Functional characterisation of synuclein-based novel genetic mouse models

Thesis submitted for the degree of
Doctor of Philosophy
Hilary Term 2011

by
Sabina Anwar
Lincoln College, University of Oxford

ABSTRACT

Functional characterisation of synuclein-based novel genetic mouse models

Thesis submitted for the degree of Doctor of Philosophy

Hilary Term 2011

Sabina Anwar, Lincoln College

Synucleins are highly conserved presynaptic proteins with unknown function. α-synuclein plays a key role regulating dopamine homeostasis and is intimately involved in Parkinson's disease (PD) pathogenesis. However, the normal/pathological role of α-synuclein remains unidentified. Studies exploring its function are limited as current transgenic mouse models do not fully recapitulate PD pathology. This thesis reports the functional characterisation of two novel synuclein-based mouse models.

I report the molecular and functional characterisation of transgenic mouse lines with wild-type or A30P-mutant human α-synuclein genomic locus carried within a bacterial artificial chromosome. *SNCA*-A30P^{+}*Snca-/-* mice exhibited a highly physiologically relevant expression pattern of the transgene, including expression in the substantia nigra pars compacta (SNpc) and a specific, age-related loss of TH^{+} cells in the SNpc, the key region of preferential cell loss in PD, compared with non-transgenic *Snca* -/- littermate controls. Analysis of dopamine signalling using fast-scan cyclic voltammetry (FCV) showed young adult *SNCA-A30P*$^{+}$*Snca-/-* mice had an approximately 20% lower evoked extracellular dopamine concentration ($[DA]_o$) compared with non-transgenic *Snca* -/- littermate controls, a decrease specific to the

dorsal striatum. This difference diminished with age and could not be attributed to changes in dopamine reuptake/content.

I detail the behavioural and neurochemical phenotype in mice lacking all three synucleins (α/β/γ). Functional compensation between synucleins emphasises the importance of studying their effects by removing all three proteins simultaneously. Triple-null mice exhibited hyperactivity in a novel environment reminiscent of a hyperdopaminergic-like phenotype, but showed no phenotype in anxiety or motor related tests. FCV revealed synuclein triple-null mice had a two-fold increase in $[DA]_o$, specific to the dorsal striatum and not attributable to changes in dopamine reuptake/content, changes in striatal nicotinic receptor activity nor calcium-dependent changes in dopamine exocytosis.

Together, the analysis from these two novel mouse models reveal synucleins play an important role in altering synaptic function in the dorsal striatum (the region selectively affected in PD) and contributes to growing evidence suggesting synucleins are negative regulators of synaptic dopamine release.

DECLARATION

Except where acknowledgement is made, all the work presented in this thesis was performed by the author at the University of Oxford. The work reported has not been submitted for any other degree in this or any other institute of learning.

ACKNOWLEDGEMENTS

There are many people I would like to thank for the help they afforded me during my time in the Wade-Martins and Cragg labs. Without them I would not have completed this thesis. First and foremost, I am extremely grateful to my supervisors, Richard Wade-Martins and Stephanie Cragg, who have both supported me throughout my thesis studies with expert knowledge, patience and encouragement. Thank you both for your invaluable guidance; I could not have wished for better supervisors. I also offer my sincere gratitude to David Bannerman and Rob Deacon for devoting their time, expert supervision and valuable advice during the behavioural work and subsequent analysis. Thank you also to the numerous labs in the department for kindly allowing me the use of their equipment, and for showing me how to use it! I would also like to express my gratitude to Denise Jelfs, Sian Polley and the excellent team in the Biomedical Sciences facility for their patience and flexibility with the demands of my experiments.

During my daily work, I was lucky enough to be surrounded by exceptional, committed and friendly colleagues. I offer a heartfelt thank you to all members of the Wade-Martins and Cragg labs, both past and present, for making my experiences thoroughly inspiring and enjoyable. From the Cragg lab, particular thanks to Richard Exley and Sarah Threlfell for their time, patience and expert guidance when teaching me the wonder that is FCV (as well as the not-so-wondrous world of electrode making!). Special thanks to Katie Jennings for all her assistance with the HPLC equipment and showing me how to analyse the outputs. There are numerous people in the Wade-Martins lab to whom I owe special thanks; Steve, Liv and Fran all for your

help and support with the animal techniques; Gillian for providing me with advice with my PCR; Michele and Javier for their constructive scientific discussions and valuable insights throughout my thesis studies; and Tara for whom my expression of gratitude for her encouragement and unfailingly sage advice does not suffice.

Most importantly, I would like to thank my family for their resolute support throughout my university studies. Mum and Dad, I wouldn't be who or where I am without you both. To Sophie and Bali, thank you for your unconditional love and constant support. Finally, to my wonderful boyfriend John, who endured my moaning and stresses with patience I didn't know was possible and for always being there to wipe my tears and tell me everything was okay.

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AADC	Aromatic L-amino acid decarboxylase
AAV2	Adeno-associated viral vector 2
AC	Anterior commissure
AC	Phosphatidylcholine
ACh	Acetylcholine
AChE	Acetylcholinesterase
AMPH	Amphetamine
AP	Action potential
BAC	Bacterial artificial chromosome
BG	Basal ganglia
ChAT	Choline acetyl transferase
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CPu	Caudate putamen
CSPα	Cysteine-string protein alpha
DA	Dopamine
DAPI	4'-6'diamidino-2-phenylindole
DAT	Dopamine transporter
DBS	Deep brain stimulation
DHβE	Dihydro-β-erythroidine hydrobromide
DLB	Dementia with Lewy bodies
DLBD	Diffuse Lewy body disease

DNA	Deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
EM	Electron microscopy
ENS	Enteric nervous system
EOPD	Early onset Parkinsons disease
EPSP	Excitatory postsynaptic potential
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
ESC	Embryonic stem cell
FABP	Fatty acid binding protein
FCV	Fast-scan cyclic voltammetry
FISH	Fluorescent *in-situ* hybridisation
GABA	γ-aminobutyric acid
GDNF	Glial-derived growth factor
GFAP	Glial fibrillary acidic protein
GPe	Globus pallidus (external)
GPi	Globus pallidus (internal)
GWASs	Genome wide association studies
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
LB	Lewy bodies
LRRK2	Leucine-rich repeat kinase 2
MAO	Monoamine oxidase
MAP	Microtubule associated protein
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSN	Medium spiny neuron
NAc	Nucleus accumbens
nAChR's	Nicotinic ACh receptors
NET	Norepinephrine transporter
NO	Nitric oxide
NOS	Nitric oxide synthase
OT	Olfactory tubercle
PA	Phosphatidic acid
PAC	P1 artificial chromosomes
PD	Parkinson's disease
PDGFβ	Platelet derived growth factor-β
PET	Positron emission tomography
PINK1	PTEN-induced putative kinase 1
PKC	Protein kinase C
PLD	Phospholipase D
PP2A	Protein phosphatase 2A
PPD	Paired pulse depression
PrP	Prion promoter
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
RRP	Readily release pool
SERT	Serotonin transporter
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SNCA	α-synuclein
SNP	Single nucleotide polymorphism

SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulate
STN	Subthalamic nucleus
TH	Tyrosine hydroxylase
UCHL-1	Ubiquitin carboxyl-terminal esterase L1
UPS	Ubiquitin-proteosome system
VMAT2	Vesicular monoamine transporter 2
VP	Ventral pallidum
VPdl	Dorsalateral ventral pallidum
VPvl	Ventrolateral ventral pallidum
VTA	Ventral tegmental area

TABLE OF CONTENTS

1. CHAPTER 1: Introduction 1
1.1. General Introduction 1
1.2. Parkinson's disease 1
1.2.1. Overview 2
1.2.2. PD Pathogenesis 2
1.2.3. The Basal Ganglia 4
1.2.4. PD Treatments: Present and Future 11
1.2.5. PD Aetiology 16
1.3. The Synuclein Family 22
1.3.1. Overview 22
1.3.2. α-synuclein 23
1.3.3. α-synuclein and PD 24
1.3.4. α-synuclein structure and expression 26
1.3.5. α-synuclein function: overview 28
1.3.6. α-synuclein and synaptic vesicle function 29
1.3.7. α-synuclein and dopamine homeostasis 33
1.3.8. Beta and gamma synuclein 39
1.3.9. β-synuclein: structure and function 39
1.3.10. γ-synuclein: structure and function 41
1.3.11. Summary 43
1.4. Mechanisms of PD Pathogenesis 43
1.4.1. Protein aggregation and PD 43
1.4.2. Oxidative stress and PD 47
1.4.3. Other factors in PD 51
1.4.4. Summary 53
1.5. Animal Models to Study PD 54
1.5.1. Overview 54
1.5.2. Synuclein knockout mouse models 55
1.5.3. Synuclein transgenic mouse models 59
1.5.4. Summary 65
1.6. Thesis Aims 66
2. CHAPTER 2: Materials and Methods 68
2.1. Generation of mice 68
2.2. DNA extraction 69
2.3. PCR 69
2.4. Protein extraction 71
2.5. Western Blotting 71
2.6. Perfusion/Fixation 72
2.7. Immunofluorescence 73
2.8. Immunohistochemistry 73
2.9. Neuronal cell counts 74
2.10. Preparation of primary fibroblasts for fluorescent in-situ hybridisation 76
2.11. Fast-scan cyclic voltammetry 77
2.12. High performance liquid chromatography (HPLC) 79
2.13. Behavioural Protocols 80
2.13.1. Multiple static rods 80
2.13.2. Rotating rod test 80
2.13.3. Non-anxiogenic open-field 81

2.13.4. Holeboard test 81
2.13.5. Home cage activity 81
2.13.6. Spontaneous alternation in a T-maze 82
2.13.7. Elevated plus maze test 83
2.13.8. Bright open field test 83
3. CHAPTER 3: Molecular characterisation of a novel transgenic mouse model of Parkinson's disease carrying the α-synuclein A30P mutation 84
3.1. Introduction 84
3.2. Results 88
3.2.1. Breeding, genotyping and colony management 88
3.2.2. Human α-synuclein protein expression in SNCA-BAC *lines* 91
3.2.3. Confirmation of transgene integrity in SNCA-A30P^{+}*Snca-/- line* 94
3.2.4. Location of transgene integration in SNCA-BAC *lines* 95
3.2.5. Characterisation of spatial transgene expression in SNCA-A30P^{+}*Snca-/- line* 97
3.2.6. Characterisation of transgene expression over time in SNCA-A30P^{+}*Snca-/- line* 108
3.2.7. Initial characterisation of pathology in the SNpc of ageing SNCA-A30P^{+}*Snca-/- line* 115
3.3. Discussion 117
4. CHAPTER 4: Neurochemical characterisation of DA synapse function in a novel transgenic mouse model of Parkinson's disease carrying the α-synuclein A30P mutation 129
4.1. Introduction 129
4.2. Results 134
4.2.1. Decreased levels of electrically evoked DA in the CPu of SNCA-A30P^{+}Snca-/- transgenic mice at ages 5 weeks, 3-4 months and 6-7 months ... 134
4.2.2. Lower evoked $[DA]_o$ in the CPu in SNCA-A30P^{+}Snca-/- transgenic mice is not due to changes in striatal DA content, which is not significantly changed with age 147
4.2.3. Lower evoked $[DA]_o$ in the CPu SNCA-A30P^{+}Snca-/- transgenic mice is not due to changes in DA uptake at striatal synapses, which does not significantly change with age 149
4.2.4. Assessment of frequency sensitivity of DA release and nicotine receptor function in SNCA-A30P^{+}Snca-/- transgenic mice 149
4.3. Discussion 154
5. CHAPTER 5: Functional alterations to the nigrostriatal system in mice lacking all three members of the synuclein family 164
5.1. Introduction 164
5.2. Results 169
5.2.1. Synuclein triple-null mice exhibit a hyperdopaminergic-like behavioural phenotype 169
5.2.2. Increased levels of electrically evoked dopamine in the dorsal striatum of synuclein triple-null mice 176
5.2.3. Decreased dopamine content in the dorsal striatum of synuclein triple-null mice 180
5.2.4. Increased levels of electrically evoked dopamine in the dorsal striatum of synuclein triple-null mice is not due to differences nicotinic receptor function 181

5.2.5. Increased levels of electrically evoked dopamine in the dorsal striatum of synuclein triple-null mice is not due to differences in uptake of DA 183
5.2.6. Increased levels of electrically evoked dopamine in the dorsal striatum of synuclein triple-null mice is not due to calcium dependent changes in exocytosis 184
5.3. Discussion 186
REFERENCES 204

1. CHAPTER 1: Introduction

1.1. General Introduction

Neurodegenerative diseases associated with advanced age represent one of the greatest challenges to the medical sciences. This challenge is rapidly growing in importance due to the ageing population. During the 20th century life expectancy in the developed world has risen by approximately 50%. Future projections suggest this trend will continue, and by 2050 the average life expectancy is predicted to reach between the ages of 80 and 85 years (Hayflick, 2000). Parkinson's disease (PD) is the second most frequently occurring neurodegenerative disease with an incidence which increases with age. At present, PD has a cumulative prevalence of one per thousand (Kuopio et al., 1999), with at least 1.5 million confirmed cases in the United States, and 6 million cases worldwide (Bower et al., 1999; de Rijk et al., 1995; Thomas and Beal, 2007).

Rapid advances in molecular biology have revolutionised neuroscience research. The combination of novel genetic and molecular research tools offers new and exciting opportunities to identify and explore fundamental neural pathways relevant to neurodegeneration. Together, these opportunities promise fresh insight into the causes and progression of these disorders and should drive the development of improved therapeutic interventions for devastating illnesses such as PD.

1.2. Parkinson's disease

1.2.1. Overview

Parkinson's disease (PD) was first described in 1817 by James Parkinson in a seminal article entitled *'An Essay on the Shaking Palsy'*. He described six patients with "involuntary tremulous motion with lessened muscular power, in parts not in action even when supported, with a propensity to bend the trunk forward and to pass from a walking to a running pace" (Parkinson, 1817). Since this description, PD is now recognised as the second most common neurodegenerative disease worldwide (Dauer and Przedborski, 2003).

PD is a progressive movement disorder characterised by the chronic and preferential degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) in the basal ganglia (BG). Despite intensive research efforts yielding a wealth of potential pathogenic mechanisms and pathways, the aetiology of PD is still poorly understood. As a result, available treatments for PD remain largely symptomatic and, to date, no treatments can slow down or halt the progression of the disease.

1.2.2. PD Pathogenesis

The cardinal motor symptoms of PD include a resting tremor, rigidity, bradykinesia and postural instability. The resting tremor presents in approximately 70% of cases and most commonly affects the upper limbs. Rigidity is often described clinically as 'cogwheel' because the muscles respond with cogwheel-like jerks to the use of force in bending the limb. Slowed movement, or bradykinesia, initially causes difficulties

with fine motor commands such as buttoning a shirt, but later progresses to disturb gross movements such as walking. Further progression results in a complete loss of movement, or akinsesia. Postural instability in patients may also be present and causes patients to have a characteristic hunched posture and shuffled gait.

The other motor symptoms in PD are common and include a 'paucity' or absence of facial expressions, difficulty eating and swallowing, speech impairments and sleep disturbances. The co-prevalence of dementia with PD is approximately 25-40% (Aarsland et al., 1996; Brown and Marsden, 1984; Hietanen and Teravainen, 1988) and patients clinically diagnosed with PD are 4-6 times more likely to become demented than control subjects (Mayeux et al., 1990; Riggs, 1993). Further, psychiatric features such as hallucinations, personality changes and, most commonly, depression can also occur (McDonald et al., 2003).

The major pathological hallmark of PD is an extensive loss of dopamine (DA) producing neurons in the SNpc, which project (via the nigrostriatal pathway) to the striatum. This results in a profound reduction in striatal DA content and leads to the consequent motor disturbances (Damier et al., 1999; Samii et al., 2004). The neuronal circuitry involved in coordinated movement is complex and is explained in more detail in section 1.2.3. There may also be a reduction, though to a lesser extent, in other populations of brainstem neurons, leading to further neurotransmitter deficits. Indeed, noradrenaline, serotonin and acetylcholine are reduced in PD due to the loss of neurons in the locus coeruleus, dorsal raphe nuclei and nuclei basalis of Meynert, respectively (Mayeux et al., 1984). Deficits in these non-DA neurons are likely to contribute to many of the non-motor PD symptoms.

A second pathological feature of PD is the presence of intracytoplasmic eosinophilic inclusions called Lewy bodies (LBs) in surviving dopaminergic neurons (Takahashi and Wakabayashi, 2001). LBs were first described in 1812 by Friedrich Lewy in cholinergic neurons of the substantia innominata (Gibb and Poewe, 1986). In 1963, biochemist Oleh Hornykiewicz discovered PD was accompanied by a dramatic reduction striatal dopamine levels (approximately 80-90%), therefore linking the disorder to DA cell loss in the substantia nigra for the first time (Hornykiewicz, 1963). 'Classical' LBs range from 15-30 μm in diameter and have a distinctive laminated spheroid appearance. Ultrastructurally, they are composed of filamentous and granular material (Forno et al., 1996) with the major fibrillary component comprising of a protein called α-synuclein. LB pathology is not restricted to the substantia nigra in PD as it can be present in other brain regions such as the amygdala, locus coeruleus, the dorsal nucleus of the vagus, the thalamus and the cortex (Braak et al., 2002; Forno, 1996).

1.2.3. The Basal Ganglia

The basal ganglia (BG) comprise a collection of interconnected nuclei that lie deep within the cerebral hemispheres and are closely involved in a variety of processes including motor, associative and cognitive functions. The principal nuclei are the caudate nucleus and putamen (together known as the 'striatum'), the globus pallidus (GP) which is divided into internal (GPi) and external (GPe) segments, the subthalamic nucleus (STN) and the substantia nigra (SN) which consists of the substantia nigra pars compacta (SNpc) and the substantia nigra pars reticulata (SNpr).

