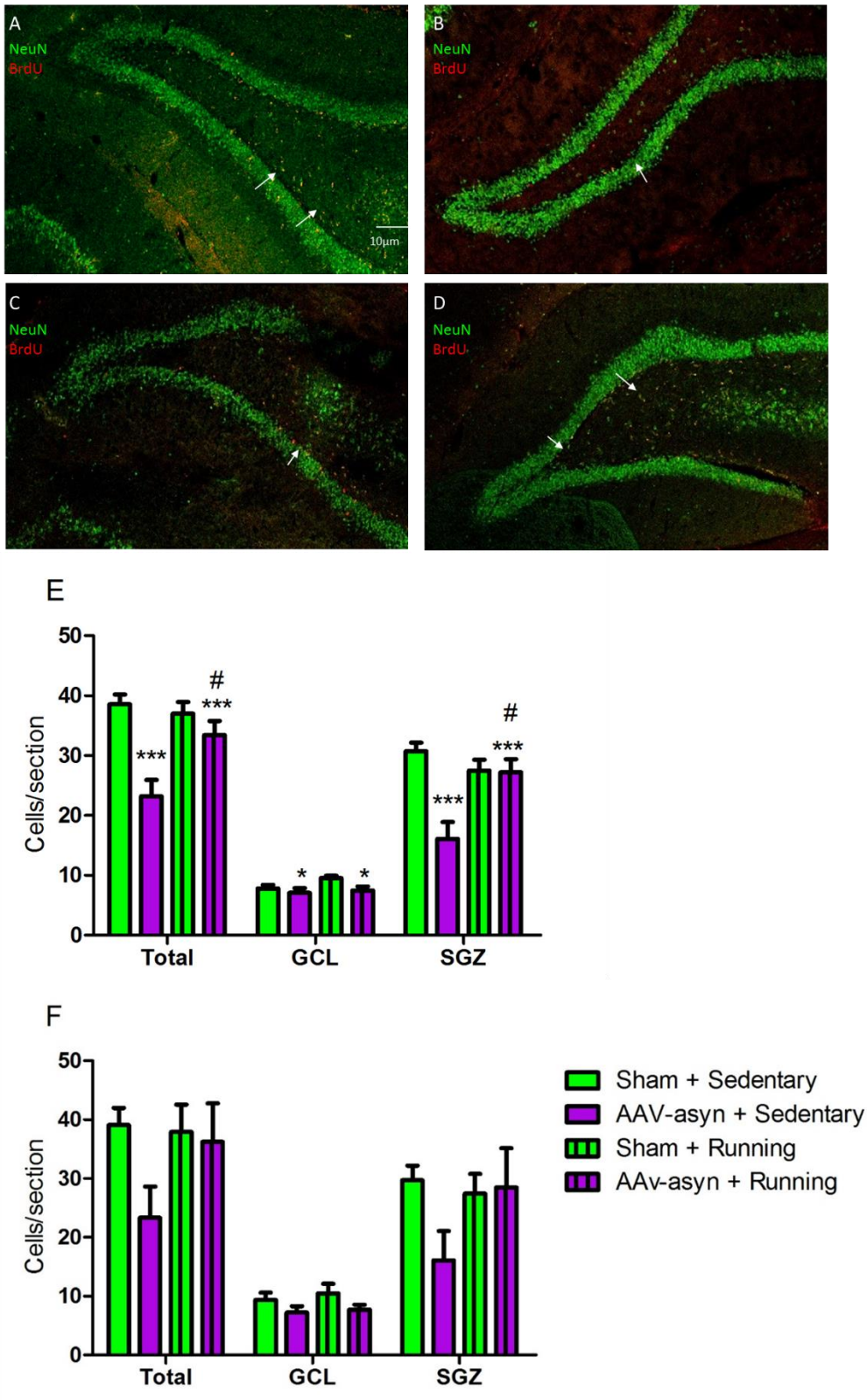

*Figure 6.10 BrdU+ neurons (indicated by the arrows) in the dentate gyrus in (A) sham + sedentary, (B) AAV-α-synuclein + sedentary, (C) sham + running and (D) AAV-α-synuclein + running groups. BrdU+ neurons in the granule cell layer (GCL) and subgranular zone (SGZ) in the (E) dorsal (F) and ventral dentate gyrus. Data are shown as mean ± SEM and analysed using 2-way factorial ANOVA and post hoc Fisher's LSD, *p<0.05, ***p<0.001 vs corresponding saline control, #p<0.05 vs α-synuclein sedentary. Scale bar represents 100 µm.*

6.5 Discussion

A growing body of evidence supports the Braak hypothesis (Heiko Braak et al., 2003), which states that the aggregation and propagation of α-synuclein throughout the brain is responsible for the array and progression of motor and NMS experienced by PD patients. Moreover, it has been proven that the neuropathological staging of the disease correlates with cognitive decline (Braak et al., 2006b). In this study, we show that bilateral overexpression of α-synuclein in the SN not only causes degeneration of nigral dopaminergic neurons and motor impairment, but that it also leads to deficits in hippocampal-associated memory tasks, specifically in tasks assessing the ability to "pattern separate". Furthermore, we show that these deficits are linked to alterations in adult hippocampal neurogenesis in the dentate gyrus.