The striatum is the major input nucleus, or 'receiving area' of the BG. The name (meaning "striped body") is given because the axon fascicles that pass through the caudate and putamen give them a striped appearance when cut in cross section. Although an unequivocal boundary has not been defined (Ikemoto, 2007; Voorn et al., 2004), the striatum can be divided into dorsal and ventral portions based on connectivity and function. The dorsal portion consists of the caudate nucleus and the putamen (caudate putamen or CPu) whilst the ventral portion mainly comprises the nucleus accumbens (NAc) and portions of the olfactory tubercle (OT) as well as the ventral conjunction of the caudate and putamen. Since studies in this thesis have focussed on the CPu and NAc, further anatomical descriptions are provided below.

The neuronal makeup of the CPu and NAc within the striatum are similar. Medium spiny neurons (MSNs) are the biggest population, comprising approximately 90-95% of all striatal neurons (Fallon and Moore, 1978; Oorschot, 1996). MSNs have extensive dendritic processes and are inhibitory, using γ-aminobutyric acid (GABA) as their principal neurotransmitter. Although MSNs are distributed throughout the striatum, they occur in clusters of cells called 'patches' or 'striosomes' and in a surrounding 'matrix' of neurochemically distinct cells (Gerfen, 1992). The distinction between the patch and matrix neurons was originally based on differences in the types of neuropeptides contained by the MSNs in the two regions, but the cell types are now also known to differ according to their cortical inputs and in their outputs to other parts of the BG. In broad terms, even though most cortical areas project to both 'patch' and 'matrix' MSNs, limbic areas of the cortex (such as cingulate gyrus) project more heavily to the patches, whereas motor and somatic sensory areas project

preferentially to neurons in the matrix. This arrangement provides an insight into the degree of complexity and functional subdivision within the striatum. The remaining 5-10% of striatal neurons comprises a diverse collection of interneurons, including a division of large cholinergic interneurons which are discussed in more detail below. Despite having a similar neuronal composition, the CPu and NAc are proposed to have very different functions. The CPu is traditionally proposed to have sensorimotor functions whilst the NAc is believed to fulfil limbic-motor or motivation-action commands.

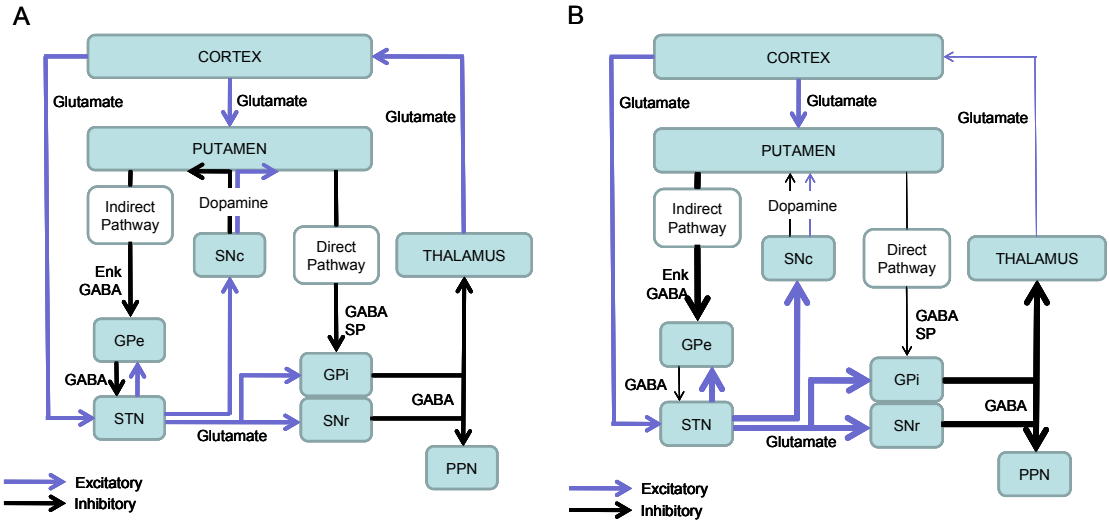


Figure 1.1 ***Basal ganglia circuitry in normal conditions and in PD.*** (A) In the normal brain, parallel neuronal networks of the striatum connect and integrate functions between BG nuclei, the cerebral cortex and the thalamus; (B) in PD, loss of DA leads to an abnormal levels of activity within the BG circuitry. Changes in the PD brain are depicted by the differing thickness of arrows, which represents the relative degree of activation in each projection: reduced output is shown by narrower arrows and enhanced output is shown by bolder arrows.

The circuitry of the CPu is important for understanding movement disorders such as PD. The striatum receives input, via glutamatergic synapses on dendritic spines of MSNs, from the cerebral cortex and thalamus. The CPu also receives dense

dopaminergic innervations from neurons in the SNpc and to a lesser extent from neurons in the ventral tegmental area (VTA) (Björklund and Hoekfelt, 1984a, b; Haber et al., 2000) via the nigrostriatal pathway. Significantly in PD, dopamine neurons in the SNpc are lost, whereas dopamine neurons in the VTA are relatively spared. The cortical input is processed and then channelled, via either a 'direct' or an 'indirect' pathway to the output nuclei of the BG, the GPi and the SNr. These output nuclei in turn transmit information, via the thalamus, back to the cerebral cortex, forming a subcortical feedback circuit, or 'loop'.

In the direct pathway, inhibitory striatal MSNs project directly to the output nuclei of the BG, the GPi and SNpr, which in turn project to the thalamic relay nuclei (Parent and Hazrati, 1995). These output projections are also inhibitory (or GABAergic) and represent the main output of the BG. An excitatory, glutamatergic projection from the thalamic nuclei back to the cortex completes the direct pathway. It is hypothesised that activation of MSNs via the direct pathway decreases the inhibitory output from the BG and, therefore, *disinhibits* the motor thalamus, consequently facilitating movement (Chevalier and Deniau, 1990).

In the indirect pathway, inhibitory striatal MSNs project to the GPe, which send GABAergic projections to the STN. Instead of projecting to structures outside the BG, the STN then projects to the BG output nuclei (GPi and SNpr) via an excitatory, gluatamatergic projection. From here, the indirect pathway follows the direct path, via the thalamus, back to the cortex. Activation of MSNs via the indirect pathway inhibits the GPe, which then sends less inhibition to the STN. This reduced inhibition allows

the STN to send more excitation to the GPi, resulting in enhanced inhibitory output from the BG to the motor thalamus, consequently impeding movement.

The dopaminergic innervation into the CPu is critical in controlling the balance of activity between the direct and indirect pathway in the BG. DA is a neuromodulatory transmitter, and DA receptors are G-protein coupled receptors and can be classified as either 'D1-like' which are excitatory, or 'D2-like' which are inhibitory. DA activates the direct pathway through acting on a population of MSNs which predominantly express excitatory D1 receptors. These activated MSNs in turn inhibit GABAergic neurons in the GPi, releasing the thalamus from inhibition and promoting movement.

DA also acts to inhibit the indirect pathway via activation of MSNs which express inhibitory D2 receptors. These MSNs project to the GPe, where they inhibit a GABAergic projection to the STN. This suppresses the glutamatergic projection from the STN to the GPi, curtailing the normally enhanced inhibitory output from the BG. In PD, the loss of dopaminergic neurons in the nigrostriatal pathway leads to a dramatic reduction in the DA content in the striatum. This results in activation of striatal outputs to the GPe (promoting the indirect pathway) and inhibition of striatal projections to the GPi and SNpr (suppressing the direct pathway). Ultimately, the consequences of the DA loss in the SNpc and striatum manifest as the hypokinetic movement dysfunction seen in PD.

The NAc is organised into two major subdivisions, the NAc core and the NAc shell. The NAc core sits around the anterior commissure (AC) whilst the NAc shell lies ventral to the NAc core. The NAc MSNs receive extensive excitatory inputs from the

prefrontal cortex, the hippocampus and the amygdala (Haber et al., 2000; Heimer, 2000). The NAc also receives dopaminergic innervation mainly from cells in the VTA, and to a lesser extent from cells in the SNpc (Haber et al., 2000) via the mesolimbic pathway. These dopaminergic projections innervate both the NAc core and NAc shell divisions with little discrimination. There is further NAc afferent input from the ventral pallidum (VP) (Groenewegen et al., 1993). Inhibitory output projections of the NAc core target the dorsalateral VP (VPdl), the SNpc and the SNpr. The outputs of the NAc shell also project to the SNpc as well as to the ventrolateral VP (VPvl), the VTA and the lateral preoptic and lateral hypothalamic areas (Usuda et al., 1998).

Although detailed circuitry and functional roles of NAc divisions are beyond the scope of this introduction, their effects on motor outcomes should be discussed. Whilst the NAc shell is believed to have a more selective role in governing motor actions in association with unconditioned stimuli, the NAc core is thought to have an important role in selecting, integrating and executing motor actions in response to stimuli which are complex, context-dependent or that have motivational significance or value (i.e. reward-related) (Di Ciano et al., 2001; Di Ciano and Everitt, 2001; Parkinson et al., 1999). To do this, a significant degree of well-defined neuronal connectivity between the mesolimbic and nigrostriatal DA pathways exists (Ikemoto, 2007). This includes NAc core and shell MSNs having inhibitory innverations to the SNpc (Heimer et al., 1991; Usuda et al., 1998) via a striato-mesencephalic circuit. This allows signals transmitted from the mesolimbic pathway in the NAc to influence the CPu via the nigrostriatal pathway (Alexander et al., 1986; DeLong, 1990) through what is proposed to be an elaborate ‘spiral’ organisation of connections between the

striatum and ventral midbrain (Haber et al., 2000). Another circuit, called the striato-pallido-mesencephalic circuit, can also send information from the NAc to the nigrostriatal DA pathway. Here, the NAc core and shell project to the VPdl and VPvl (Usuda et al., 1998) which then project to the SNpc (Groenewegen et al., 1993). The SNpc then influence motor actions via the CPu. A third, striato-pallido-thalamo-corical circuit also allows information transfer from the NAc to the CPu. Through this intricate circuitry, mediated largely by DA pathways in the BG, signals from the NAc can influence intelligent and cognition-guided movement via what is termed the 'rational motor system', consistent with the idea that the NAc serves as a limbic-motor or motivation-action interface (Nauta et al., 1978).

As discussed, the striatum has dense dopaminergic innervation, but it also has very dense cholinergic innervation (Björklund and Hoekfelt, 1984a, b; Zhou et al., 2001). Despite being only 1-3% of total striatal neurons, cholinergic interneurons, which use acetylcholine (ACh) as their principal neurotransmitter, have extensive and extremely dense axonal arborisations (compared to other striatal neurons). Consequently the striatum displays the densest staining in the central nervous system for cholinergic markers such as choline acetyl transferase (ChAT) and acetylcholinesterase (AChE) (Hebb, 1957; Holt et al., 1997; Macintosh, 1941). These neurons provide the sole cholinergic input into the striatum (Contant et al., 1996; Woolf, 1991). Dynamic and cooperative interactions between dopaminergic and cholinergic neurons are pivotal to the proper functioning of the striatum (Cragg, 2006; Rice and Cragg, 2004; Zhang and Sulzer, 2004; Zhou et al., 2001). At the presynaptic level, DA and ACh interact with each other directly, since local and systemic application of D_1-receptor agonists enhances striatal ACh release *in vivo*, while D_2-receptor agonists can reduce ACh

release (DeBoer et al., 1996; Ikarashi et al., 1997). Moreover, striatal ACh acts on nicotinic ACh receptors (nAChRs) located on dopaminergic axon to either inhibit or enhance DA release, depending on the extracellular ACh concentration and the frequency of activity in the presynaptic DA neurons (Cragg, 2005; Rice and Cragg, 2004; Zhang and Sulzer, 2004; Zhou et al., 2001). At the postsynaptic cellular level, a more clear-cut antagonistic relationship between DA and ACh exists and the two transmitters can have opposing effects on striatal output neurons (Calabresi et al., 2000; Centonze et al., 2003; Pisani et al., 2003; Reynolds et al., 2001). This ACh-DA balance hypothesis is supported by earlier reports which found that symptoms seen in PD could be alleviated by treatments which either promote striatal DA, or reduce ACh (Barbeau, 1962; Pisani et al., 2003).

1.2.4. PD Treatments: Present and Future

Currently available treatments for PD remain largely symptomatic and, to date, no treatments can slow down or halt the progression of the disease. This highlights the pressing need for a more thorough understanding of the molecular events involved in PD pathogenesis in the hope that novel targets for therapy, and even disease prevention, can be identified.

At present, the first line of treatment in PD is striatal DA replacement to alleviate motor symptoms. Levodopa (L-DOPA) remains the most effective drug for the treatment of PD symptoms. Levodopa is a transient metabolic intermediate in the DA synthesis pathway. It is synthesised from tyrosine by tyrosine hydroxylase (TH) and is converted to DA by aromatic L-amino acid decarboxylase (AADC). Levodopa is

usually administered with peripheral AADC inhibitors such as carbidopa or benserazide. This reduces the dose of levodopa required and helps to decrease the peripheral side effects, such as nausea, caused by conversion of DA in the periphery (Rascol et al., 2002). Levodopa penetrates the blood-brain barrier, whereas decarboxylase inhibitors do not, therefore levodopa can be metabolised to DA by the remaining TH^+ neurons in the SNpc and striatum. However, levodopa therapy has a number of shortcomings associated with its long-term use. Dyskinesias usually involving involuntary writhing movements, particularly of the mouth, face and limbs occur due to peak and end dose effects of DA. These can affect over half of patients on levodopa therapy after five years (Hely et al., 1994; Nutt and Nygaard, 2001). Motor fluctuations, or the 'on-off effect', are also a side effect of levodopa and refer to rapid fluctuations in the clinical state of a patient from a mobile to an immobile state, thought to be a consequence of the 'wearing off' of levodopa (Nutt, 2001). These motor fluctuations affect approximately 20-50% of patients after 5-6 years of levodopa therapy (Hely et al., 1994; Koller et al., 1999; Montastruc et al., 1994). Clearly, the margin between the beneficial effects of levodopa and the unwanted effects of the drug become progressively narrower over time.

Although levodopa remains the frontline treatment for PD, other drugs, especially in early-stage patients, may be used. DA receptor agonists, such as bromocriptine, are favoured as a first line treatment in younger patients because dyskinesias are 50% less common than with levodopa therapy (2000). There is putative evidence *in vitro* which suggests bromocriptine may have additional neuroprotective benefits by acting as a free radical scavenger and there are suggestions that, *in vivo*, it may minimise damage caused by oxidative stress by means of reducing DA turnover (Lange et al., 1994;

Olanow and Jankovic, 2005; Yamamoto, 1998). At present, however, these results remain controversial (Marek et al., 2002). DA receptor agonists are not without their side effects (Singh et al., 2007) and are not as effective as levodopa in alleviating motor symptoms such that most patients will require levodopa therapy within 2-5 years (Silver and Ruggieri, 1998).

Other DA-based pharmacological therapies include catechol-O-methyltransferase (COMT) inhibitors such as tolcapone and monoamine oxidase (MAO) inhibitors such as selegiline. Anticholinergics may also be used as therapeutic agents, and are believed to exert their effects via muscarinic receptors, by correcting the disequilibria between striatal DA and ACh. While all of these agents offer benefits in PD treatment, they are not effective alone, are not without side effects and patients taking these drugs will require a transition to levodopa therapy with time (Singh et al., 2007).

Prior to the use of levodopa and other pharmacotherapy, in the 1960's surgical interventions had been used as a treatment for PD. Now, as the shortcomings of levodopa associated with long-term use have become apparent, surgical strategies have once more become an important treatment target in PD. The aim of surgery is to restore the functional balance in the BG lost through the degeneration of the nigrostriatal pathway. The targets for surgery are the ventral intermediate nucleus of the thalamus, the GPi or the STN. Surgical techniques may be ablative, in procedures called thalamotomy, pallidotomy or subthalamotomy. In 1992, a landmark publication by Laitinen and colleagues described the relief or substantial improvement of tremor in 81% of PD patients undergoing pallidotomy and a reduction by up to 92% of rigidity and bradykinesia, alongside concurrent relief of levodopa-induced dyskinesias

(Laitinen et al., 1992). Most recently, technical advances have made an intervention called deep brain stimulation (DBS) the preferred surgical tool for PD treatment. DBS is preferred to structural lesioning techniques as they create 'functional' lesions which are adjustable for the needs of the patient, non-destructive and reversible (Lang, 2000). In DBS, an implanted electrode delivers a high frequency electrical current, reversibly ablating the target structure. The current preferred targets for DBS are the GPi and STN. Surgical procedures are not without their shortcomings, and although rare, surgical complications with DBS reportedly arise in 1-2% of patients. Side effects which may occur include a worsening of motor symptoms, although this can often be relieved by adjusting the stimulation parameters. Deterioration in cognition and effects on mood, caused by inadvertent stimulation of the limbic system, have also been noted with DBS (Parsons et al., 2006; Saint-Cyr et al., 2000).

Experimental and novel therapeutic avenues for treating PD include cell replacement strategies, gene therapy and neuroprotection. PD is a popular candidate for cell-replacement because of the specificity of the lesion and the homogeneity of the degenerating cell population. In the 1980s, PD patients received striatal cell-grafts from various sources, including fetal tissues and porcine fetal SNpc neurons. Although some open-label studies using mesencephalic fetal grafts showed some success in treating PD symptoms in humans, placebo controlled clinical trials failed to exhibit overall clinical benefits (Freed et al., 1992; Freed et al., 2001; Freeman et al., 1995; Lindvall et al., 1990; Lindvall et al., 1992; Olanow et al., 2003). Further, the best result of any transplant study in PD is not greater than the clinical successes resulting from DBS of the STN or parenteral delivery of levodopa and apomorphine (Obeso et al., 2010; Olanow et al., 2009). Coupled with the costs, risks associated

with the procedure, and the difficulties obtaining adequate donor tissue volumes, fetal graft therapy for PD arguably no longer offers the potential it once promised. More recently, there has been attention focused in the use of human stem cells as a cell-replacement strategy alternative. Studies have shown stem cells can survive, and have some success in forming innervations and even in reversing motor symptoms in rodent and monkey models of PD (Bjorklund et al., 2002; Cai et al., 2009; Redmond et al., 2007). For example, Bjorklund and colleagues reported that transplanted undifferentiated embryonic stem cells (ESCs) provided some behavioural recovery in 6-OHDA lesioned rodents, however several subjects developed tetromas (Bjorklund et al., 2002). Furthermore, the use of ESCs has raised ethical concerns and still faces many of the challenges associated with fetal grafts such as immune rejection of the transplant. The use of induced pluripotent stem cells (iPS cells) offer potential as they do not come with the same ethical concerns as ESCs and allow the delivery of cells of an autologous origin, therefore removing adverse immune responses which can occur after transplantation (Kiskinis and Eggan, 2010; Soldner et al., 2009). When evaluating the future use of iPS cells and other cell-replacement strategies, clear advantages over existing treatments need to exist, such as improved efficacy, if they are to be viable future therapeutic avenues and, at present, further experimental data is required before this can be assessed.

Gene therapy approaches for PD treatment involve either attempting to restore DA synthesis through the delivery of necessary enzymes or stopping the neurodegenerative process through the delivery of neuroprotective agents such as glial-derived growth factor (GDNF). There are clinical trials currently in progress testing both approaches which have shown some success. For example, adeno-

associated viral vector 2 (AAV2) mediated delivery of AADC aims to make the conversion of levodopa to DA more efficient, giving optimal therapeutic benefits on lower doses (thus reducing levodopa induced side effects) and has been through successful phase I clinical trials (Eberling et al., 2008). Phase I trials for the AAV2-mediated delivery of neurturin, a functional analog of neuroprotective agent GDNF, have also been met with success (Marks et al., 2008) after showing promise in rodent and primate models (Gasmi et al., 2007; Kordower et al., 2006). Planned trials will soon show whether these approaches can deliver further success in phase II trials. Other agents with proposed neuroprotective potential in PD include MAO-B inhibitors such as selegiline, anti-apoptotic agents, glutamatergic antagonists, promitochondrial drugs such as coenzyme Q10 and growth factors such as GDNF. However, none of these candidates have so far shown definitive neuroprotective success in clinical trials (LeWitt and Taylor, 2008).

Clearly, the advancement and future success of experimental strategies for PD treatment will markedly benefit from an improved understanding of the aetiology and pathogenesis of PD and will allow the identification of novel potential therapeutic candidates for targeting neurodegeneration.

1.2.5. PD Aetiology

Despite many years of research, the aetiology of PD remains unknown. Age is the largest independent risk factor for the development of PD (de Rijk et al., 1995), whilst the relative contributions of genetic versus environmental factors have been intensely debated. Early cross sectional studies examining PD heritability in twins have not

shown concordance between monozygotic (MZ) and dizygotic (DZ) twins (Marttila et al., 1988a; Tanner et al., 1999; Ward et al., 1983) and suggest that environmental factors lead to PD. Several environmental hypotheses have been proposed, one of the earliest being a potential infectious aetiology linked to influenza A (Takahashi and Yamada, 1999). The best established environmental hypothesis relates to the effects of environmental toxins on the development of PD. In 1982, a group of young drug users developed a rapidly progressive parkinsonian syndrome as a result of the accidental injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a by-product of synthetic heroin (Langston et al., 1983). MPTP crosses the blood brain barrier, where it is converted to the active toxin MPP+ by MAO-B. MPP+ is a high affinity substrate for the DA transporter (DAT). Once inside DA neurons, MPP+ acts as a potent mitochondrial complex I inhibitor. Consequently, MPTP-based rodent and primate animal models have become common and important research tools for studying PD pathogenesis. Subsequently, long-term exposure to other chemically similar pesticides, such as rotenone and paraquat have been linked to an increased risk of developing PD in humans (Brown et al., 2006; Engel et al., 2001; Liou et al., 1997) and have been shown to cause parkinsonism in animal models. Other environmental factors associated with PD include exposure to heavy metals (Gorell et al., 1999a; Gorell et al., 1999b) and solvents (McDonnell et al., 2003). There are also possible protective factors, including a history of cigarette smoking and caffeine use (Checkoway et al., 2002; Hancock et al., 2007; Paganini-Hill, 2001; Powers et al., 2008).

The identification of inherited forms of PD shifted research attention back towards genetic factors. In the last decade, several causative genes and susceptibility factors

have been identified in rare families with Mendelian inheritance and 5-10% of patients are now known to have monogenic forms of PD (Lesage and Brice, 2009). Even though early twin studies showed poor concordance rates for PD, studies using ^{18}F-DOPA based positron emission tomography (PET) to detect early, subclinical loss of DA neurons have revealed substantial (up to 75%) concordance in MZ twins and an approximate 28% concordance in DZ twins, indicating an interaction between genetic and environmental factors exists (Burn et al., 1992; Piccini and Brooks, 1999). To date, linkage analysis in families has identified 13 loci and 9 genes associated with autosomal dominant and autosomal recessive PD (Lesage and Brice, 2009). These have been summarised in Table 1.1. They include: *SNCA* (*PARK1* and *PARK4*; *PARK4* corresponds to a triplication of the gene locus) (Polymeropoulos et al., 1997; Singleton et al., 2003), *PARKIN* (*PARK2*) (Kitada et al., 1998), *UCHL-1* (*PARK5*) (Leroy et al., 1998a; Leroy et al., 1998c), *PINK1* (*PARK6*) (Valente et al., 2004), *DJ1* (*PARK7*) (Bonifati et al., 2003), *LRRK2* (*PARK8*) (Funayama et al., 2002; Zimprich et al., 2004), and *ATP13A2* (*PARK9*) (Ramirez et al., 2006).

Locus	Gene	Map position	Forms of PD	Protein Function	Associated mutations	References
PARK1/PARK4	SNCA	4q21	EOPD AD and sporadic		A30P, A53T, E46K, genomic duplications / triplications	(Polymeropoulos et al., 1997)
PARK2	Parkin	6q25.2-q27	Juvenile and EOPD AR and sporadic	Ubiquitin E3 Ligase	>100 mutations (point mutations, exonic rearrangements)	(Kitada et al., 1998)
PARK3	Unknown	2p13	LOPD AD	Unknown	Not identified	
PARK5	UCHL1	4p14	LOPD AD*	Ubiquitin Esterase	One mutation in single PD sibling pair	(Leroy et al., 1998a)
PARK6	PINK1	1p35-p36	ARPD	Mitochondrial kinase	>40 point mutations, rare large deletions	(Valente et al., 2004)
PARK7	DJ-1	1p36	EOPD AR	Uncertain, putative antioxidant properties	>10 point mutations and large deletions	(Bonifati et al., 2003)
PARK8	LRRK2	12q12	LOPD AD and sporadic	Protein kinase	>40 missense variants, >7 of them pathogenic including the common G2019S	(Funayama et al., 2002)
PARK9	ATP13A2	1p36	Juvenile AR Kufor-Rakeb syndrome and EOPD	P-type ATPase	>5 point mutations	(Ramirez et al., 2006)

PARK10	Unknown	1p32	Unclear	Unclear	Not identified	
PARK11?	GIGYF2	2q36-q37	LOPD AD		Seven missense variants	
PARK12	Unknown	Xq	Unclear		Not identified	
PARK13	Omi/HRTA2	2p13	Unclear	Anti-apoptotic	Two missense variants	(Strauss et al., 2005)
PARK14?	PLA2G6	22q13.1	Juvenile AR levodopa responsive dystonia-parkinsonism		Two missense mutations	
PARK15?	FBXO7	22q12-q13	EO AR parkinsonian-pyramidal syndrome		Three point mutations	
N/A	NR4A2/NURR1	2q22-23	AR	DA neuron differentiation		(Le et al., 2003; Xu et al., 2002b; Zheng et al., 2003)
N/A	Synphilin-1, SNCAIP1	5q23.1-.3	Unclear	Protein aggregation		(Chung et al., 2001; Marx et al., 2003)
Not assigned**	SCA2	12q24.1	Unclear, dominant for SCA2		Low range interrupted CAG expansions in SCA2	

Not assigned**	GBA	1q21	Unclear, recessive for GD	Heterozygous GD-associated mutations increase risk for PD

Table 1.1 ***Summary of genes associated with PD***. EO, early onset; LO, late onset; AD, autosomal dominant; AR, autosomal recessive; PD, Parkinson's disease; GD, Gaucher's disease; SCA2, spinocerebellar ataxia type 2; SNCA, α-synuclein; UCHL1, ubiquitin carboxy-terminal hydrolase L1; PINK1, PTEN-induced kinase 1; LRRK2, Leucine-Rich Repeat Kinase 2; GIGYF2, GRB10-interacting GYF protein 2; PLA2G6, group VI phospholipase A2; GBA, β glucocerebrosidase. *Kindred analysis indicates incomplete penetrance; **PD-associated genes proposed by candidate gene approach. (Modified from (Lesage and Brice, 2009)).

While pathogenic mutations associated with these genes only account for a small percentage of all PD cases, they provide valuable insights into the pathogenic dysfunctions and molecular pathways associated not only with genetic forms of the disorder, but of sporadic PD as well. Mutations in these PD associated genes have been linked to molecular cascades which eventually lead to cell death. These cascades include oxidative stress, impaired mitochondrial dysfunction, excitotoxicity, protein misfolding and aggregation, impaired lysosome and chaperone mediated autophagy, all central areas of PD research for elucidating the mechanisms of cell death (Dauer and Przedborski, 2003; Hindle, 2010; Zhang et al., 2006). For example, the gene product of *PARKIN*, parkin, is an E3-ubiquitin protein ligase which identifies and targets proteins, via a ubiquitin tag, to the proteosome for degradation. Mutations in parkin abolish this E3 ligase activity (Kitada et al., 1998; Shimura et al., 2000). Meanwhile, *UCHL-1* has been identified as an enzyme involved in the de-conjugation of ubiquitin and mutations in this protein are believed to lead to the loss of recycling of ubiquitin monomers. Together, mutations in these genes indicate abnormal handling of misfolded proteins occurs and identifies a role for the ubiquitin-proteosome system in the pathogenesis of PD. Among the different genetic forms of PD, mutations in the *SNCA* gene, which codes for the protein α-synuclein, has received the most attention. α-synuclein, along with β-synuclein and γ-synuclein, belong to a small family of proteins called synucleins.

1.3. The Synuclein Family

1.3.1. Overview

The synuclein family of proteins arise from three distinct genes and, so far, have only been described in vertebrates. They are small (113-143 amino acids), closely related proteins which are expressed primarily in neural tissue and are particularly enriched in presynaptic terminals. Structurally, all three proteins are distinctive and highly conserved, sharing a common design. The first two thirds of the sequence (~90 residues) are organised around an amphipathic motif, which include a variable number of 11-residue repeats. The carboxy-terminal is less well conserved but is generally acidic, with a preponderance of glutamate residues (Clayton and George, 1998). Despite intense research efforts, the normal functions of these proteins are still to be elucidated. The proteins will be discussed further below, with emphasis on α-synuclein, given its intimate association with PD pathogenesis.

1.3.2. α-synuclein

α-synuclein was first isolated in 1988 from the Pacific electric ray *Torpedo californica* using an antiserum raised against purified cholinergic terminals (Maroteaux et al., 1988). It was given the name synuclein because of its localisation to the nuclear envelope of neurons and to presynaptic nerve terminals (Lavedan, 1998; Maroteaux et al., 1988). The proteins' association with nuclear membranes has not been consistently observed since, but the name has been retained. In 1993, Ueda and colleagues reported that a short peptide, termed non-amyloid component (or NAC) from amyloid plaques from the brains of Alzheimer's disease patients was derived from a larger precursor protein, called the non-amyloid component precursor (or NACP). The NACP was identified as homologous to that of rat α-synuclein (Jakes

et al., 1994; Ueda et al., 1993). This finding provided the first link for a potential role for synuclein proteins in neurodegeneration.

1.3.3. α-synuclein and PD

Since the initial link with neurodegeneration, there have been a number of seminal findings over the past decade which has placed α-synuclein firmly at the centre of research into PD pathogenesis. The first was the identification of three missense mutations in the *SNCA* gene which caused autosomal dominant forms of PD in a number of families (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). All three mutations cause early onset PD (EOPD) with a high penetrance. The second link came with the discovery that α-synuclein was present in LBs in post mortem brain tissue of patients with sporadic forms of PD (Spillantini et al., 1997). Thirdly, short chromosomal duplications or triplications containing the *SNCA* gene, plus relatively short flanking regions of chromosome 4, were discovered to cause autosomal dominant PD (Chartier-Harlin et al., 2004; Singleton et al., 2003). Finally, recent genome-wide association studies further support the notion that α-synuclein is critically linked to PD (Edwards et al., 2010; Pankratz et al., 2009; Satake et al., 2009; Simon-Sanchez et al., 2009).

In 1997, a point mutation (G209A) which caused an alanine to threonine amino acid residue substitution at point 53 (A53T) in the sequence of the *SNCA* gene was reported in a large Italian-American family and three smaller unrelated Greek families with autosomal dominant EOPD (Polymeropoulos et al., 1997). Since then, two more mutations have been found in unrelated families with autosomal dominant forms of

PD; an alanine to proline substitution at position 30 (A30P) was identified in a German kindred (Kruger et al., 1998) and a glutamate to lysine substitution at position 46 (E46K) (Zarranz et al., 2004) was identified in kindred with either classical PD or PD with pathological features of dementia associated with widespread Lewy pathology, termed dementia with Lewy bodies (or DLB).

Soon after the first genetic link between α-synuclein and PD, it was found that antibodies against α-synuclein resulted in strong staining of the protein in post-mortem brain tissue of patients with sporadic forms of PD (Spillantini et al., 1998; Spillantini et al., 1997). LBs are a key pathological feature in sporadic PD and the discovery that they contain α-synuclein suggests a common pathogenesis of both sporadic and familial PD.

More recently, duplication and triplication of the *SNCA* locus has been shown to cause autosomal dominant PD. Singleton and colleagues identified a triplication of *SNCA* locus in a large Iowa family with severe EOPD (Singleton et al., 2003). *SNCA* duplication and triplication mutations were subsequently reported in independent families with autosomal dominant PD (Chartier-Harlin et al., 2004; Farrer et al., 2004). These gene multiplications cause increased expression of α-synuclein, the degree to which correlates to the severity of PD symptoms. For example, individuals carrying triplication mutations in the *SNCA* locus had an earlier age of PD onset and more severe and widespread degeneration than individuals carrying *SNCA* gene duplications (Chartier-Harlin et al., 2004; Eriksen et al., 2005).

Most recently, two large genome wide association studies (GWASs) have demonstrated a strong association of sporadic PD with common single nucleotide polymorphisms (SNPs) within the *SNCA* locus, in two populations with different ancestry (Satake et al., 2009; Simon-Sanchez et al., 2009). These findings support previous reports that variation at the *SNCA* locus increases susceptibility to PD (Maraganore et al., 2006; Maraganore et al., 2005; Pankratz et al., 2009). Most recently, another GWAS identified an identical *SNCA* variant previously reported by Simón-Sánchez et al. in a different population (Edwards et al., 2010). The association of common variants of *SNCA* with an increased PD risk in three independent GWASs strongly implies that a pathological role for α-synuclein in PD is not confined to rare, familial forms of PD, but also in sporadic forms of the disorder.

1.3.4. α-synuclein structure and expression

The human *SNCA* gene maps to chromosome 4q21.3-q22 (Chen et al., 1995; Shibasaki et al., 1995; Spillantini et al., 1995) where it spans ~111 kb of genomic DNA. The gene is comprised of six exons, the first of which is untranslated (Touchman et al., 2001; Xia et al., 2001). The mouse *SNCA* gene has been mapped to chromosome six and the intron/exon structure of both human and mouse *SNCA* are highly conserved; the human and murine protein sequences are 95.3% identical (Lavedan, 1998; Touchman et al., 2001). The human *SNCA* gene encodes a 140 amino acid protein of approximately 19 kDa (Touchman et al., 2001). The protein comprises an amphipathic N-terminal region which is characterised by a series of 11 imperfect amino acid repeats containing the core sequence KTKEGV (residues 1-61), a hydrophobic central domain which corresponds to the NAC peptide (residues 61-95)

and an acidic C-terminus (residues 96-140). α-synuclein exists in a variety of conformations depending on the environment (Uversky, 2003). In aqueous solution, α-synuclein is "natively unfolded" and as such, α-synuclein belongs to a class of intrinsically unstructured proteins which may act to facilitate protein-protein interactions, (Davidson et al., 1998; Eliezer et al., 2001; Jakes et al., 1994; Weinreb et al., 1996). Other proteins in this family include tau and microtubule associated protein-2 (MAP2), both of which bind and influence cytoskeletal elements. In contrast, α-synuclein adopts a more ordered α-helical structure in the presence of acidic phospholipids. The amphipathic 11-residue N-terminal repeating structure of α-synuclein has a very similar organisation to the class-A_2 helix family of apolipoproteins which are capable of lipid interactions. Consistent with this, α-synuclein reversibly binds phospholipid membranes which occurs together with a large change in the structure of the protein from approximately 3% to over 70% α-helical (Li et al., 2001; Perrin et al., 2000). In LBs, α-synuclein assumes a fibrillary β pleated-sheet conformation as insoluble aggregations, and binds other proteins such as synphilin-1, parkin, and the antiapoptotic chaperone 14-3-3. The protein possibly exists in further conformations in high molecular weight aggregates (George et al., 1995; Kawamoto et al., 2002; Maroteaux and Scheller, 1991; Nakajo et al., 1990; Schlossmacher et al., 2002; Wakabayashi et al., 2001). The acidic C-terminus of α-synuclein remains free and unfolded in solution, and does not associate with vesicles. It appears to be critically involved in mediating the chaperone-like activity of α-synuclein, which is lost if the C-terminal is removed (Eliezer et al., 2001; Ostrerova et al., 1999).

The α-synuclein protein is predominantly expressed in neurons throughout the central nervous system (CNS). In the adult brain, it is expressed in the neocortex, hippocampus, olfactory bulb, striatum, substantia nigra, thalamus, amygdala, nucleus accumbens, and cerebellum (Galvin et al., 2001; George et al., 1995; Iwai et al., 1995; Jakes et al., 1994; Maroteaux and Scheller, 1991; Masliah et al., 1996; Mori et al., 2002; Shibayama-Imazu et al., 1993). On an intracellular level, α-synuclein expression seems to change during the development of the CNS. It is initially confined to the cell bodies of neuronal precursors, but is redistributed to presynaptic nerve terminals later in development where it exists in close proximity to, though not bound with, synaptic vesicles (Hsu et al., 1998; Iwai et al., 1995; Nakajo et al., 1994). The loose association of α-synuclein to synaptic vesicles is confirmed by the finding that α-synuclein is present in crude synaptosomal fractions, but it is absent from highly purified synaptic vesicle preparations (Irizarry et al., 1996; Kahle et al., 2000b; Shibayama-Imazu et al., 1993).