AAV-mediated overexpression of α-synuclein has been well-characterised in the literature as a viable animal model of PD (reviewed by Lindgren et al. 2012). It is the only model to date that replicates all of the neuropathological, neurochemical and behavioural features of the disease, including the formation of α-synuclein aggregates in nigral neurons (Kirik et al., 2003). The majority of the work published to date employs the model unilaterally, using lateralised tasks such as the cylinder test and the stepping test to measure motor dysfunction and allowing the contralateral hemisphere to act as a control in *post mortem* analyses. Here, we show that bilateral administration of α-synuclein induces bilateral nigral dopaminergic

degeneration and a decrease in striatal TH innervation, which subsequently causes bilateral motor dysfunction that can be measured on a rotarod. Although the degree of striatal denervation was not as profound as that seen in animal with acute lesions of the nigrostriatal system such as those induced by stereotaxic injections of 6-OHDA, it was sufficient to result in a significant degree of motor impairment. Previous work by Kirik and colleagues (1998) described the different thresholds of TH-positive fibre loss in the rat striatum that are necessary to induce motor impairment on specific laboratory tasks. They showed that a 40-50% reduction in TH innervation was required to induce detectable deficits in the apomorphine-induced rotations task, but that 70-80% reduction was necessary for significant motor impairment in the stepping test and in a modified paw-reaching test (Kirik et al., 1998). It is worth noting that this work was carried out using the unilateral 6-OHDA model, and so although the results cannot be directly compared to the present study, the underlying premise that different degrees of striatal denervation are required to detect motor deficits on different tasks remains valid.

In the present study, voluntary running did not alleviate motor dysfunction, nor did it rescue the partial degeneration of dopaminergic nerve terminals in the striatum. Interestingly, voluntary running did protect against TH-positive neuronal loss in the SN immediately proximal to the administration site of the viral vector. However, this neuroprotective effect was not sufficient to improve motor function. To our knowledge, this is the first study to investigate the effects of voluntary running on motor and NMS in an α-

synuclein model of PD. Although several reports have employed exercise as an intervention in other animal models of PD including the MPTP model (Jang et al., 2017), the rotenone model (Shin et al., 2017) and the 6-OHDA model (Landers et al., 2013; Tillerson et al., 2003), results are highly variable and again, these models are not the most representative of the human disease when compared to the AAV-α-synuclein model (reviewed by Volpicelli-Daley et al., 2016).

The rotarod protocol used in this study was a fixed speed test. A previous study, albeit one using the 6-OHDA model, has shown that an accelerating rotation rate on the rotarod allows a more specific correlation between motor deficits and lesion size (Monville et al., 2006). This is supported by the recent publication of a study by Wang and colleagues, who demonstrated that despite modest loss of TH-positive fibres in the striatum after intra-striatal administration of 6-OHDA (approximately 20% less than control animals), an accelerating rotarod protocol was sufficiently sensitive to detect motor dysfunction (Wang et al., 2013). Nonetheless, our results are in line with previously published work where bilateral administration of α-synuclein induced TH-positive cell loss in the SN and corresponding TH-positive fibre loss in the adult rat striatum, coupled with mild motor deficits on a ledged beam-walking test (Caudal et al., 2015). Furthermore, this also highlights the variability in tests used to measure motor impairment in bilateral models of PD. There are a number of different rotarod protocols currently published, which use distinct training paradigms and different testing speeds (Goes et al., 2014; Marei et al., 2015; Wang et al., 2013); this

makes it difficult to extrapolate between the results of these studies. Gait disturbances and postural instability are classic motor symptoms of PD (reviewed by Sveinbjornsdottir 2016) and recent evidence suggests that gait analysis may be a more sensitive and specific method of detecting motor dysfunction in animal models of movement disorders (Vandeputte et al., 2010). Studies carried out on rats with bilateral 6-OHDA lesions confirms that these animals display a wide variety of gait disturbances post-lesion, including alterations in stride length, swing speed and stance duration; furthermore these symptoms are sensitive to L-dopa therapy (Westin et al., 2012). Moreover, gait disturbances can be used to differentiate between lesions induced by 6-OHDA administration into the striatum, into the MFB and into the SN, and some aspects of gait impairments positively correlate with the extent of loss of TH-positive cells in the SN (Zhou et al., 2015). Similarly, a time-course of gait analysis after a unilateral 6-OHDA lesion has been shown to be more sensitive to motor deficits and compensatory mechanisms than the apomorphine-induced rotations test (Hsieh et al., 2011). To our knowledge, there is currently no data investigating gait disturbances in the AAV-α-synuclein rat model, however the technology is available and has been relatively well characterised and so this remains a possible avenue for future investigation.

Overexpression of α-synuclein in this study resulted in a hyperactive phenotype, exemplified by animals travelling greater distances and at higher velocities than sham animals. This confirms previous work by Aldrin-Kirk and colleagues (2014), who showed that administration of an AAV6 viral vector

overexpressing α-synuclein into the forebrain of SD pups resulted in hyperactive behaviour in the open field after 40 weeks (9 months). This was coupled with a significant degeneration of cholinergic interneurons in the striatum (Aldrin-Kirk et al., 2014). Although the exact role of cholinergic interneurons in the pathophysiology of PD is unclear, growing evidence suggests that these cells can modulate dopamine transmission in the striatum (Johnson et al., 2017; Kosillo et al., 2016), as well as modulating motor symptoms in animal models of PD (Kondabolu et al., 2016; Maurice et al., 2015; Ztaou et al., 2016). Interestingly, overexpression of α-synuclein in either a transgenic mouse line or through AAV administration in rats has been shown to reduce striatal dopamine levels, and it was suggested that this was *via* a direct effect on striatal cholinergic interneurons (Tozzi et al., 2015).

Cognitive impairment is a highly prevalent NMS in PD (Domellöf et al., 2015; Yarnall et al., 2014), and it can significantly impact upon the quality of life of PD patients (Bugalho et al., 2016; Kwon et al., 2016). Although there is a wealth of studies that have investigated cognitive impairment in animal models of PD, such as the 6-OHDA and MPTP models (reviewed by Lindgren & Dunnett 2012), relatively little has been published to date using the AAV-α-synuclein rat model. One report by Campos and colleagues (2013) used bilateral nigral overexpression of α-synuclein in adult rats to investigate cognitive dysfunction, and showed that α-synuclein had no effect on performance in three versions of the MWM task, which measured working and spatial reference memory as well as cognitive flexibility (Campos et al.,

2013). However, in that study, cognitive testing was carried out 7-8 weeks post-surgery and α-synuclein expression in the hippocampus was not confirmed, so it is possible that the testing point was too early to ensure that viral integration, α-synuclein expression and subsequent propagation to the hippocampus had occurred. In the present study, we show that although overexpression of α-synuclein had no effect on spatial working memory measured by a discrete alternations task, it significantly impaired the ability of animals to perform a pattern separation task. Pattern separation refers to the organisation of similar, overlapping episodic memories so that they may be recognised as singular and separate representations of each memory (Bekinschtein et al., 2013), and it has been shown to be uniquely dependent on adult hippocampal neurogenesis in the dentate gyrus (Clelland et al., 2009). Moreover, increasing levels of adult hippocampal neurogenesis in rodents by environmental enrichment or voluntary running can also enhance performance in a pattern separation task (reviewed by Bekinschtein et al. 2011). In this study, we have demonstrated that long-term voluntary running can rescue α-synuclein-induced deficits in a small pattern separation task, and that this is likely to be mediated by alterations in adult hippocampal neurogenesis. Overexpression of α-synuclein caused significant decreases in the number of immature neurons (DCX+ cells) and the survival of newly born neurons (BrdU+/NeuN+) in the dorsal, and not the ventral, dentate gyrus. Similarly, voluntary running increased the survival of neurons only in the dorsal dentate gyrus. This effect was localised to the subgranular zone, which has been shown to be the specific region of the

dentate gyrus associated with adult hippocampal neurogenesis (reviewed by Goncalves et al. 2016). The dentate gyrus is known to be involved in various cognitive and emotional processing, and it is functionally divided along its longitudinal axis whereby the dorsal dentate gyrus is associated with learning and memory and the ventral region regulates anxiety and stress resilience (reviewed by O'Leary & Cryan 2014).