1.3.5. α-synuclein function: overview

Despite over a decade of intense study, the normal physiological functions of α-synuclein remain largely unknown. It has been shown to interact with a wide range of proteins and consequently has been implicated in several biochemical pathways, making its precise function difficult to elucidate. α-synuclein can interact with cytoskeletal-associated proteins such as tau (Jensen et al., 1999), mitogen activated protein 1B (MAP1B) (Jensen et al., 2000), MAP2 (D'Andrea et al., 2001), tubulin (Payton et al., 2001), actin (Alim et al., 2004; Zhou et al., 2004) and synphilin-1 (Engelender et al., 1999; Kawamata et al., 2001). α-synuclein has also been shown to

interact with numerous protein kinases and phosphatases such as extracellular regulated kinase (ERK), protein kinase C (PKC) (Iwata et al., 2001; Li and Lee, 2005; Ostrerova et al., 1999). Perhaps the most intriguing is α-synuclein's considerable sequence similarity with the phospho-dependent signalling chaperone 14-3-3 (Yaffe, 2002). This indicates that α-synuclein may function as a molecular chaperone, which could explain α-synuclein's functional pleiotropy in different cellular processes. Indeed, α-synuclein can bind to 14-3-3 and interacts with many known binding partners of 14-3-3, including PKC and ERK (Ostrerova et al., 1999). In addition, α-synuclein has been shown to interact with molecular components associated with lipid transport, fatty acid metabolism and membrane turnover, such as phospholipases D1 and D2 (Jenco et al., 1998). Finally, α-synuclein can associate with proteins involved in DA synthesis and regulation, such as tyrosine hydroxylase and DA transporter (DAT) (Fountaine and Wade-Martins, 2007; Lee et al., 2001; Peng et al., 2005; Perez et al., 2002; Wersinger and Sidhu, 2003; Yu et al., 2004) and proteins involved in the regulation of other catecholamines such as the norepinephrine transporter (NET) and the serotonin transporter (SERT) (Wersinger et al., 2006a; Wersinger et al., 2006b).

Two keys aspects of its function which continue to receive interest are its potential regulatory roles in intracellular vesicle function and molecular interactions with key aspects of dopamine synthesis and metabolism.

1.3.6. α-synuclein and synaptic vesicle function

Consistent with its presynaptic localisation, several studies implicate a role for α-synuclein in modulating synaptic transmission, the vesicle recycling and neuronal

plasticity. Indeed, in humans, rats and mice, α-synuclein expression reaches its highest concentration per synapse during early embryogenesis; a period of maximal neuronal plasticity (Galvin et al., 2001; Hsu et al., 1998). α-synuclein upregulation occurs after synaptogenesis during development, indicating the protein is not essential in the formation of neurons. Confirmation of this is provided by the fact that knockout synuclein mouse models are not lethal and that brain morphology in these models is normal (Abeliovich et al., 2000; Cabin et al., 2002; Chandra et al., 2005). Even though α-synuclein is not essential for synapse formation or cell survival, studies in α-synuclein knockout mice have been instrumental in revealing an essential role for the protein in synaptic transmission, vesicle trafficking and recycling and synaptic maintenance and integrity.

A study by Abeliovich and colleagues showed that α-synuclein knockout mice showed an enhanced rate of recovery during paired-pulse stimulations in striatal slices which could be mimicked by increased concentrations of calcium (Abeliovich et al., 2000). This suggested that α-synuclein may act as an activity-dependent, negative regulator of neurotransmitter release that may restrict the trafficking of vesicles from the reserve pool to the readily release pool (RRP) of vesicles. It is also reported that loss of α-synuclein decreased the number of reserve pool synaptic vesicles in hippocampal neurons in vitro (Murphy et al., 2000) and *in vivo* where a corresponding impairment of neurotransmitter release from hippocampal neurons occured in response to prolonged, high frequency stimulation, where an altered response to amphetamine (AMPH) was also observed (Cabin et al., 2002). A higher rate of vesicle depletion following tetanic stimulation has also been reported (Yavich et al., 2004). Further, α-synuclein-depleted cells exhibit decreased levels of synapsin, a protein

essential for synaptic vesicle recycling (Cabin et al., 2002; Lotharius et al., 2002). Together, these studies imply a role for α-synuclein in the formation and/or maintenance of a subset of a reserve pool of presynaptic vesicles and the regulation of vesicle transport. That said, studies on knockout mouse models which are deficient in α- and β-synuclein failed to show differences in the size of the vesicle pools or in synaptic transmission. However, changes in the levels of the signalling molecules complexin and 14-3-3 proteins were seen, indicating α-synuclein may not be essential components of synaptic machinery but contribute subtle regulatory roles (Bellani et al., 2010; Chandra et al., 2004). Further studies on mice deficient in α- and γ-synuclein reported a two-fold increase in neurotransmitter release from striatal slices without corresponding changes in transmitter content or uptake (Senior et al., 2008). Taken together, these data highlight that α-synuclein may have altered functions depending on whether other members of the closely related synuclein family of proteins are present.

There are suggestions that α-synuclein may even act as a positive regulator of synaptic transmission, which come from a study on cysteine-string protein (CSPα) knockout mice (Chandra et al., 2005). These mice show a rapidly progressive neurodegeneration because CSPα deletion inhibits soluble NSF attachment receptor (SNARE) complex assembly. Surprisingly the phenotype is partially rescued by transgenic expression of α-synuclein. Its mode of action in this case is unclear since α-synuclein does not directly function in place of CSPα and does not bind to SNARE proteins. It is possible that α-synuclein, in keeping with its homology to 14-3-3 proteins, acts as a molecular chaperone which complements CSPα - facilitating SNARE protein function and maintaining synaptic integrity.

The role of α-synuclein as an adaptor or chaperone protein and the potential molecular mechanisms by which it may regulate vesicular recycling, are exhibited through its proposed interaction with phospholipase D (PLD). α-synuclein physically binds to and inhibits the activity of PLD2, and to a lesser extent to PLD1, through its highly conserved N-terminal repeat domain (Ahn et al., 2002; Jenco et al., 1998).

PLD2 is located on the plasma membrane where it has an important role in regulating synaptic vesicle recycling. It functions as a membrane bound enzyme that hydrolyses phospholipids such as phosphatidylcholine (AC) into phosphatidic acid (PA) (Exton, 2002a, b; Jones et al., 1999). PA, in addition to acting as an intracellular messenger, can promote the recruitment of cytosolic adaptor molecules at membrane surfaces, which in turn facilitates the recruitment of clathrin coat proteins and initiates vesicle biogenesis (Ktistakis et al., 1996; Liscovitch et al., 2000). Therefore, by modulating PLD2 activity, α-synuclein may have a key role in regulating vesicular recycling. Most recently however, one study failed to show that α-synuclein had a robust inhibitory interaction with PLD2, either directly or indirectly (Rappley et al., 2009).

Reports also suggest α-synuclein has lipid binding properties and can regulate vesicular recycling through modulation of fatty acid metabolism, a crucial aspect of vesicle function and membrane turnover. As well as having significant homology with class-A_2 apolipoproteins and C1-3, which are involved in lipid transport and are components of LBs (Clayton and George, 1998; Ueda et al., 1993), short amino acid stretches in the N- and C-termini of α-synuclein share considerable homology with the cytosolic fatty acid binding motif present in fatty acid binding proteins (FABPs). As such, α-synuclein can bind to fatty acids in a dose-dependent and competitive

manner (Sharon et al., 2001). α-synuclein may therefore shuttle fatty acids, which are highly enriched at synaptic vesicles, to vesicle formation sites. It is also possible that α-synuclein acts as a lipid chaperone to regulate the turnover or local organisation of polyunsaturated fatty acid acyl groups thought to be involved in clathrin-mediated endocytosis and therefore in vesicle recycling (Heuser and Reese, 1973; Schmidt et al., 1999).

1.3.7. α-synuclein and dopamine homeostasis

It is important to understand the relationship between α-synuclein and DA homeostasis. This is because PD pathology preferentially targets DA neurons in the SNpc, and in turn, α-synuclein has been shown to affect various components of DA biology: the synthesis of DA by TH, the storage and release of DA, and the reuptake of DA by the DAT.

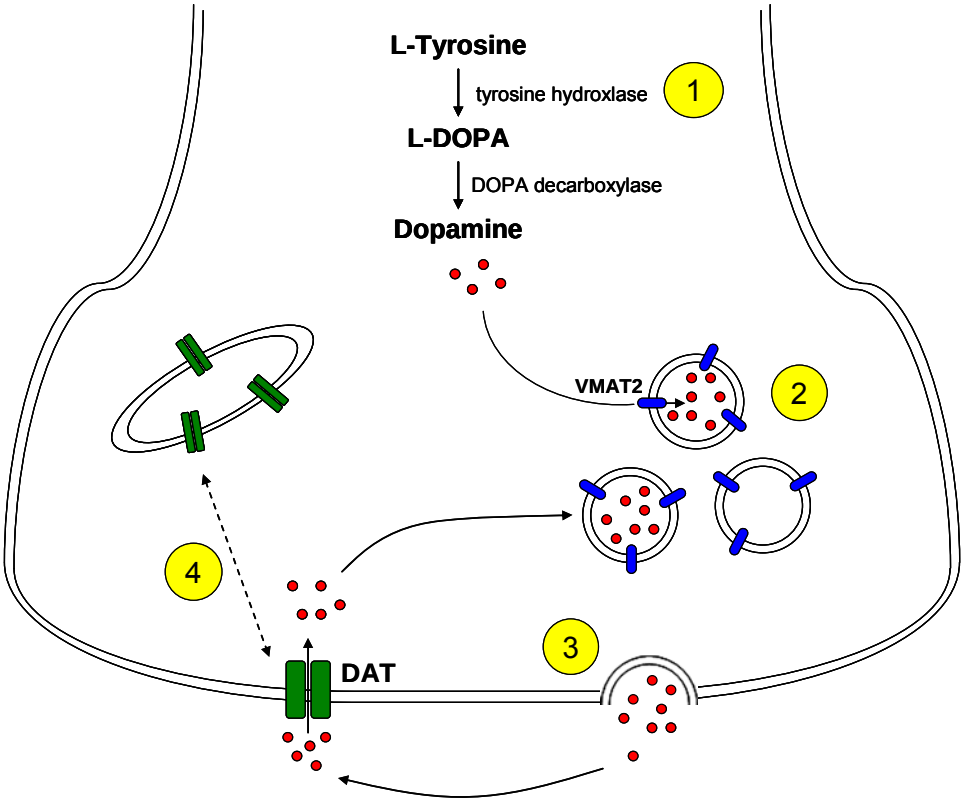


Figure 1.2 ***α-synuclein and DA homeostasis***. DA synthesis from L-tyrosine occurs in the cytoplasm, via the actions of tyrosine hydroxylase (TH) and DOPA decarboxylase (1). α-synuclein has been shown to regulate DA synthesis via interactions with the TH promoter and direct interactions with TH (Gao et al., 2007; Peng et al., 2005; Perez et al., 2002). DA is then sequestered, by vesicular monoamine transporter 2 (VMAT2), into synaptic vesicles where it is stored (2). α-synuclein has been shown to be involved in the regulation of synaptic vesicle function, vesicular transport and regulation of the resting/reserve vesicle pools (Yavich et al., 2004). DA release occurs via Ca^{2+} mediated exocytosis (3). α-synuclein has shown to regulate DA release, possibly through interference with late steps in exocytosis (Larsen et al., 2006; Yavich et al., 2005). DA action is terminated by reuptake via the dopamine transporter (DAT) (4). α-synuclein has been shown to interact with DAT, with increasing evidence suggesting α-synuclein is involved in the facilitation of DAT localisation to the cell surface membrane (Fountaine and Wade-Martins, 2007; Lee et al., 2001).

DA synthesis occurs via a multistep process in which the amino acid tyrosine is converted to L-DOPA by phosphorylated TH. The enzymatic activity of TH is the rate limiting step in the production of DA. L-DOPA undergoes conversion to DA by AADC in dopaminergic neurons. α-synuclein has been shown to act as a negative regulator of DA synthesis through effects both on the TH promoter and an interaction

with TH directly. The TH promoter has been shown to be significantly less active in cells which overexpress α-synuclein compared to controls (Gao et al., 2007). Correspondingly, cells overexpressing α-synuclein exhibit reduced levels of TH mRNA and protein (Baptista et al., 2003; Yu et al., 2004). Studies *in vitro* also suggest that α-synuclein can directly bind to and reduce the activity of TH (Perez et al., 2002). TH activity is dependent on phosphorylation of regulatory serine residues in the N-terminus of the protein (Campbell et al., 1986; Haycock, 1990). Phosphorylation activates TH by calcium-calmodulin dependent protein kinase II in response to a depolarisation-dependent influx of calcium. This process has been shown to be modulated by 14-3-3 proteins, which bind to phosphorylated TH and maximise TH phosphorylation (Ichimura et al., 1988; Ichimura et al., 1987; Xu et al., 2002a). Conversely, α-synuclein has been shown to bind to dephosphorylated TH and maintain the enzyme in an inactive form, causing an overall net reduction in TH activity and DA synthesis (Perez et al., 2002). In addition, α-synuclein can bind to, and promote the activity of protein phosphatase 2A (PP2A), an enzyme which is responsible for deactivating TH through dephosphorylation of a key serine residue (Peng et al., 2005). The view that α-synuclein acts as a negative regulator of DA synthesis is further supported by studies *in vitro* which demonstrate that the activity of AADC is reduced in the presence of α-synuclein overexpression (Tehranian et al., 2006). *In vivo* studies support the notion that α-synuclein may regulate DA production. In one line of α-synuclein knockout mice, an approximately 20% reduction in striatal DA content was reported (Abeliovich et al., 2000), although this finding was not supported in an independently generated line of α-synuclein knockout mice (Robertson et al., 2004; Schluter et al., 2003). This highlights the potential phenotypic variability which may exist between mouse lines generated on different

strain backgrounds or with a different methodology. The age of the mice is also very important, since whilst no changes were seen in the latter mentioned cohort of α-synuclein knockout mice at a young age, a significant reduction was observed in α-synuclein knockout mice at 24-26 months (Al-Wandi et al., 2010). Furthermore, DA levels in α- and β-synuclein double knockout mice were decreased by approximately 20% (Chandra et al., 2004), although no reduction in DA content were observed in striatum of α- and γ-synuclein double knockout mice (Senior et al., 2008).

Once DA is synthesised, it is rapidly sequestered into synaptic vesicles through the activity of vesicular monoamine transporter 2 (VMAT2) where it is stored until it is released in a stimulus-evoked fashion from presynaptic terminals by calcium-dependent exocytosis. As described, α-synuclein has effects on vesicular function and studies in α-synuclein mouse models have been useful in determining the influence α-synuclein may have on the storage and release of DA. Abeliovich and colleagues showed that α-synuclein knockout mice showed subtle changes in DA release in a paired stimulation paradigm. Under normal conditions, if a neuron is stimulated twice in rapid succession, the amount of DA released on the second stimulation will be significantly lower than on the first. This is known as paired pulse depression (PPD). α-synuclein knockout mice showed an enhanced rate of recovery from PPD at DA terminals in the striatum (Abeliovich et al., 2000). Furthermore, Yavich and colleagues demonstrated that mice overexpressing α-synuclein exhibited a decrease in DA release (Yavich et al., 2005), a finding supported in cell culture models which have shown α-synuclein overexpression impairs DA release, possibly through interference with a late step in exocytosis (Larsen et al., 2006; Lotharius et al., 2002). Studies on α- and γ-synuclein double knockout mice have shown a two-fold increase

in evoked extracellular DA concentration after discrete stimulation in striatal slices compared with wild-type, α-synuclein or γ-synuclein single knockout control mice. This occurred without corresponding changes in transmitter content or uptake and an increased recovery from PPD was not observed in any of the knockout mouse lines (Senior et al., 2008). Deficits in neurotransmitter release or changes in paired stimulation paradigms were not observed in α- and β-synuclein double knockout mice, although these observations were made in hippocampal slices (Chandra et al., 2004).

Studies *in vitro* indicate α-synuclein may also have a role in DA sequestration and storage. In adrenal cells, α-synuclein overexpression altered vesicular pH gradients important for the correct functioning of the VMAT2 which corresponded to an increase in cytoplasmic catecholamine levels (Mosharov et al., 2006). Overexpression of A53T mutant α-synuclein in human dopaminergic (MESC2.10) cells leads to a decrease in the levels of VMAT2, a decrease in DA release and an increase in AMPH stimulated DA extrusion via DAT (Lotharius et al., 2002). In a study of α-synuclein knockdown in another human neuronal cell model, a decrease in the total number of intracellular vesicles was observed, although an increase in the density of the VMAT2 per vesicle was reported (Fountaine et al., 2008). Therefore, α-synuclein may influence the number of DA storage vesicles or alter DA storage ability, leading to increases in cytosolic DA levels.

Postsynaptic DA action is terminated by removal of DA from the extracellular synaptic cleft. This may occur by diffusion of DA away from the cleft, or by degradation of DA by enzymes; however, the primary mechanism of rapid DA

removal is by re-uptake of DA into the presynaptic terminal by DAT. DAT function on the cell surface has a crucial role in regulating intracellular and extracellular DA levels and as such is closely regulated through a variety of intracellular signalling cascades (Bannon, 2005). α-synuclein interacts directly with DAT and is believed to have an important role in the regulating transporter number through the trafficking of DAT between the cell surface and internal plasma membranes. It has been shown in tissue culture that α-synuclein acts to traffic DAT towards the cell surface (Lee et al., 2001). This observation is supported by the important finding that α-synuclein knockout mice are resistant to MPTP toxicity (Dauer et al., 2002; Robertson et al., 2004; Schluter et al., 2003). MPP+, the active metabolite of MPTP, enters DA neurons through the DAT, where it potently inhibits mitochondrial complex I. Dauer and colleagues showed that in α-synuclein knockout mice, MPTP was less able to reach the mitochondria, indicating less DAT localisation to the cell surface for MPP+ uptake (Dauer et al., 2002). Similarly, α-synuclein knockout mice exhibit reduced locomotor hyperactivity in response to AMPH, whose action is partially dependent upon the DAT (Abeliovich et al., 2000). Consistent with this, α-synuclein knockdown in human neuroblastoma cell lines protects against MPP+ toxicity by reducing the amount of DAT present on the cell surface (Fountaine and Wade-Martins, 2007). Taken together, the data largely support a role for α-synuclein in facilitating DAT localisation to the cell surface. In further support of this view, it has been shown that overexpression of α-synuclein blocks ER-golgi trafficking (Cooper et al., 2006; Gosavi et al., 2002).

Whilst the precise cellular role of α-synuclein is not known, it is clear that the protein, potentially together with the other synucleins, acts to regulate synaptic transmission,

vesicle trafficking and recycling and synaptic maintenance at presynaptic nerve terminals. There is also strong evidence to suggest α-synuclein is involved in regulating and maintaining DA homeostasis within the cytoplasm, with reported actions on the synthesis, sequestration and storage, release and reuptake of the neurotransmitter.

1.3.8. Beta and gamma synuclein

Less is known about the other two synuclein family members, β-synuclein and γ-synuclein. There is considerable sequence homology between the synuclein family members, indicating that a high degree of functional redundancy potentially exists between the family members. The lack of an overt phenotype in α-synuclein single knockout mice is likely caused by this functional compensation and may have obscured the detection of an essential role of α-synuclein. Indeed, β-synuclein has been shown to be upregulated in α- and γ-synuclein double knockout mice (Robertson et al., 2004), whilst γ-synuclein expression is increased in α- and β-synuclein double knockout mice (Chandra et al., 2004). Therefore, the functions of β-synuclein and γ-synuclein must be taken into consideration if future studies of α-synuclein knockout mice studies are to be more informative. In particular, their potential role in the development and progression of neurodegeneration has been poorly studied.

1.3.9. β-synuclein: structure and function

The human β-synuclein gene (*SNCB*) maps to chromosome 5q35 (Spillantini et al., 1995), and the mouse β-synuclein gene is found on chromosome 13 (Sopher et al.,

2001). Both the human and mouse β-synuclein gene consist of six exons separated by five introns. The human protein is 134 amino acids in length and shares 97.8% homology with mouse β-synuclein. Structurally, β-synuclein lacks 11 amino acid residues present in the highly amyloidic hydrophobic domain in the N-terminal region of α-synuclein, which may explain why β-synuclein has a poor propensity to self-aggregate and form oligomers and fibrils (Uversky et al., 2002). β-synuclein shares 61% sequence homology with α-synuclein and, like α-synuclein, is expressed predominantly in the CNS where it is concentrated in the cytoplasmic matrix of the presynaptic axon terminals (Jakes et al., 1994; Nakajo et al., 1994). β-synuclein is not exclusively located in nerve terminals; it is also present in the perikarya of neurons (Giasson et al., 2001; Mori et al., 2002).

β-synuclein has not been found to co-localise with α-synuclein in LBs (Lavedan et al., 1998a) although, like γ-synuclein, the protein has been associated with hippocampal axon pathology in PD and DLB (Galvin et al., 1999). Further reports support a more specific role for β-synuclein in modulating α-synuclein toxicity. Two independent reports investigating α-synuclein aggregation *in vitro* have demonstrated that β-synuclein is able to inhibit α-synuclein aggregation and fibril formation (Park and Lansbury, 2003; Uversky et al., 2002). It has also been reported that in mice which overexpress both α-synuclein and β-synuclein, β-synuclein overexpression can reduce α-synuclein aggregation and toxicity *in vivo* (Hashimoto et al., 2001). Another study reported that mice which overexpress wild-type β-synuclein can reduce the endogenous expression of α-synuclein protein, without altering the mRNA levels (Fan et al., 2006). These mice are phenotypically normal and do not exhibit a

neuropathological phenotype. When crossed with an α-synuclein transgenic mouse carrying the A53T mutation under the control of the PrP promoter (Giasson et al., 2002), a marked improvement in motor performance, neuropathology and survival is seen, which corresponds to a decrease in α-synuclein protein expression (Fan et al., 2006). Taken together, growing evidence suggests a possible protective role of β-synuclein in neurodegenerative diseases, potentially through its ability to modulate α-synuclein neurotoxicity. In support of this, Ohtake and colleagues performed a genetic screen of patients with genetic and familial forms of DLB for alteration in the β-synuclein gene. They discovered two different amino acid alterations in the *SNCB* gene in unrelated cases of DLB. These amino acid substitutions occur at conserved residues in a highly conserved region of β-synuclein. However, although widespread LB pathology and α-synuclein aggregation was present in these patients, no β-synuclein aggregation was detected (Ohtake et al., 2004). This report provided the first genetic link to a potential role for β-synuclein in neurodegeneration, and led to the suggestion that defects in the *SNCB* gene may reflect a loss of β-synuclein's ability to inhibit α-synuclein toxicity.

1.3.10. γ-synuclein: structure and function

The human γ-synuclein gene (*SNCG*) has been mapped to chromosome 10q23 (Lavedan et al., 1998b), and the mouse γ-synuclein gene is located on chromosome 14 (Alimova-Kost et al., 1999). The human γ-synuclein gene is comprised of five exons which encodes a 127 amino acid protein. The human protein is 87.7% identical to mouse γ-synuclein protein (Lavedan, 1998). Structurally, γ-synuclein is similar to α-

synuclein, and can adopt a free-state residual secondary structure, and unlike β-synuclein, γ-synuclein can also aggregate and form fibrils *in vitro* (Sung and Eliezer, 2007). When it was initially identified, γ-synuclein was termed persyn, owing to its expression in the peripheral nervous system, for example in sympathetic neurons and in motor neurons, as well as the CNS (Buchman et al., 1998b). Little is known about the normal function of γ-synuclein and there is considerable research which focuses on its potential role in cancer formation or progression, specifically breast cancer (Ahmad et al., 2007; Guo et al., 2007; Ji et al., 1997; Jia et al., 1999). Although mutations in the *SNCG* gene have not been identified in PD patients and the protein is not present in LBs (Lavedan et al., 1998a), there is growing evidence associating γ-synuclein with a potential role in neurodegeneration. Like β-synuclein, γ-synuclein has been associated with axonal pathology in PD and DLB patients (Galvin et al., 1999) and has also been shown to inhibit the aggregation of α-synuclein (Biere et al., 2000; Uversky et al., 2002). Further, acute overexpression of γ-synuclein in neuronal cell culture does not cause cell death but causes an impairment in the neurofilamentary network (Buchman et al., 1998a). Recently, Ninkina and colleagues have more comprehensively assessed the effects of γ-synuclein overexpression on the nervous system in transgenic mice expressing high levels of γ-synuclein under the control of the Thy-1 promoter. They found these mice develop a severe age- and transgene dose-dependent neuropathological phenotype throughout the nervous system which is prematurely fatal (Ninkina et al., 2009). Aggregation of γ-synuclein and the accumulation of various inclusions in neuronal cell bodies and processes occurs, most prominently in the spinal cord, leading to the loss of spinal motor neurons and corresponding severe motor deficits (Ninkina et al., 2009). These findings support the view that γ-synuclein may have an as yet unidentified function in

certain types of neurodegenerative diseases. Most recently, a pilot study screening a cohort of patients with diffuse LB disease (DLBD), consistent with a clinical diagnosis of PD or DLB, was carried out to determine the associations of *SNCA*, *SNCB* and *SNCG*. They identified one SNP in the γ-synuclein gene which was significantly associated with the disease (Nishioka et al., 2010). This provides further evidence of a potential role of this protein in neurodegeneration.

1.3.11. Summary

It is clear that a better understanding of the normal and pathological functions of α-synuclein, β-synuclein and γ-synuclein, and the relationship between them will be key to more fully understanding the mechanisms of PD pathogenesis, and of the pathogenesis of related neurodegenerative disorders.

1.4. Mechanisms of PD Pathogenesis

1.4.1. Protein aggregation and PD

The accumulation of abnormal protein aggregates is a common pathological feature of several neurodegenerative disorders, including PD, where the deposition of insoluble α-synuclein into LBs is a characteristic hallmark of the disorder. However, it is not clear how these protein aggregations are linked to the disorder. A growing body of evidence indicates the aggregates are not a prerequisite for neurodegeneration, since there is no correlation between inclusion formation and cell death. Firstly, PD patients with *PARKIN* mutations can have nigral degeneration in the absence of LBs (Kitada

et al., 1998). Secondly, in post-mortem brain tissue from patients with PD, most cells undergoing apoptosis do not exhibit LB pathology (Tompkins and Hill, 1997). Furthermore, the number of LBs present in post-mortem brains does not correlate with neuronal loss (Gomez-Isla et al., 1999). Finally, many individuals who have not experienced neurological illness during their lives are found to have widespread LBs post-mortem (Parkkinen et al., 2005a; Parkkinen et al., 2005b) and in animal models, inclusions can occur without associated neurodegeneration (Lo Bianco et al., 2002). Therefore rather, it is proposed that inclusion formation may arise from active processes meant to sequester insoluble misfolded proteins from the cytoplasm, and toxicity arises through dysfunction of these processes. Aggregate formation has even been suggested to be cytoprotective, acting as a defensive response aimed at sequestering toxic species to prevent cellular damage.

Several lines of evidence indicate that dysfunction of the ubiquitin-proteosome system (UPS), which is responsible for marking and degrading appropriate proteins, may be causal in PD. LBs contain multiple molecular components of the UPS such as ubiquitin (Lennox et al., 1989a; Lennox et al., 1989b), parkin (Choi et al., 2000) and UCHL-1 (Lowe et al., 1990). Further, a 40% decrease in proteosome activity selectively in the SNpc has been observed in the brains of PD patients at autopsy (McNaught and Jenner, 2001). In addition, proteosome activity decreases with normal ageing, particularly in the SNpc, which may contribute to the selective vulnerability of this region in PD (Zeng et al., 2005). The strongest evidence for UPS dysfunction in PD comes from the identification of several PD-associated genes which are directly linked to UPS impairment. These include mutations in the conjugating E3 ligase enzyme parkin, which abolish the proteins ability to tag target proteins with ubiquitin

(Kitada et al., 1998; Shimura et al., 2000) and mutations in UCHL-1, an enzyme involved in the de-conjugation of ubiquitin, which are believed to lead to the loss of recycling of ubiquitin monomers (Leroy et al., 1998b; Leroy et al., 1998c).

Impairment of the UPS has been closely linked with α-synuclein. For example, overexpression of A53T mutant α-synuclein in a cell culture produced ubiquitinated, cytoplasmic inclusions and exhibited reduced proteosome activity (Stefanis et al., 2001). This suggests that α-synuclein aggregation, or an increased propensity to aggregate, into LBs may be an important step in mediating cellular toxicity. Monomeric α-synuclein is readily soluble, and under certain conditions, such as oxidative stress, overexpression or genetic disruption, forms small oligomers and protofibrils that elongate into mature insoluble fibrils which then readily coalesce into LBs. Indeed, α-synuclein mutations are associated with an increased tendency for the protein to aggregate in aqueous solutions (Conway et al., 1998; Conway et al., 2000a; Conway et al., 2000b; Giasson et al., 1999) and *in vivo* (Feany and Bender, 2000). Increasing evidence indicates that toxicity may not be mediated by the insoluble aggregates, but by the soluble non-fibrillar oligomers or protofibrils. In support of this, protofibrils are increased in the brains of patients with PD and DLB (Sharon et al., 2003) and have been identified in cells overexpressing α-synuclein and in transgenic mice where they are associated with cellular toxicity (Gosavi et al., 2002; Masliah et al., 2000). Furthermore, increased protofibril formation, but not insoluble fibril formation, is promoted by A30P and A53T α-synuclein mutations (Conway et al., 2000a; Conway et al., 2000b; Lashuel et al., 2002; Volles and Lansbury, 2002). In fact, the conversion of protofibrils into insoluble fibrils is slower in the A30P mutated α-synuclein form than in the wild-type form.

The mechanism by which α-synuclein protofibrils mediate cellular toxicity may relate to its effects on lipid membranes. The protofibrillar forms of α-synuclein exhibit tighter lipid binding than monomeric or fibrilar forms (Volles et al., 2001), and α-synuclein protofibrillar structures have been shown to resemble a class of bacterial toxins capable of forming pore-like structures in lipid membranes. These observations support the finding that α-synuclein protofibrils can destabilise vesicular membranes (Lashuel et al., 2002; Volles and Lansbury, 2002; Zhu et al., 2003) and lead to the leakage of small molecules such as cations (Furukawa et al., 2006; Tsigelny et al., 2007) and catecholamines (Lotharius and Brundin, 2002a; Mosharov et al., 2006) from intracellular compartments, notably calcium ions (Ca^{2+}) and DA.

A number of studies *in vitro* have observed that DA itself may modulate the aggregation of α-synuclein (Cappai et al., 2005; Kuhn et al., 1999; Li et al., 2004; Norris et al., 2005; Xu et al., 2002a). DA has been shown to induce the aggregation of both wild-type and A53T mutant α-synuclein in a temperature dependent fashion, with mutated α-synuclein having an increased propensity to form aggregations in the presence of DA in human neuroblastoma M17 cells (Moussa et al., 2008). The coexpression of α-synuclein with TH in SH-SY5Y cells leads to an increased cytosolic DA which facilitates the formation of soluble oligomeric intermediates, although these oligomers are not toxic to the cells (Mazzulli et al., 2006). It has been demonstrated that addition of DA to preformed fibrils can lead to their disaggregation into small, non fibrillar soluble oligomers (Li et al., 2004). Some of the strongest evidence comes from Xu and colleagues, who demonstrated that endogenous inhibition of DA production inhibits α-synuclein-induced apoptosis (Xu et al., 2002a).

Iron (III) (Fe^{3+}) is able to promote α-synuclein aggregation into fibrils, and DA has been shown to completely inhibit this ability (Cappai et al., 2005; Uversky et al., 2001). Taken together, this evidence suggests DA may be an important modulator of α-synuclein aggregation, and increased levels of DA in the cytosol may enable DA to contribute to toxic α-synuclein protofiril formation. However, the precise molecular mechanism by which this modulation occurs remains unclear (Conway et al., 2001; Norris et al., 2005). In support of this, elevated cytosolic DA levels, together with high cytosolic Ca^{2+} concentrations and α-synuclein expression have recently been proposed to be central to the selective vulnerability of SNpc neurons in PD. Mosharov and colleagues showed that elevated cytosolic DA levels were toxic to cells and interventions which lowered DA levels provided neuroprotection. The application of L-DOPA raised cytosolic DA levels 2- to 3-fold more in SNpc neurons than in VTA neurons, a response which was shown to depend on dihydropriridine-sensitive Ca_v 1.3 Ca^{2+} channels; leading to an increased vulnerability of SNpc neurons to L-DOPA mediated neurotoxicity. Further, in neurons lacking α-synuclein, cytosolic DA levels were not changed but the cells were resistant to L-DOPA induced cell death (Mosharov et al., 2009).

Increased cytosolic levels of Ca^{2+} and catecholamines also lead to the increased production of reactive oxygen species (ROS) which increase oxidative stress in cells.

1.4.2. Oxidative stress and PD

There is indisputable evidence that oxidative stress plays a central role in PD pathogenesis. Oxidative stress is a pathological condition which results from an

imbalance between the production of oxidative species and their clearance by anti-oxidant mechanisms. Furthermore, PD-associated oxidative stress cascades appear to have an important and complex relationship with α-synuclein; ROS can promote α-synuclein aggregation and in contrast, α-synuclein can influence the production of ROS (Cole et al., 2005; Hashimoto et al., 1999a; Kanda et al., 2000; Kim et al., 2002). Studies in post-mortem brain tissue have confirmed signs of oxidative damage in the SNpc of PD patients, including increased lipid perioxidation, increased activity of mitochondrial superoxide dismutase, increased protein oxidation and increased iron deposition (Dexter et al., 1989; Jenner, 2007; Jenner et al., 1992; Marttila et al., 1988b; Saggu et al., 1989). Further, PD-associated genes DJ1 and PINK1 are both associated with oxidative stress. DJ1 is an atypical peroxiredoxin-like peroxidase with an important role in the cellular response to oxidative stress. The protein shows antioxidant activity in tissue culture (Taira et al., 2004) and the loss of DJ1 in flies and mice does not produce neuronal cell death but renders them more sensitive to oxidative stressors (Park et al., 2005; Yamaguchi and Shen, 2007; Yang et al., 2005). PINK1 is a mitochondrial protein linked to PD (Silvestri et al., 2005; Valente et al., 2004) and has been shown to protect cells against oxidative stress-evoked apoptosis by preventing the release of cytochrome c, which can promote α-synuclein aggregation, from the mitochondria (Hashimoto et al., 1999b).

Mitochondrial dysfunction, which occurs commonly in PD patients, can lead to the insufficient ATP production and the generation of ROS, such as superoxide, leading to a rise in oxidative stress (Schapira et al., 1998). Mitochondrial impairment is thought to result from a complex I deficit (Schapira et al., 1990; Schapira et al., 1989). Indeed, a reduction in complex I activity in the SNpc of PD patients has been

observed (Schapira et al., 1992; Swerdlow et al., 1996), although complex I activity spans a large range and the majority of patients exhibit normal levels. Furthermore, PD-causing environmental toxins, including MPTP, are mitochondrial complex I inhibitors and cause the selective loss of DA neurons (Manning-Bog et al., 2002). Mitochondrial dysfunction is also linked to α-synuclein. While α-synuclein overexpression in mice leads to an increased sensitivity to MPTP (Richfield et al., 2002), α-synuclein knockout mice are resistant to the pathogenic effects of the toxin (Dauer et al., 2002; Drolet et al., 2004; Fornai et al., 2005; Robertson et al., 2004). Similarly, *in vitro*, mitochondrial inhibition lead to an upregulation of α-synuclein expression and aggregate formation (Kalivendi et al., 2004; Lehmensiek et al., 2006; Lehmensiek et al., 2002) while RNAi mediated knockdown of α-synuclein in human neuroblastoma cells protects them from MPTP toxicity (Fountaine and Wade-Martins, 2007).