In this study, voluntary running did not ameliorate anxiety-like behaviour in the α-synuclein groups, measured by thigmotaxis behaviour in the open field or by behaviour in the elevated plus maze. Moreover, voluntary running increased the amount of time spent by animals in the closed arms of the elevated plus maze when compared to sedentary controls. Although running has been widely regarded to confer anxiolytic benefits in animals (Duman et al., 2008; Greenwood et al., 2003), these findings are becoming increasingly controversial. Recent work, albeit in mice, has shown that voluntary running can induce anxiety-like behaviour in a number of behavioural tasks including the open field task, the light/dark box and the FST (Fuss et al., 2010a). Moreover, deletion of running-induced hippocampal neurogenesis by focalized irradiation was sufficient to reverse the anxious phenotype (Fuss et al. 2010), suggesting that the enhanced levels of neurogenesis somehow played a role in generating the anxious behaviour. Although we found no evidence that overexpression of α-synuclein had impaired neurogenesis in the ventral dentate gyrus, α-synuclein overexpression did induce an anxiety-like phenotype in both the open field and the elevated plus maze, suggesting alternative mechanisms in the regulation of these behaviours.

Olfactory discrimination is primarily functionally linked to the olfactory bulbs, however there is growing evidence to support a role for the olfacto-hippocampal network in olfactory discrimination tasks (Gourevitch et al., 2010; Knafo et al., 2005; Martin et al., 2007; Restivo et al., 2006; Uva and De Curtis, 2005). In this study, we show that overexpression of α-synuclein did not impact on an animal's ability to discriminate between their own odour and a fresh bedding odour. However, voluntary running significantly affected the capacity for discrimination between novel and familiar odours. Although the olfactory bulb is an established site for adult neurogenesis in rats, the role of the newly-born neurons in olfactory processes and memory remains inconclusive (Bardy and Pallotto, 2010). Ablation of adult neurogenesis in the olfactory bulb has not consistently resulted in deficits in odour discrimination (reviewed by Kageyama et al. 2012 and Lazarini & Lledo 2011), and so conversely enhancing neurogenesis such as in the present study may not lead to improved performance in an olfactory discrimination task. Perhaps a more sensitive task, such as an odour delayed nonmatching to sample task that has previously been shown to be impaired in patients with hippocampal lesions (Levy et al., 2004) would be more appropriate in future studies.

To conclude, in this study we have shown that bilateral administration of AAV-α-synuclein into the adult rat SN results in bilateral degeneration of nigral dopaminergic neurons and of TH-positive fibres, as well as bilateral motor impairment measured on the rotarod. This model can also be used to replicate some of the primary NMS of PD, including hippocampal-associated

learning and memory tasks as well as anxiety-like behaviours. Moreover, we have shown that administration of AAV-α-synuclein into the adult rat SN can lead to the propagation and expression of α-synuclein in the dentate gyrus of the hippocampus where it can affect markers of adult hippocampal neurogenesis. Finally, we have shown that α-synuclein-induced deficits in a pattern separation task and associated alterations in hippocampal neurogenesis can be alleviated by the neuroprotective effects of voluntary running.

Chapter 7

7.0 General discussion

Parkinson's disease is the second most common neurodegenerative disease in the world (Ascherio and Schwarzschild, 2016), and given an ageing global population the incidence is expected to double by 2010 (Dorsey et al., 2007). Although there are a variety of therapies currently available to treat both motor and NMS of the disease, these are only used for symptomatic relief and do nothing to halt the disease process itself. Moreover, by the time patients begin to display motor symptoms, it is estimated that up to 30% of the dopaminergic neurons in the SN have already been lost (reviewed by Burke and O'Malley, 2013). Thus, research into effective preventative strategies is highly desirable.

Given the difficulty in researching disease mechanisms in humans, a range of animal models of PD have been developed to allow comprehensive investigations of the neuropathological, neurobiological and neurochemical features of the disease. Each model has their own relative advantages and disadvantages (reviewed by Bové and Perier, 2012), however a recurring theme is the inability of any model to fully reproduce the progressive nature and Lewy body pathology that are characteristic of PD (reviewed by Lindgren et al., 2012 and Volpicelli-Daley et al., 2016), as well as the broad range of NMS that are also inherent to the disease (reviewed by Schapira et al., 2017). Moreover, the failure of many of the classic neurotoxin models to accurately predict translational success of neuroprotective compounds in clinical trials further hampers progress (reviewed by Athauda and Foltynie, 2015). The development of the AAV-α-synuclein model by Kirik and colleagues (2002) was in many ways a turning point, as it was able to replicate the behavioural