The mitochondria therefore represent one source of ROS in oxidative stress. Another source of ROS is DA. DA is a highly reactive molecule and, if not effectively sequestered, will readily oxidise to form ROS such as hydrogen peroxide and superoxide, as well as reactive DA quinones which are toxic to cells (Graham, 1978). Several reports confirm that catecholamines such as DA are linked to oxidative stress-related toxicity both *in vitro* and *in vivo* (Filloux and Townsend, 1993; Graham et al., 1978; Hastings et al., 1996; Michel and Hefti, 1990; Rosenberg, 1988). Cytosolic DA and α-synuclein are intimately linked in bringing about oxidative stress in PD since α-synuclein influences cytosolic DA levels in several ways, including influencing DA synthesis via effects on TH (Perez et al., 2002), DA sequestration and storage through effects on VMAT2 (Lotharius et al., 2002) and DA reuptake into the cytoplasm via

the regulation of DAT (Lee et al., 2001; Wersinger et al., 2003). α-synuclein-mediated changes in these aspects of DA homeostasis may ultimately lead to DA toxicity in cells (Lotharius and Brundin, 2002b). Furthermore, protofibrillar α-synuclein may increase membrane permeability, leading to DA leakage from synaptic vesicles into the cytoplasm (Caughey and Lansbury, 2003).

In addition to oxidative stress, nitrative stress is implicated in the pathogenesis of PD. Reactive nitrogen species, which are responsible for nitrative stress, are produced from nitric oxide (NO). Under normal conditions, NO functions as an intracellular messenger and neurotransmitter (Garthwaite et al., 1988), but in pathological situations it can form toxic reactive nitrogen species, such as peroxynitrite which is a very labile and toxic species (Jenner, 2007). Reactive nitrogen species are capable of causing protein modifications and have been linked to PD in a number of ways. For example, the S-nitrosylated form of parkin, which has reduced enzyme activity, has been shown to be increased in post-mortem brain tissue of PD patients (Chung et al., 2004). Further, inhibitors of nitric oxide synthase (NOS), the enzyme which generates NO, can protect against MPTP toxicity in animals, and mice which lack the neuronal or inducible forms of NOS (nNOS or iNOS, respectively) are more resistant to MPTP (Liberatore et al., 1999). Nitrative stress also affects α-synuclein. Nitration of α-synuclein leads to enhanced formation of protofibrils, a lower affinity for binding lipid vesicles and reduced degradation by proteosomes (Hodara et al., 2004; Paxinou et al., 2001; Souza et al., 2000; Uversky, 2007). Further, in human post-mortem brain samples of PD patients, α-synuclein is nitrated more than other proteins with α-synuclein in LBs showing particularly high levels of nitration (Duda et al., 2000; Giasson et al., 2000).

1.4.3. Other factors in PD

There are a number of other factors which have been implicated in the pathogenesis of PD, including potential roles for the endoplasmic reticulum (ER) stress pathway and for Ca^{2+} dysregulation.

The ER is a major signal transducing organelle that senses and responds to changes in cellular homeostasis. Conditions interfering with ER function lead to ER stress. The ER stress pathway (or unfolded protein response, UPR) is induced by the accumulation of unfolded protein aggregates and uses a number of different mechanisms to reduce this load of aberrant proteins. Firstly, protein translation is reduced via phosphorylation and activation of an ER sensor kinase called PERK, which in turn phosphorylates and inactivates eIF2-α. This results in a reduction in the amount of new protein which needs to be folded. Secondly, activation of the ATF6 transcription factor induces the expression of genes involved in protein folding. Thirdly, ER-associated protein degradation involves the transport of misfolded proteins from the ER into the cytoplasm for proteosomal degradation. There are a number of studies which have linked ER stress to PD, and once more, to α-synuclein. One *in vivo* study has reported the presence of ER stress markers, phosphorylated PERK and phosphorylated eIF2-α in the SNpc of PD patients but not in controls (Hoozemans et al., 2007). Parkin expression has been shown to be increased by ER stress, and parkin overexpression is reportedly protective against ER stress induced apoptosis (Takahashi et al., 2003; Wang and Takahashi, 2007). High levels of cytosolic DA induce the expression of GADD153, a transcription factor associated

with the ER stress pathway (Gomez-Santos et al., 2005). In addition, in cell culture, the A53T-mutated form of α-synuclein caused toxicity which was mediated by the mitochondrial and ER stress pathways, resulting in the activation of pro-apoptotic caspases. Blocking the UPR partially protected the cells against the α-synuclein mediated toxicity (Smith et al., 2005). Overexpression of α-synuclein has been shown to be toxic in yeast, neuronal cell culture, *Drosophilia melanogaster* and *Caenorhabditis elegans* α-synuclein models, with accompanying signs of ER stress. α-synuclein overexpression leads to toxicity by blockade of ER-golgi transport and fragmentation of the Golgi apparatus, which interferes with the trafficking of misfolded proteins from the ER into the cytoplasm for proteosomal degradation. Furthermore, upregulation of genes involved in mediating this transport have been shown to be suppressors of this toxicity (Cooper et al., 2006; Gitler et al., 2008; Gosavi et al., 2002; Lashuel and Hirling, 2006).

The theory that Ca^{2+} dysregulation has a role in PD pathogenesis has also been proposed. Ca^{2+} is an important intracellular messenger in neurons and its cytoplasmic levels are tightly controlled via numerous mechanisms which include Ca^{2+} sequestration and buffering in intracellular compartments, including the ER and mitochondria. Excess cytosolic Ca^{2+} triggers glutamate excitotoxicity and causes cell death through a number of potential pathways, including mitochondrial dysfunction, oxidative stress and ER dysfunction. In PD, it is thought that SNpc neurons may experience excessive glutamate signalling through overactive STN neurons, which could make them particularly vulnerable to cell death. Indeed, there is some evidence to suggest glutamate receptor blockers can attenuate MPTP toxicity in animal models, though this is not a consistent finding (Jenner, 2007). There are other reports which

support the view that SNpc neurons may be selectively at risk to Ca^{2+} mediated toxicity. In adulthood, SNpc dopaminergic neurons have spontaneous "pacemaker" activity which is driven by dihydropiridine-sensitive Ca_v 1.3 Ca^{2+} channels. It is proposed that having to continuously handle high levels of intracellular, oscillating Ca^{2+} increases the vulnerability of these cells to excitotoxicity (Surmeier, 2007). Significantly, blocking Ca_v 1.3 Ca^{2+} channels results in a marked protection from MPTP toxicity (Chan et al., 2007). Together, this data implies that Ca^{2+} dysregulation may be an exacerbating factor that enhances the underlying pathological mechanisms in PD.

1.4.4. Summary

It is important to emphasise that it is very unlikely that proteosomal dysfunction, abnormal protein aggregation, oxidative stress and mitochondrial damage occur as independent cellular events to cause cell death. Rather, it is highly probable that there is a synergistic interaction between them, which in turn reciprocally promote each other. Indeed, accumulation of α-synuclein through ineffective proteosomal activity may occur, from which protofibrils arise and cause increased levels of Ca^{2+} and DA in the cytoplasm, which can then form ROS. Conversely, ROS and nitrative species arising from a number of sources, including cytosolic DA and mitochondrial dysfunction, promote the aggregation of α-synuclein, which can then lead to further oxidative damage. PD may therefore result from the dysfunction of several molecular players, which together lead to a self-amplifying, pathological cycle to induce cell death.

1.5. Animal Models to Study PD

1.5.1. Overview

Animal models of PD are fundamental tools which are required for advancing our understanding of the mechanisms of disease pathogenesis, for the identification of novel therapeutic targets and for the screening and testing of novel therapies (Dawson et al., 2010; Savitt et al., 2006). To this end, there are many PD-based animal models spanning a range of organisms, including primates, rats, mice, *Drosophilia melanogaster* and *Caenorhabditis elegans*. These models can be toxin-based, in which a neurotoxin, such as MPTP or 6-OHDA, is administered either peripherally or locally to selectively destroy dopaminergic neurons (Dauer and Przedborski, 2003). These models recapitulate many of the key features of PD and have been very useful in helping to understand the consequences of DA neuronal cell loss and for testing potential therapies. However they have several drawbacks, for example only a few PD cases are caused by neurotoxin exposure and there is no clear evidence that their acute mechanism of action is the same in animal models as it is in humans. Furthermore, these models do not reproduce the pathology which is seen in other brain regions or the non-motor symptoms seen in PD.

Following the identification of PD-associated genes, numerous genetic-based animal models have been generated over the past ten years (Melrose et al., 2006). These include transgenic, knockout and virus-based models of the disease, and have been created for the range of genes associated with PD. Due to the prominent role of α-synuclein in PD and related synucleinopathies, many α-synuclein animal models have

been created and extensively characterised. In the *Drosophilia melanogaster* model, overexpression of wild-type, A30P or A53T α-synuclein induces many of the characteristics seen in PD, including, importantly, an age-dependent selective loss of DA neurons, LB-like filamentous inclusions which contain α-synuclein, and locomotor deficits which are responsive to L-DOPA (Auluck and Bonini, 2002; Feany and Bender, 2000; Pendleton et al., 2002). However, this model is limited since *Drosophilia melanogaster* lack the physiological complexity of vertebrates and do not show signs of the principal symptoms of PD, such as bradykinesia, tremor and rigidity. Therefore, their value is mainly restricted to identifying potential agents which may modify α-synuclein-induced DA cell loss. Indeed, this model has become an important tool for the rapid screening of genetic or pharmacological interventions that may alter the neurodegenerative process. At present, however, the most widely available α-synuclein-based genetic animal models are mice because they are relatively easy to genetically manipulate.

1.5.2. Synuclein knockout mouse models

Several lines of α-synuclein knockout mouse models have been characterised in an attempt to elucidate the functional effects of the loss of the protein (Abeliovich et al., 2000; Cabin et al., 2002; Dauer et al., 2002; Schluter et al., 2003; Specht and Schoepfer, 2001). These studies have yielded some success, with varied reports on phenotype (Table 1.2).

Knockout Model	Phenotype	References
α-synuclein knockout	• Normal development, fertile • No impairment of basic brain morphology or survival • Increased rate of recovery from paired pulse stimulations in striatal slices • Increased rate of vesicle depletion following tetanic stimulation • Reduction in reserve pool of synaptic vesicles in hippocampus • No change in DA release or reuptake after discrete stimulation in striatal slices • Reduced rearing in open-field (not replicated) • Attenuation of locomotor response to AMPH • AMPH induced DA depletion • Partial or complete resistance to acute and chronic MPTP treatment, not attributed to changes in DAT function • Reduced striatal DA levels, though levels of DA and DA metabolites in whole brain reportedly unchanged • Significant reduction in DA levels at 24-26 months • Reduced number of DA neurons SNpc but not in VTA of early post-natal and adult without significant changes in striatal DA or DA metabolite levels and motor dysfunction • Decreased levels of synapsin protein • No reported changes in the levels of β- or γ- synuclein • No LBs or signs of major neuronal loss	(Abeliovich et al., 2000; Al-Wandi et al., 2010; Cabin et al., 2002; Dauer et al., 2002; Robertson et al., 2004; Schluter et al., 2003; Senior et al., 2008; Specht and Schoepfer, 2001; Yavich et al., 2004)
β-synuclein knockout	• Normal development, fertile • No impairment of basic brain morphology or survival • No evidence of neurodegeneration in aged mice • No change in DA levels in striatum or brain	(Chandra et al., 2004)
γ-synuclein knockout	• Normal development, fertile	(Robertson et al., 2004)

	• No impairment of basic brain morphology or survival • No change in DA release or reuptake after discrete stimulation in striatal slices • No change in DA levels in striatum or brain • Reduced number of DA neurons SNpc but not in VTA of early post-natal and adult without significant changes in striatal DA or DA metabolite levels and motor dysfunction	(Senior et al., 2008)
α- and β-synuclein double knockout	• Normal development, fertile • No impairment of basic brain morphology or survival • No significant changes in synaptic ultrastructure or plasticity • No significant changes in vesicle pool size, replenishment or recycling of synaptic vesicles • Release and uptake kinetics from isolated nerve terminals normal • activity-dependent synaptic kinetics not assessed • ~20% decrease in DA levels in brain • ~50% increase in expression levels of remaining γ-synuclein • ~30% increase in 14-3-3ε protein and ~30% decrease in 14-3-3ζ protein • ~30% increase in complexin protein • Causes accelerated pathology in CSPα knockout mice than in single synuclein knockouts	(Chandra et al., 2004; Chandra et al., 2005)
α- and γ-synuclein double knockout	• Normal development, fertile • No impairment of basic brain morphology or survival • Two-fold increase in DA concentration after discrete stimulation in striatum • No change in uptake kinetics of DA in striatum • No difference in recovery from paired pulse stimulations in striatal slices • No change in DA levels in striatum • Increase in expression levels of remaining β-synuclein • Reduced number of DA neurons SNpc but not in VTA of early post-natal and adult	(Robertson et al., 2004; Senior et al., 2008)

	without significant changes in striatal DA or DA metabolite levels and motor dysfunction • Increased rearing in non-anxiogenic open field • Decrease in spontaneous alternation in T-maze
β- and γ-synuclein double knockout	N/A

Table 1.2 ***Summary of current synuclein knockout mouse models.***

Overall, α-synuclein knockout mice are viable and fertile and do not appear to have an overt phenotype, implying the protein is not critical to the development or maintenance of DA neurons. However, the mice do exhibit subtle perturbations in DA homeostasis and behaviour, and responses to various neurotoxins. The lack of an obvious phenotype in these mice could be due to the presence of the remaining synuclein proteins, β-synuclein and γ-synuclein, with which α-synuclein shares considerable sequence homology. This high level of conservation implies functional redundancy exists between the synuclein proteins, meaning the lack of α-synuclein may by compensated for by its family members. Consequently, this has prompted the generation and analysis of single β-synuclein and γ-synuclein knockout mice, and mice which lack a combination of the proteins. These mice, along with the α-synuclein knockout mice, have been summarised in Table 1.2. Again, these mice do not present with an overt phenotype and no impairments in basic brain morphology or survival have been reported. However, once more subtle changes in DA biology, including changes in DA content and release, and changes in behaviour, have been observed. In both α- and β-synuclein, and α- and γ-synuclein double knockout mice, increased expression of the remaining synuclein family member was observed, implying that once more functional compensation may be occurring (Chandra et al., 2004; Robertson et al., 2004).

1.5.3. Synuclein transgenic mouse models

Several lines of α-synuclein-based transgenic mouse models are also available that overexpress wild-type, A30P or A53T forms of human α-synuclein under the control of a variety of promoters. These promoters include the platelet derived growth factor-

β (PDGFβ) (Masliah et al., 2000), the prion promoter (PrP) (Giasson et al., 2002; Gomez-Isla et al., 2003; Lee et al., 2002; Maskri et al., 2004; Yavich et al., 2005), the Thy-1 promoter (Kahle et al., 2000b; Rockenstein et al., 2002; van der Putten et al., 2000), the TH promoter (Matsuoka et al., 2001) and the chicken β-actin promoter (Maskri et al., 2004). The characteristic features of these transgenic models has been summarised in Table 1.3.

Reference	Promoter	Transgene	Expression Levels	Motor phenotype	Neuropathology
(Masliah et al., 2000)	PDGF-β	hu-wt	10-80% of human controls	Middle age onset, mildly impaired performance on rotarod.	↓ striatal TH (levels and activity); ↓ DA levels observed in highest expressors. α-syn and ubiquitin positive inclusions.
(van der Putten et al., 2000)	murine Thy-1	hu-A53T		Early onset severe, progressive motor decline.	Widespread, prominent degeneration of motor neurons and spinal cord. α-syn and ubiquitin positive inclusions. No modification of nigrostriatal system.
(Kahle et al., 2000b)	murine Thy-1	hu-wt, hu-A30P	2-fold end. α-syn	None.	α-syn positive inclusions.
(Matsuoka et al., 2001)	rat TH	hu-wt, hu-A30P hu-A53T		N/A.	No pathology despite high levels of expression in SNpc.
(Giasson et al., 2002)	mouse prion	hu-wt, hu-A53T	2.5 - 30-fold end. α-syn	A53T mice: middle age onset, severe complex, progressive motor impairment.	Widespread prominent degeneration, particularly in spinal cord. Sparing of motor cx and SNpc. α-syn positive inclusions.
(Lee et al., 2002)	mouse prion	hu-wt, hu-A30P hu-A53T	4-15 x > non Tg	A53T mice: Late onset, severe motor decline. Earlier onset in highest expressors. A30P mice: none despite comparable levels of transgene expression.	A53T mice: Widespread axonal degeneration. α-syn positive inclusions. No alteration in dopaminergic markers. Exacerbated pathology in highest expressors. A30P mice: none despite comparable levels of transgene expression.

(Richfield et al., 2002)	rat TH	hu-wt hu-A30P + A53T	30-50% of mouse α-syn	Middle age onset, mild. ↓ locomotion and coordination.	↓ DAT density; ↓ DA, DOPAC, HVA
(Kahle et al., 2002)	proteolipid	hu-wt	0.1-1,2 ng/ug	None.	Insoluble oligodendroglial inclusions similar to those seen in MSA.
(Rockenstein et al., 2000) (Fleming et al., 2004) (Fleming et al., 2006)	murine Thy-1	hu-wt, hu-A30P	10-fold human levels	hu-wt mice: Early onset reduced olfaction not attributed to motor impairments. Early onset, progressive deficits in behaviours specifically testing nigrostriatal function.	α-syn positive inclusions.
(Gomez-Isla et al., 2003)	hamster prion	hu-wt, hu-A30P hu-A53T	5 – 15 fold end. α-syn	early/middle age onset, severe motor decline. Exacerbated in highest expressors.	No alteration in dopaminergic markers. α-syn positive inclusions.
(Gispert et al., 2003)	mouse prion	hu-wt, hu-A53T	5 – 20-fold end. α-syn	Motor decline in both lines correlating with expression levels in both lines.	Altered neuronal morphology. Diffuse accumulation of α-syn, with no α-syn aggregation.
(Maskri et al., 2004)	ch-β-actin, mouse prion, mouse TH	hu-wt, hu-A30P hu-A53T	High for all promoters. Not quantified.	N/A.	Demonstration of significant variation in expression pattern between lines expressing from the *same* promoter. More replicable expression from TH promoter.