and neuropathological aspects of PD while also resulting in a progressive degeneration of dopaminergic neurons (Kirik et al., 2002). Since then, the model has enabled a wide range of critical preclinical research to be completed that would not have been possible in other neurotoxic models. The progressive nature of this model is one of its key features, as it has allowed for a more in-depth interrogation of the mechanisms of synaptic dysfunction when compared to other neurotoxin models such as 6-OHDA (reviewed by Volpicelli-Daley et al., 2016). Moreover, the AAV-α-synuclein overexpression model has also provided a more comprehensive overview of the role of α-synuclein in the pathogenesis of PD. For example, despite promising *in vivo* animal studies investigating the neuroprotective effects of NTFs such as GDNF, these results did not translate to successful clinical trials (reviewed by Olanow et al., 2015). By employing the AAV-α-synuclein model, Decressac and colleagues (2012c) established that overexpression of α-synuclein in nigral dopaminergic neurons blocks GDNF signalling, providing a novel insight that would have been otherwise undiscovered in the neurotoxin models. Additionally, given that the AAV-α-synuclein model is most representative of the human disease, it has allowed for a more thorough investigation into putative therapeutic targets (reviewed by Volpicelli-Daley et al., 2016).

The primary hypothesis of this thesis was that:

The propagation of α-synuclein throughout the brain in PD is linked to the presence of NMS.

To test this hypothesis, this thesis employed the AAV-α-synuclein model overexpressing human wild-type α-synuclein to explore the ability of this model to replicate the proliferation of α-synuclein throughout the brain, and whether this could be linked to the pathogenesis of NMS. In study 1, we show that both unilateral and bilateral administration of AAV-α-synuclein induce distinct patterns of nigrostriatal degeneration. Interestingly, animals that received bilateral injections of AAV-α-synuclein demonstrated a more robust degeneration of dopaminergic neurons and TH-positive fibres in the striatum, confirming that this administration paradigm is a viable model of PD. We confirmed that both unilateral and bilateral overexpression of α-synuclein in the SN induced bilateral propagation of α-synuclein throughout distinct regions of the brain, including the hippocampus, and that it can affect some aspects of hippocampal-associated behaviours. Given this data, we then focused on the impact of α-synuclein overexpression on specific cognitive and emotional tasks that are dependent on adult hippocampal neurogenesis. We also exmained the neuroprotective effects of exercise on α-synuclein-induced deficits in these tasks. In study 2, we show that bilateral administration of AAV-α-synuclein into the adult rat SN results in bilateral degeneration of nigral dopaminergic neurons and of TH-positive fibres, as well as bilateral motor impairment measured on the rotarod. Similarly, this model can be used to replicate some of the NMS associated with PD, including impairments in hippocampal-associated learning and memory tasks as well as anxiety-like behaviours. We demonstrated that administration of AAV-α-synuclein into the adult rat SN can lead to the

propagation and expression of α-synuclein in the dentate gyrus of the hippocampus where it can affect markers of adult hippocampal neurogenesis. Finally, we showed that α-synuclein-induced deficits in a pattern separation task and associated alterations in hippocampal neurogenesis can be alleviated by the neuroprotective effects of voluntary running. To summarise, we have proven our initial hypothesis; namely, this thesis confirms that the propagation of α-synuclein throughout the brain, a pathological feature inherent to PD, is linked to the pathogenesis NMS. More specifically, we have shown that overexpression of α-synuclein in the hippocampus results in significant impairment in hippocampal-associated tasks, some of which are dependent on neurogenesis.

However, there are a number of limitations to the work presented in this thesis. One of the most difficult aspects of the present studies was finding suitable behavioural tasks that were sufficiently sensitive to detect the subtle effects of α-synuclein overexpression. Moreover, we also had to take into account significant expected motor impairment, and so initially we selected tasks that did not overly rely on the locomotor ability of each animal. In study 1, although we detected α-synuclein in the hippocampus in animals that were administered with both unilateral and bilateral AAV-α-synuclein, there were no clear behavioural effects as a result of this. Similarly, the extent of motor impairment that we observed was not directly comparable to previously published studies. In light of this, for study 2 we increased the dose of viral vector administered, and focused on behavioural tasks that assessed specific cognitive domains, such as pattern separation.

More generally, there are difficulties in extrapolating the data from this thesis to both the human condition and the wider community. Under experimental conditions, it is possible to link specifics of the AAV-α-synuclein model, for example *post* mortem analysis of the extent of dopaminergic neuronal degeneration, to motor dysfunction. Similarly, in the exercise paradigm, an output such as distance run daily/weekly/monthly or levels of neurogenic markers can be correlated to performance in cognitive tasks. However, to compare results gleaned from an experimental model in an outbred rat strain to the heterogenous nature of PD in the human population is obviously quite a leap. Nevertheless, any information that can be gained from preclinical research in some way contributes to the greater knowledge in the field, and the more representative the animal model is, the higher the translational impact of the work.

Furthermore, despite the many advantages of the AAV-α-synuclein model when compared to other neurotoxic animal models of PD, it is not without its caveats. Firstly, despite both genetic and pathological evidence that α-synuclein is inherently linked with the disease process (Polymeropoulos et al., 1997; Spillantini et al., 1997), there is still no known endogenous function for the protein. Its localisation at presynaptic nerve terminals and its interaction with membrane proteins suggest that it plays a role in neurotransmitter release (Bendor et al., 2013). Additionally, it has been shown to be involved in vesicular trafficking (reviewed by Lautenschlager et al., 2017) and dopamine transmission (Butler et al., 2016), all of which are dysregulated in PD. Secondly, the mechanism of α-synuclein-induced cell

death is highly debated. The Braak hypothesis (Braak et al., 2003; Braak et al., 2003b), which was widely accepted, suggested that α-synuclein pathology originates outside of the CNS, and once present in the gastro-intestinal tract it can propagate in a prion-like manner along a vulnerable neuronal network to eventually reach the brain, where the proliferation and accumulation of α-synuclein into Lewy bodies causes degeneration of dopaminergic neurons. However, in recent years, a number of publications have precipitated a critical re-think of this hypothesis. Engelender and Isacson recently published their "Threshold Theory for Parkinson's Disease" (Engelender and Isacson, 2017), where they suggest that α-synuclein pathology is present in both the central and enteric nervous systems at the same time, and that the time of onset of motor and NMS are different due to variances in neuronal vulnerability and the presence of extensive compensatory networks in the brain that are not present in peripheral neurons (Engelender and Isacson, 2017). Similarly, Benskey and colleagues (2016) put forward their 'loss of function' hypothesis, where they postulated that the accumulation of endogenous α-synuclein into LBs and LNs, and accompanying shift in subcellular localisation of α-synuclein from presynaptic terminals to cell soma, impedes the ability of α-synuclein to carry out its normal cellular functions and results in neuronal toxicity as seen in PD (Benskey et al., 2016). Thirdly, there is a growing body of evidence that indicates that the various clinical and pathological features of the synucleinopathies are increasingly dependent on the structure of the α-synuclein that is present. *In vitro* studies have demonstrated the existence

of structurally different α-synuclein strains that display different levels of toxicity and seeding propensity (Bousset et al., 2013; Guo et al., 2013). This was built upon by Peelaerts and colleagues (2015), who fully characterised the distinct histopathological and behavioural phenotypes that are induced *in vivo* after administration of specific α-synuclein strains (Peelaerts et al., 2015). Different strains of α-synuclein have been shown to trigger distinct inflammatory reactions (Gustot et al., 2015) and have also demonstrated species-specific effects (Abdelmotilib et al., 2017).

Taken together, what is clear is that despite the overwhelming evidence that links α-synuclein to the pathophysiology of PD, there are vast gaps in our knowledge. Nonetheless, targeting α-synuclein as a potential therapy has shown promise and currently there are several different strategies that are being investigated, including immune targeting, reducing α-synuclein aggregation or synthesis, blocking the propagation of α-synuclein or enhancing the clearance and degradation of α-synuclein (reviewed by Olanow and Kordower, 2017 and Wong and Krainc, 2017). Of these, immune targeting is currently the most developed method, and a series of animal studies that used either active or passive immunisation of α-synuclein have repeatedly demonstrated significant reductions in α-synuclein-induced neurodegeneration and propagation as well as ameliorating motor deficits (Covell et al., 2017; Games et al., 2014; Spencer et al., 2017; Tran et al., 2014). This has led very recently to the publication of data from the first-in-human trial of PRX002, a monoclonal antibody targeting α-synuclein, which demonstrated not only that the drug has a favourable safety, tolerability and

pharmacokinetic profile but that it can reduce the amount of free serum α-synuclein in a dose-dependent manner (Schenk et al., 2017). Future work is focusing on the completion of a double-blind placebo-controlled trial in patients with PD (Schenk et al., 2017).