(Yavich et al., 2005)	mouse prion	hu-A30P	1.5-fold endo α-syn	middle age onset; ↓ locomotion, deteriortating coordination and balance. Increased stereotypy.	No gross morphological changes, no Lewy-like inclusions, no loss of TH immunoreactivity. No change in lifespan. Decrease in evoked dopamine release, despite no alteration in dopaminergic markers.

Table 1.3 ***Summary of current α-synuclein transgenic mouse models*** *(Adapted and updated from (Fernagut and Chesselet, 2004))*

In general, these mice have been successful in reproducing some aspects of neurodegeneration, including neuronal toxicity and behavioural impairments but to date no α-synuclein-based transgenic mouse accurately represents the essential features of PD. The phenotype varies from none (Matsuoka et al., 2001), to a severe and widespread pathology which does not selectively target DA neurons in the SNpc, a key pathological hallmark in PD (Giasson et al., 2002; Masliah et al., 2000; van der Putten et al., 2000). The phenotypic outcome in these transgenic mice depends heavily on the promoters used to drive the transgene expression. On the whole, more severe phenotypes have consistently been observed in lines with the highest levels of transgene overexpression, and in the lines with α-synuclein mutations, supporting the emerging view that expression levels may have a significant role in disease progression and manifestation (Singleton et al., 2003). The promoters used in these models have specific drawbacks related to their expression patterns and levels in the CNS. For example, broad promoters, such as the PrP, exhibit particularly high levels of expression in neurons that are not typically affected in PD, such as motor neurons, which result in patterns of cell death which are not typical of those seen in patients (Fernagut and Chesselet, 2004). Conversely, using the TH promoter only drives α-synuclein expression in catecholaminergic neurons, whereas α-synuclein is widely expressed in human neurons and α-synuclein PD neurodegeneration is often more widespread in the brain. The site of integration of the cDNA cassette also appears to have effects on the transgene (Maskri et al., 2004), as does the background strain of the mouse, which reportedly can modify the pattern of transgene expression driven by the same promoter (Chesselet et al., 2008). In addition, the presence of endogenous α-synuclein in these models may be a problem. A study by Cabin and colleagues demonstrated that the presence of endogenous mouse α-synuclein protects against the

deleterious pathogenicity of the human A53T mutant α-synuclein protein (Cabin et al., 2005). Furthermore, it should be noted that the endogenous mouse *Snca* gene sequence already has a threonine at position 53. In summary, α-synuclein-based transgenic mice have relied on cDNA-based expression cassettes driven by strong constitutive promoters, which are unlikely to result in physiologically relevant levels of α-synuclein or allow regulated, temporal and spatially specific transgene expression. Indeed, the overexpression levels of human α-synuclein in these transgenic mice have been estimated to range from 0.5 to 30 fold compared with endogenous α-synuclein, which bears little or no physiological relevance (for references see Table 1.3). Therefore, while these models may represent a useful model for α-synuclein toxicity, it is possible the observed phenotypes may produce some artefacts which arise due to high levels of transgene expression.

1.5.4. Summary

What is clear from the discussions of α-synuclein-based knockout and transgenic mouse models is that novel approaches are required to study the normal functions of the synuclein proteins and to investigate more specifically the role of α-synuclein, and its mutations, in PD pathogenesis. The problem of intra-synuclein compensation will only be overcome with the generation and functional analysis of mice which lack all three members of the synuclein family. This approach will allow a more informed and thorough understanding of both the normal roles of the synuclein proteins, and their potential roles in neurodegenerative diseases. Further to this, a novel approach to generating α-synuclein-based genetic transgenic models must be adopted, which can overcome the shortcomings of the current cDNA-based models. These models must

then be functionally characterised to assess more rigorously the potential roles of α-synuclein in PD. The need for thorough analysis of synuclein triple knockout mice, and the need for functional characterisation of novel α-synuclein-based transgenic mice form the basis of this thesis.

1.6. Thesis Aims

The first aim of this thesis is to study the functional effects of mutations in α-synuclein when expressed from the complete *SNCA* locus in a novel transgenic mouse model, and is described in Chapters 3 and 4. Chapter 3 focuses on the molecular characterisation of a *SNCA*-BAC transgenic mouse line which carries the PD-associated A30P mutation. More specifically, the Chapter describes the breeding strategy for the *SNCA*-BAC transgenic mouse lines and confirms the protein expression, transgene integrity and location of the *SNCA* locus in the *SNCA*-BAC transgenic mouse lines. Further, the Chapter characterises spatial expression of the transgene and expression of the transgene in the SNpc and VTA over time in the *SNCA*-A30P^{+}*Snca-/-* line. A preliminary analysis of any early molecular pathology in the *SNCA*-A30P^{+}*Snca-/-* line is also described. Chapter 4 focuses on the neurochemical characterisation of DA synapse function with age in *SNCA*-A30P^{+}*Snca-/-* transgenic mice. The Chapter assesses differences in the levels of electrically evoked DA in the dorsal and ventral striatum of *SNCA*-A30P^{+}*Snca-/-* transgenic mice compared to non-transgenic littermate control mice (*Snca-/-*) with increasing age, using fast scan cyclic voltammetry at carbon fibre microelectrodes, and assesses whether any changes in DA releasability are regional specific within the CPu. Further, differences in uptake and changes in DA content are investigated in

CPu in *SNCA*-A30P^{+}*Snca-/-* transgenic mice compared to *Snca-/-* control mice with increasing age, and preliminary investigations into whether differences in frequency sensitivity and nicotinic receptor function occur in young adult *SNCA*-A30P^{+}*Snca-/-* transgenic mice compared to *Snca-/-* control mice are described.

The second aim of this thesis is to investigate the behaviour and DA neurotransmission in a novel mouse model lacking all three members of the synuclein gene family, and is described in Chapter 5. Behavioural testing focuses on assessing whether abnormalities in activity-, motor- or anxiety-related behaviours are exhibited in synuclein triple-null mice when compared to age-matched, wild-type control mice. Further, the Chapter reports on the results of assessing differences in the levels of electrically evoked DA in the dorsal and ventral striatum of synuclein triple-null mice compared to control mice and investigations into whether there are differences in DA content in the CPu and NAc in synuclein triple-null mice compared to control mice. The Chapter describes the results of experiments performed to assess candidate mechanisms for potential differences in DA neurotransmission and DA content in these mice, including potential differences in frequency sensitivity and nicotinic receptor function, DA uptake, or calcium dependent changes in exocytosis.

2. CHAPTER 2: Materials and Methods

2.1. Generation of mice

SNCA-BAC transgenic mice characterised in Chapters 3 and 4 were generated previously in the Wade-Martins group by Dr Steven Senior. Briefly, the 146 kb PAC clone 27M07 containing the complete 111 kb *SNCA* genomic locus was obtained. From this, a 135 kb region which contained the 111 kb locus and approximately 18 kb of upstream and 6 kb of downstream sequence was subcloned into a BAC vector by homologous recombination. BAC constructs were then modified to contain the A30P point mutation using combined techniques of homologous recombination in *E. coli* and site-directed mutagenesis. BAC constructs containing human SNCA locus and the A30P mutated form were microinjected into the pronuclei of fertilized C57Bl6 mouse ova using standard pronuclear injection methods at the MRC Mary Lyons Centre, to generate *SNCA*-BAC transgenic mice. Further detail on the breeding strategy is discussed in Section 3.2.1.

Triple synuclein-null mice characterised in Chapter 5 were generated previously at the University of Cardiff. Briefly, α-γ-synuclein double knockout mice were generated as described previously (Robertson et al., 2004). Heterozygous β-synuclein knockout mice (Chandra et al., 2004) on C57Bl6 background were further backcrossed with pure C57Bl6 mice for a further six generations before breeding with α-γ-synuclein double knockout mice. Resulting triple heterozygous animals were intercrossed to produce founders of triple synuclein-knockout and wild-type colonies. Therefore, all mice were on the same C57Bl6 genetic background.

2.2. DNA extraction

All transgenic and knockout littermate control mice used in Chapters 3 and 4 were ear or tail biopsied shortly after weaning to obtain tissue for DNA extraction. Genomic DNA was extracted using the following standard protocol. The biopsy was digested in 450 µl (for ear tissue) or 700 µl (for tail tissue) of a lysis buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris pH 8.5, 0.45% NP40 (Terigitol), 0.45% Tween-20) and 260 µg (for ear tissue) or 400 µg (for tail tissue) of proteinase K (10 µg/ml, Sigma). The biopsy was digested for two to three hours at 55ºC. The sample was then heated to 95ºC for 20 minutes to inactivate the proteinase K. The sample was then incubated on ice for one minute, and then centrifuged at 7 000 rpm for one minute in a bench-top centrifuge. The supernatant was then stored at 4ºC and used for genotyping.

2.3. PCR

Genotyping was carried out to identify the mice carrying the BAC-*SNCA* transgene. PCR was used to amplify the wild-type or knock-out *Snca*$^{-/-}$ alleles, and to amplify the junctions between the BAC vector and the *SNCA* genomic DNA insert. Exon PCR was used to confirm all six exons from the BAC insert were present. Separate master mixes were prepared for each primer pair. The primer sequences used were previously designed in our laboratory and are listed in Table 2.1.

Name	Forward primer	Reverse primer	Product size
Mouse *Snca* WT	CAGCTCAAGTTCAGCCACGA	AAGGAAAGCCGAGTGATGTAC	485 bp
Mouse *Snca* KO	CAGCTCAAGTTCAGCCACGA	ATGGAAGGATTGGAGCTACG	550 bp
BAC-*SNCA* 5' junction	CACTGAGTATTGTTCTGGTAAC	TCAAACATGAGAATTGGTCG	450 bp
BAC-*SNCA* 3' junction	GGCCTCTGTCGTTTCCTTTCTCTG	ACGTGCATAGGCAGAGCTAAAACCT	450 bp
SNCA Exon 1	ATCCGAGATAGGGACGAGGAG	AATAGGAAAGAAGAAGGAAAAGGAG	465 bp
SNCA Exon 2	CCGAAAGTTCTCATTCAAAGTG	TGACATTTGGGGTTTACCTACC	206 bp
SNCA Exon 3	GAAAACTAGCTAATCAGCAATTTAAGG	AATCTTGAATACTGGGCCACAC	182 bp
SNCA Exon 4	CCACCCTTTAATCTGTTGTTGC	CACAAAACGTACACAGCCATAC	203 bp
SNCA Exon 5	TCATCATGTTCTTTTTGTGCTTC	TGTGACAATGACAGGTTTTTGG	182 bp
SNCA Exon 6	AACAGTGTGTGCTGTCTTTTTG	AGATTGAAGCCACAAAATCCAC	317 bp

Table 2.1. ***List of primer sequences used for PCR.*** All PCR primer sequences are given 5' – 3'.

A PCR master mix was prepared as follows (for each 20 µL PCR reaction):

10x Buffer (Qiagen)	2 µL
dNTP's (8 Mm)	0.4 µL
Forward and reverse primer mix (10 mM)	2 µL
Hotstar Taq (Qiagen)	0.1 µL
Autoclaved MilliQ H_2O	13.5 µL

Master mixes for the individual primer pairs were added to the desired number of wells of a 96-well plate. 2 µL of template DNA was added to each of the mixes.

DNA amplification was performed using a PCR Peltier Thermocycler (PTC-225, MJ Research) under the following PCR programme:

1. 95ºC for 15 minutes

2. 95ºC for 30 seconds
3. 60ºC for 30 seconds
4. 72ºC for 1 minute per kb of PCR product
5. Repeat steps 2 – 4 for a further 44 cycles (35 cycles for exon PCR)
6. 72ºC for 3 minutes

PCR products were analysed by gel electrophoresis of 7 μL of PCR product on 2% agrose gels.

2.4. Protein extraction

Protein was extracted from mouse brain tissue using the following standard protocol. Brain tissue samples were weighed to the nearest 0.01g. The sample was then added to 3 mL/g sample ice-cold RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP40 (Tergitol), 0.5% sodium deoxycholate, 0.1% SDS, 1x protease inhibitor cocktail) and homogenised using a glass dounce. The samples were then sonicated briefly and left for one hour on ice. Samples were then centrifuged at 14 000 rpm for 10 minutes at 4ºC using a desk-top centrifuge. The supernatant containing the protein was then removed from the pellet and both were stored at -80ºC until required. Protein quantification was performed using a BCA assay according to the manufacturer's instructions (Sigma).

2.5. Western Blotting

For western blotting, 10 μg of protein was boiled in 1x Laemelli buffer for 5 minutes. Proteins were then separated by SDS-polyacrylamide gel electrophoresis and transferred onto a 0.45 μm PVDF membrane (Millipore). The membrane was blocked in WB blocking buffer (tris buffered saline (TBS), 1% Tween-20, 5% milk) and probed using primary antibody suspended in WB antibody buffer (TBS, 1% Tween-20, 1% milk) for one hour at room temperature. The membrane was washed three times with TBS, 1% Tween-20 and then incubated with an appropriate horse radish perioxidase-conjugated secondary in WB antibody buffer for one hour at room temperature. The membrane was then washed four times with TBS, 1% Tween-20. Protein bands were visualised by chemiluminescence using an ECL^{+} kit (Amersham). Images were taken using a charge couple device camera and Quantity One Image software (Bio-Rad). The antibodies used were: mouse monoclonal anti-human α-synuclein (Abcam) and goat anti-mouse IgG HRP-conjugated (Bio-Rad).

2.6. Perfusion/Fixation

Mice were administered an overdose of anaesthetic by intraperitoneal injection, using ketamine (0.6 mg/g) and xylazine (0.06mg/g). After cessation of reflexes, the mice were transcardially perfused with 50 ml of 0.2 M phosphate buffer (PB) followed by 50 ml of 4% paraformaldehyde (PFA) in PB (pH 7.4). Brains were removed from the skull and post-fixed in 4% PFA in PB for 24 hours at 4ºC. The brains were then cyroprotected by transferring into 30% sucrose solution (in PBS) for 24-48 hours at 4ºC. Brain sections of 35 μm thickness were cut on a freezing microtome (Leica). The brain sections were stored in 96-well plates at -20ºC in antifreeze solution (0.1 M PB,

4 g sodium chloride, 250 ml ethylene glycol, 250 ml glycerol in 1 L distilled water) until required.

2.7. Immunofluorescence

Immunoflorescence staining was carried out on 35 µm thick free floating sections. Sections were washed three times in 1x PBS, treated with 50 mM glycine in PBS for 15 minutes and then washed a further three times in 1x PBS. The sections were blocked for one hour in PBS+ (PBS, 10% donkey serum, 0.5% Triton X-100) at room temperature on a shaker table. Sections were then incubated overnight in PBS+ containing the primary antibodies (Table 2.2) at 4ºC on a shaker table. After incubation the sections were washed three times in 1x PBS. All procedures from this point were performed ensuring the sections were covered from light. The sections were incubated for 1 hour in PBS+ with the appropriate secondary antibodies at room temperature on a shaker table (goat anti-rabbit, 1:500, Alexa 594, Invitrogen; goat anti-mouse, 1:500, Alexa 488, Invitrogen). After washing a further three times in 1x PBS, the sections were DAPI stained for 10 minutes (40 mg/ml DAPI stock solution 1:1000 in PBS). Sections were washed a final three times in 1x PBS and mounted on slides using Flouromount-G (Southern Biotech) and coverslipped. Sections were imaged using fluorescent microscopy (Nikon).

2.8. Immunohistochemistry

All immunohistochemical staining was performed using a Vector mouse-on-mouse (M.O.M) Peroxidase kit (Vectorlabs), following the manufacturers' instructions.

The primary antibody used is listed in Table 2.2. Sections were imaged using an Olympus BX41 microscope (Olympus, UK).

Epitope	Host	Supplier	Dilution
Human α-synuclein	Mouse monoclonal	Abcam, Ab1904	1:500
Mouse α-synuclein	Mouse monoclonal	BD Transduction Labs, 610787	1:200
Tyrosine hydroxlyase	Rabbit polyclonal	Millipore, Ab152	1:2000

Table 2.2. ***Primary antibodies used for immunostaining.***

2.9. Neuronal cell counts

Three cohorts of male mice, aged 3-6 months, 12 months and 21 months of age, were used for cell counting analysis in the SNpc and VTA. Each cohort comprised of three *SNCA*-A30P^{+}*Snca-/-* transgenic mice and three non-transgenic *Snca-/-* control mice, which together totalled 18 mice for the study. All mice were perfused and fixed, as described above. Immunoflorescence staining was carried out on 35 μm thick free floating sections, as described above. For the SNpc and VTA regions, the slices were confirmed to be: 1) complete, i.e. there were no slices missing; 2) intact, i.e. no slices had folded over when mounting or had fractured apart such that the region could not be used for counting analysis; and 3) immunolabelled i.e. no slices had been poorly stained such that the region could not be used for counting analysis. All brain slices used met these criteria.

The anterior and posterior extent of the SNpc and VTA were identified based on a standard mouse atlas (Paxinos and Franklin, 2001), using landmarks described by Nelson and colleagues (Nelson et al., 1996). Immunostaining was performed on slices which began (as accurately as possible) from Bregma -2.80, where the rostral portion of the SNpc starts with the first TH+ cells near the end of the STN and is lateral to the TH+ cells in the medial forebrain bundle. From here, ten 35 μm alternating sections (i.e. every other section) were immunostained. The caudal SNpc ended where the retrorubal field became visible. Counting included the SN lateralis, and the dorsal portion of the SNpr defined the ventrolateral boundary of the SNpc. The regions ended (as accurately as possible) at Bregma -3.80. Where slices were slightly unequally sectioned (i.e. one hemisphere was more anterior than the other), the left hemisphere defined the start point. Delimitation between the VTA and SNpc was determined using the medial terminal nucleus of the accessory optic tract as a landmark (Bensadoun et al., 2000; Lee et al., 1996). Identification of SNpc and VTA neurons was further defined by the fact DA neurons of the SNpc are larger than VTA DA neurons (Nelson et al., 1996), and SNpc DA neurons are orientated along the SNpc axis.