α-synuclein is also showing increasing potential as a biomarker. Levels of α-synuclein in the cerebrospinal fluid (CSF) have repeatedly been shown to be elevated when compared to healthy age-matched controls (reviewed by Parnetti et al., 2013). More specifically, it appears that alterations in levels of the oligomeric form of α-synuclein in CSF are more sensitive (Aasly et al., 2014; Tokuda et al., 2010) and has been used to differentiate between PDD and Alzheimer's disease (Hansson et al., 2014). Recently, a combination of antibodies to detect both oligomeric and phosphorylated α-synuclein in CSF has been employed and been shown to successfully discriminate between PD patients and controls (Majbour et al., 2016b). Importantly, this combination of antibodies was also sufficiently sensitive to detect longitudinal changes in α-synuclein that reflect the progressive nature of PD (Majbour et al., 2016a). Nevertheless, lumbar punctures to collect CSF are invasive and high-risk procedures, and so alternative methods of biomarker detection would be highly worthwhile. Outside of the CNS, α-synuclein has been detected in the enteric nervous system (ENS) (Braak et al., 2006a), the olfactory mucosa (Duda et al., 1999), the submandibular gland (Adler et al., 2016) and skin (Michell et al., 2005; Rodríguez-Leyva et al., 2014), although the evidence for using any of these as peripheral biomarkers remains controversial (reviewed by Malek et al., 2014 and Schneider et al., 2016).

In this thesis, we focused on the neuroprotective effects of voluntary exercise to ameliorate both motor and NMS of PD. Increasingly, lifestyle factors are being investigated for their relevance in decreasing the risk of developing PD, but also in the pathogenesis of the disease itself. Large-scale epidemiological studies have repeatedly shown that exercise can protect against the possibility of developing PD (Sasco et al., 1992; Shih et al., 2016; Yang et al., 2015). Moreover, acculumating evidence demonstrates that exercise can also alleviate both motor and NMS of PD (Cusso et al., 2016; LaHue et al., 2016). In this thesis, we showed that voluntary exercise ameliorated hippocampal-associated learning and memory tasks, and that this was likely mediated by modulation of adult hippocampal neurogenesis. The beneficial effects of exercise are thought to be mediated by a wide range of mechanisms, including enhanced hippocampal neurogenesis (reviewed by Ma et al., 2017), attenuation of neuroinflammation (reviewed by Ryan and Nolan, 2016), enhancement of antioxidant species (reviewed by Boccatonda et al., 2016 and de Sousa et al., 2017) and upregulation of the expression of neurotrophic factors (reviewed by Dinoff et al., 2016). Although neurotrophic factors such as GDNF and NRTN have shown some promising results in clinical trials (reviewed by Olanow et al., 2015), data from animal studies demonstrates that the trophic effects of GDNF are blocked by α-synuclein-induced downregulation of the transciption factor Nurr1 and it's downstream receptor, Ret (M. Decressac et al., 2012). Given that in this thesis we show that exercise can ameliorate nigral degeneration as well as motor function in the AAV-α-synuclein model, further

investigation into alternative mechanisms of exercise-induced neuroprotection could provide new insight into novel downstream signalling pathways.

7.1 Final conclusions

In summary, this thesis demonstrates that the AAV-α-synuclein model is a reproducible and robust model of PD that can replicate both the motor symptoms and some of the primary NMS associated with the disease. Moreover, the model can be used to investigate potential therapeutic interventions such as voluntary exercise. There are currently no licensed medical therapies that alter the progression of the disease, and promising data from animal studies investigating neurotrophic factors and cell-based therapies did not translate to human clinical trials. Thus, an animal model that can replicate the primary features of PD could be used to screen potential drug compounds, contribute to knowledge needed to interrogate signalling mechanisms and enhance the translational impact of novel therapeutic compounds and interventions.

Chapter 8

8.0 Future perspectives

This thesis has demonstrated that the AAV-α-synuclein rat model is a consistent and reliable animal model of PD that can be used to reproduce some of the NMS associated with the disease. Moreover, we show that overexpression of AAV-α-synuclein is a viable method to investigate potential therapeutic interventions such as voluntary exercise. These findings represent significant contributions to the present understanding of the neuroprotective effects of exercise, and give an insight into the cellular mechanisms behind these effects.

One of the advantages of employing animal models of PD is the ability to comprehensively investigate cellular signalling and mechanisms that would be otherwise impossible in tissue from patients with PD. The discovery that overexpression of α-synuclein can block the trophic effects of GDNF (M. Decressac et al., 2012) may go some way to explaining the inconsistent results observed in clinical trials of neurotrophic factors (Olanow et al., 2015), and highlights the critical importance of using a viable and representative animal model of PD in pre-clinical research. In this thesis, we show that despite overexpression of α-synuclein, voluntary running can significantly improve dopaminergic survival and performance on hippocampal-associated tasks, which is at least partly due to enhancing adult hippocampal neurogenesis. However, further work is required to fully elucidate the neuroprotective effects of voluntary running in this study. The neurotrophic factor GDF-5 has been shown *in vitro* to mediate its trophic effects through a different signalling pathway compared to other NTFs (Hegarty et al., 2013) and recently an *in vivo* study further demonstrated its

ability to protect hippocampal neurons against toxic insult (Zhao et al., 2017). However, the interplay between GDF-5 and α-synuclein, and other neuropromoting agents, has yet to be investigated.

Overexpression of α-synuclein has been demonstrated to induce a robust neuro-inflammatory reaction (Alvarez-Erviti et al., 2011; Wilms et al., 2009), and the propagation of α-synuclein throughout the brain may be in part due to deficits in microglial activity (Bliederhaeuser et al., 2015). Given that exercise is known to attenuate neuroinflammation (reviewed by Ryan and Nolan, 2016; Spielman et al., 2016), it is also possible that some of the neuroprotective effects seen in this thesis could be due to modulation of microglial responses, and this could be a future direction for this work.

From a more clinical perspective, there are a number of outstanding questions that remain unanswered, both specific to the relationship between α-synuclein and PD, and also about PD itself. Deweerdt recently listed the four big questions about PD (Deweerdt, 2016), namely 1) how does PD begin; 2) what is the role of α-synuclein in PD; 3) what is the role of the gut in PD and 4) what is the best way to divide people with the disease into subtypes.

Taken together, it is clear that for any major therapeutic advances in PD, a viable, robust and reproducible animal model is necessary to comprehensively identify molecular, cellular and behavioural contributions, which in turn will serve to optimise and enhance the translational impact of preclinical research in the area.

Chapter 9

9.0 Bibliography

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Chapter 10

10.0 Publications arising from this work

Journal articles

Crowley EK, Nolan YM and Sullivan AM, 2017. Neuroprotective effects of voluntary running on cognitive dysfunction in an α-synuclein rat model of Parkinson's disease. (*Submitted to Neurobiology of Aging).*

Crowley EK, Nolan YM and Sullivan AM, 2017. Exercise as a therapeutic intervention for motor and non-motor symptoms in Parkinson's disease: evidence from rodent models. *(Submitted to Progress in Neurobiology).*

Abstracts

SM O'Donovan, EK Crowley, OF O'Leary, S Timmons, PW O'Toole, DJ Clarke, NP Hyland, SA Joyce, AM Sullivan, C O'Neill. The brain ↔ gut axis in Parkinson's disease (PD): Altered gut pathology and increased gut inflammation in the rAAV-α-synuclein rat model of PD. International Conference on Alzheimer's & Parkinson's Diseases, Vienna, April 2017.

SM O'Donovan, EK Crowley, OF O'Leary, S Timmons, PW O'Toole, DJ Clarke, NP Hyland, SA Joyce, AM Sullivan, C O'Neill. The brain ↔ gut axis in Parkinson's disease: defining gut pathology in the rAAV-α-synuclein rat model of PD. New Horizons Conference, University College Cork, December 2016.

SM O'Donovan, EK Crowley, OF O'Leary, S Timmons, PW O'Toole, DJ Clarke, NP Hyland, SA Joyce, AM Sullivan, C O'Neill. The brain ↔ gut axis in Parkinson's disease (PD): Altered gut pathology and increased gut inflammation in the rAAV-α-synuclein rat model of PD. The Biochemistry Society Conference, Maynooth, November 2016

SM O'Donovan, EK Crowley, OF O'Leary, S Timmons, PW O'Toole, DJ Clarke, NP Hyland, SA Joyce, AM Sullivan, C O'Neill. The brain ↔ gut axis in Parkinson's disease: defining gut pathology in the rAAV-α-synuclein rat model of PD. APC Microbiome Institute Symposium, UCC, September 2016

Dolan EK, Nolan YM and Sullivan AM. Characterisation of cognitive deficits in an α-synuclein model of Parkinson's disease. New Horizons conference, University College Cork, December 2015.

Dolan EK, Nolan YM and Sullivan AM. Characterisation of cognitive deficits in an α-synuclein model of Parkinson's disease. Network for European CNS Transplantation and Restoration, Lund, Sweden, December 2015

Dolan EK, Nolan YM and Sullivan AM. Characterisation of cognitive deficits in an α-synuclein model of Parkinson's disease. Society for Neuroscience Annual Meeting, Chicago USA. October 2015.

Dolan EK, Nolan YM and Sullivan AM. Motor effects of unilateral and bilateral lesions induced by viral vector-mediated overexpression of alpha-synuclein in a rodent model of Parkinson's disease. Euron: New targets in neurodegenerative diseases, University of Minho, Braga, Portugal, September 2014.

Dolan EK, Nolan YM and Sullivan AM. Characterisation of cognitive deficits in an α-synuclein model of Parkinson's disease. Molecular Medicine Ireland Education and Training Annual Meeting, Trinity College Dublin, March 2014.

Chapter 11

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