Quantitative analysis of the number of TH-positive cells, α-synuclein-positive cells and TH^+/α-synuclein double positive cells was performed using fluorescent microscopy. Low power (4x) images were taken for each hemisphere and used as a map to define the SNpc and VTA boundaries. Images were then taken at a 10x magnification of the SNpc and VTA for direct cell counting. Where two images were required to capture the entire SNpc region for certain hemispheres, the images were overlapped slightly to ensure no part was left out. Accurate matching of the images

then ensured that cells were only counted once. All cells with a clearly defined nucleus were counted. All fluorescent microscopy and cell counting was performed blind, by a single investigator (myself). Direct cell counting of TH-positive cells, α-synuclein-positive cells and TH^+/α-synuclein double positive cells was performed using ImageJ software.

2.10. Preparation of primary fibroblasts for fluorescent in-situ hybridisation

The protocol used for extraction of primary fibroblasts was modified from Kulnane et al. (Kulnane et al., 2002) by Dr. Ben Davies at the Wellcome Trust Centre for Human Genetics (WTCHG), University of Oxford and performed with the help of Dr Franziska Denk, Department of Physiology, Anatomy and Genetics, University of Oxford. Briefly, ear punches were placed into Ca^{2+} and Mg^{2+} -free 500 µl HBSS (Gibco) on a petri-dish and diced into small pieces using a razor blade. The ear pieces were then placed in microfuge tube and 500 µl collagenase (type XI-S, 2000 units/ml in HBSS) was added. The samples were then incubated for at 37ºC for 25 minutes and then centrifuged at 1 000 rpm for five minutes. The supernatant was removed and the tissue was incubated in 500 µl 0.05% trypsin-EDTA for 20 minutes. Finally, cell medium was added (complete DMEM), and the fibroblasts were seeded straight into a T25 flask. The medium was replaced after 24 hours, and then as required. Once confluent, the cells were split and half were frozen down whilst half were given to Molecular Cytogenetics, Core Services, WTCHG, University of Oxford, UK, where the generation of metaphase spreads and FISH analysis was performed. BAC DNA was supplied as a probe for FISH.

2.11. Fast-scan cyclic voltammetry

Mice were sacrificed by cervical dislocation, decapitated and their brains removed over ice. 350 μm coronal striatal slices were cut using a vibrotome (Leica Microsystems, UK) in ice-cold HEPES-buffered physiological saline saturated with 95% O_2/5% CO_2 and maintained in a bicarbonate-buffered artificial cerebrospinal fluid (aCSF, containing 2.4 mM Ca^{2+}) as described previously (Cragg, 2003; Rice and Cragg, 2004). Extracellular dopamine concentration was monitored and quantified in the dorsal striatum using FCV as previously described (Cragg, 2003) with 10 μm-diameter carbon-fibre microelectrodes (exposed tip length ~50-100 μm fabricated in-house) and a Millar Voltammeter (Julian Millar, Barts and the London School of Medicine and Dentistry, UK). The applied voltage was a triangular waveform, with a voltage range of -0.7 V to 1.3 V to -0.7 V vs. Ag/AgCl at a scan rate of 800 V/s. The sampling frequency was 8 Hz. Data was acquired and analysed using Strathclyde Whole Cell Program (University of Strathclyde, UK). Electrodes were positioned in striatal slices to a minimum depth of 100 μm. The evoked current signal was confirmed as DA by comparing the potentials for peak oxidation and reduction currents with those of DA in calibration media (+500-600 and -200mV vs. Ag/AgCl, respectively). Electrodes were calibrated in 1 - 2 μM DA in experimental media. DA release was evoked by a surface, concentric bipolar Pt/Ir electrode (25 μm diameter, FHC, USA) as described previously (Rice and Cragg, 2004). Stimulus pulses generated out-of-phase with FCV scans and were applied at the lowest current that generated maximal DA release with a single stimulus pulse in wild-type animals (650 μA, 200-μs pulse duration).

FCV experiments assessed extracellular DA concentration ($[DA]_o$) evoked by discrete stimuli in the caudate putamen (CPu) and nucleus accumbens (NAc). Recording sites classed as CPu were located dorsal to the anterior commissure (AC); NAc was ventral to the AC.

Data were collected through one of two experimental designs. Firstly, several sites were sampled per slice (6-8 CPu, 2 NAc) in both genotypes on the same experimental day. Stimuli in these experiments consisted of either a single pulse or burst pulses (4 pulses at 100 Hz). In the second stimulation paradigm, recordings were taken at a single recording site at 2.5 minute intervals, to ensure consistent release, and consisted of either single pulses or trains of 5 pulses at a range of frequencies (1-100 Hz) in randomized order. These frequencies span the full range of dopaminergic neuron firing frequencies reported *in vivo*. For these experiments in CPu, recording sites were in the dorsal half of the striatum. Data are means ± SEM, and the sample size, n, is the number of observations. The number of animals in each data set was greater than 3. Comparisons for differences in means were assessed by one-way ANOVA and post hoc Bonferroni multiple-comparison t test or unpaired t test using GraphPad Prism.

DA uptake was assessed by comparing the decay phases of DA transients evoked by a single pulse between the two genotypes. DA transients were concentration-matched for a peak of 1.0 ± 0.01 μM from five animals in each genotype. DA uptake via DAT is the principal factor governing DA decay in these evoked transients (Giros et al., 1996). The rate of DA uptake by the DAT obeys Michaelis-Menton kinetics and is therefore proportional to V_{max} and varies with $[DA]_o$. Comparison of the decay phase of DA transients matched for similar peak $[DA]_o$ eliminates differences in uptake rate

caused by differences in $[DA]_o$, therefore prevailing differences in V_{max} should be apparent (Cragg et al., 2000).

2.12. High performance liquid chromatography (HPLC)

Following FCV experiments, the CPu and NAc from two striatal slices per animal were dissected and stored at -80^{o}C in 200 µL of 0.1 M $HClO_4$. For analysis, the samples were thawed, homogenised and centrifuged at 16 000 g for 15 min at 4^{o}C. The supernatant was then analysed for DA and DOPAC by HPLC with electrochemical detection. For each sample, the supernatant was diluted 1 in 15 for measurable analyte detection and 50 µL of the sample was loaded for analysis. Compounds were separated on a 100-mm Microsorb C18 reverse-phase column (Varian) and detected using an electrochemical detector (Decade II SDC, Antec, Leyden) and a carbon working electrode held at +0.7 V vs a Ag/AgCl reference electrode. The mobile phase consisted of 14.5% methanol, 0.1 M NaH_2PO4, 0.8 mM EDTA and 3.2 mM sodium octane sulphonate, pH 2.85. The flow rate was fixed at 1 mL/min. The chromatorgram peaks were identified as DA and metabolites by the retention time of standard mixtures prepared in 0.1 M $HClO_4$ (5 nM final concentrations of DA, DOPAC, HVA, 5-HIAA prepared by serial dilution, stored at 4˚C for up to two weeks). The calibration of standard mixtures was carried out separately every 20 samples to adjust peaks occurrences. Tissue sample size was estimated by protein quantification using a BCA protein quantification kit (Sigma-Aldrich). Analyte measurements were then normalised to protein measurements for each sample.

2.13. Behavioural Protocols

Prior to behavioural testing, mice were left in the testing room for 5-10 minutes to optimise their state of arousal for testing.

2.13.1. Multiple static rods

Performance on the multiple static rods was measured as described previously (Deacon et al., 2002b). Briefly, a mouse was placed on a wooden rod suspended approximately 30 cm above a cushioned surface, facing the end of the rod. Two paradigms were measured: (1) the time taken for the mouse to turn through 180˚ and; (2) the time taken to traverse the rod and step onto an end platform. The experiment was carried out using three rods of decreasing diameter (rod 1 – 35 mm, rod 2 – 22 cm, and rod 3- 9 mm).

2.13.2. Rotating rod test

The rotating rod test was performed as described elsewhere (Guenther et al., 2001). Briefly, mice were tested on an accelerating rotating rod every 90 minutes, four times a day for three consecutive days. The rod was set to rotate at 4 rpm and a mouse was placed onto the rod nose first. After ten seconds, the rod accelerated at 20 rpm to a maximum speed of 40 rpm. The speed at which the mouse fell was recorded and averaged for each day. If the mouse did not fall, the speed was recorded as 40 rpm. Performance across days was compared by two-way ANOVA.

2.13.3. Non-anxiogenic open-field

The non-anxiogenic open-field test was performed as described elsewhere (Deacon et al., 2002b). Briefly, a mouse was placed in one corner of a grey PVC open field measuring 50 × 30 × 18 cm, divided into 10 cm squares, and behaviour was observed for 3 min. Three parameters were assessed: (1) the latency of the mouse to rear for the first time; (2) the number of rears; and (3) the total number of squares entered with all four paws. The apparatus was cleaned between each experimental trial.

2.13.4. Holeboard test

The holeboard test was performed as described elsewhere (Deacon et al., 2002a) to assess spontaneous exploratory behaviour in mice. The apparatus consisted of a black box which measured 40 x 40 x 26 cm on a false floor 7 cm above the surface. The floor was divided into sixteen 10 cm squares, eight of which had 1.5 cm diameter holes in the centre. Four of the holes were in 'peripheral' squares and four were in 'central' squares. A mouse was placed into a corner peripheral square and behaviour was observed for three minutes. Three parameters were assessed: (1) the number of rears, (2) the total number of peripheral hole-dips; and (3) the total number of central hole-dips. Hole-dips were defined as the mouse's head entering the hole until its ears touched the floor.

2.13.5. Home cage activity

Mice were housed individually overnight with free access to food and water. Activity was measured using pressure sensors over a 15 hour period from approximately 5.30 pm until 8.30 am using the Threshold activity monitoring system (Med Associates, Georgia, Vermont). The number of times a pressure threshold was crossed was used as an index of activity. Total number of threshold crossings was assessed.

2.13.6. Spontaneous alternation in a T-maze

Spontaneous alternation in an enclosed T-maze was performed as described previously (Contet et al., 2001; Deacon and Rawlins, 2006). The apparatus consisted of a black T-shaped wooden maze made of arms, measuring 30 cm in length, 10 cm in width, and 29 cm in height. The 'goal' arm entrances were provided with sliding guillotine doors. A central partition wall, extending 7 cm into the start arm, divided the choice point into two goal arms. The purpose of this was to ensure that the mouse did not receive any sensory input from the non-visited arm. A mouse was placed in the start arm of the T-maze and allowed to choose a goal arm. The mouse was then confined to the goal arm by sliding the guillotine door down. The arm entered (left or right) was recorded and the mouse was allowed to explore the goal arm for 25 seconds. The mouse was returned to the start arm, with the guillotine doors re-opened and the central partition removed, and allowed to explore again. The goal arm entered on the second run was recorded (left or right) and the mouse was then returned to its home cage. Each mouse was tested twice each day for a total of 20 trials. The percentage of trials in which the mouse entered a different goal arm on the second run (i.e. alternated) was calculated.

2.13.7. Elevated plus maze test

The maze was plus-shaped and elevated 70 cm above the ground. The maze had two closed arms in the north-south position and two open arms in the east-west position. The arms measured 35 x 6 cm, leading out from a 6 x 6 cm centre square. The walls of the closed arms were at a height of 20 cm. A mouse was placed in the centre square of the maze and allowed to freely explore for 5 minutes. Measurements were made using Ethovision XT (Noldus). Parameters measured were (1) the latency to leave centre square; (2) the number of entries into open and closed arms; and (3) the time spent in open and closed arms. Total distance travelled was also taken into account for any locomotor differences. Time spent in the open arms was used as the primary measure of anxiety.

2.13.8. Bright open field test

A brightly illuminated circular white open field with a diameter of 60 cm and a high-walled circumference was used to assess anxiety in a bright open field. Ethovision XT (Noldus) software was used to define two zones: an open, central zone with a radius of 10 cm and an outer zone between the inner zone and the high-walled parameter. A mouse was placed in the open field next to the perimeter wall and was allowed to explore for five minutes. Parameters measured were (1) the latency to enter central zone; (2) the number of entries into the central zone; and (3) the time spent in each of the zones. Total distance travelled was also recorded. Duration in the central zone was used as the primary measure of anxiety.

3. CHAPTER 3: Molecular characterisation of a novel transgenic mouse model of Parkinson's disease carrying the α-synuclein A30P mutation

3.1. Introduction

The creation of transgenic mouse models to study α-synuclein function is currently dominated by the use of cDNA 'mini-gene' constructs driven by strong heterologous promoters. These transgenic mice have a number of shortcomings. Firstly, exogenous promoters are unlikely to reproduce physiologically relevant spatial or temporal levels and patterns of transgene expression, or alternative splicing. This is especially crucial for α-synuclein given the importance of regulated expression levels of the protein for normal function (Fountaine and Wade-Martins, 2007) and in PD (Chartier-Harlin et al., 2004; Singleton et al., 2003). α-synuclein is also temporally regulated throughout development (George et al., 1995) and is alternatively spliced (Kalivendi et al., 2010; Touchman et al., 2001). Further, the regions with the most severe pathology in existing α-synuclein transgenic mice, such as motor neurons and spinal cord (Giasson et al., 2002; van der Putten et al., 2000), do not correlate with the typical regions of preferential pathology observed in PD. In addition, this pathology is frequently seen in mice with the highest levels of transgene overexpression, which can reach levels 30-fold higher than endogenous α-synuclein (Giasson et al., 2002). Transgene toxicity is also highly dependent on the promoter used to drive expression (Maskri et al., 2004). Therefore, α-synuclein cDNA based transgenic mice are likely to exhibit non-physiological, unregulated, spatially inaccurate transgene expression which is not representative of the physiological expression of the gene. Secondly, the site of

integration of the cDNA cassette also appears to have effects on the transgene (Maskri et al., 2004). Different lines of transgenic mice were analysed, which used the chicken β-actin promoter, the PrP promoter or the TH promoter to drive α-synuclein expression from the same construct. Considerable line-to-line variability in the pattern and level of transgene expression was observed, despite the lines carrying the same construct. This makes phenotypic results from these transgenic mice difficult to interpret since they are likely to be sensitive to site-of-integration effects. Thirdly, the presence of endogenous α-synuclein in these models may influence the results, given that expression levels of α-synuclein influence its function. Furthermore, the presence of endogenous mouse α-synuclein may augment the effects of the transgene (Cabin et al., 2005). It should also be noted that the endogenous mouse *Snca* gene sequence already has a threonine at position 53, which may make interpreting the effects of a human mutant A53T α-synuclein transgene more complicated. Finally, many existing α-synuclein transgenic mice are bred to homozygosity (Kahle et al., 2000a; Masliah et al., 2000). During transgenesis, which relies on random insertion of the transgene, it is possible that the transgene will disrupt and inactivate another gene by integrating into it. Breeding the transgenic mouse to homozygosity can therefore result in the creation of a homozygous knockout for an unknown gene. Whilst this can be guarded against by creating several lines of transgenic mice, caution is still required when interpreting the data. It is generally better practice to avoid this potential artefact by maintaining transgenes in a hemizygous state.

Taken together, it is clear that in order to more reliably study the normal and pathogenic functions of α-synuclein, an improved approach to creating transgenic mice is required. The Wade-Martins group has previously used high-capacity

bacterial artificial chromosome (BAC) RP1-27M07 containing the complete 111 kb *SNCA* genomic DNA locus plus 18 kb of upstream and 7 kb of downstream sequence in a 135 kb insert to successfully create several lines of transgenic mice, in which human wild-type or PD-associated A30P α-synuclein is expressed from the entire *SNCA* genomic locus. These transgenic mice were then backcrossed onto a mouse α-synuclein knockout (*Snca* -/-) background to eliminate any potential interactions with the mouse protein. The BAC transgenic approach overcomes the problems associated with existing cDNA based transgenic mice because whole genomic loci transgenes offer stable, regulated and tissue-specific expression at physiologically relevant level.

These mice therefore provide a novel model to study the functional effects of mutations in α-synuclein when expressed from the complete *SNCA* locus. This chapter describes the molecular characterisation of a *SNCA*-BAC transgenic mouse line which carries the PD-associated A30P mutation.

Specifically, the aims of this chapter are:

1. The continuation of the breeding strategy for the *SNCA*-BAC transgenic mouse lines
2. To confirm protein expression, integrity and location of the *SNCA* locus in the *SNCA*-BAC transgenic mouse lines
3. To characterise the spatial expression of the transgene in the *SNCA*-A30P^{+}*Snca*-/- line
4. To characterise the expression of the transgene in the SNpc and VTA over time in the *SNCA*-A30P^{+}*Snca*-/- line

5. A preliminary analysis of any early molecular pathology in the *SNCA*-A30P^{+}*Snca*-/- line

3.2. Results

3.2.1. Breeding, genotyping and colony management

Five *SNCA*-BAC transgenic mouse lines had been previously produced in which wild-type (*SNCA*-WT) or mutant (*SNCA*-A30P^{+}*)* α-synclein is expressed. At the start of my project, each of the lines was at different stages of backcrossing onto an *Snca-/-* background, and onto a pure C57Bl6 background.

Two *SNCA-A30P*$^{+}$ lines had been produced using pure C57Bl6 embryos using pronuclear injection. Transgenic founders were backcrossed with the *Snca-/-* line for two generations to produce lines hemizygous for the *SNCA*-A30P transgene on a pure C57Bl6 *Snca-/-* background, named *SNCA-A30P*$^{+}$*Snca-/-*. The presence of mRNA and protein expression for one line had been previously confirmed, but for the second line, although mRNA was present, no protein was detectable by western blot. The breeding of this line was therefore discontinued.

Three *SNCA*-WT lines carrying the entire human wild-type *SNCA* locus had also been produced. These lines are control transgenic lines for studying the effects of the mutation the *SNCA*-A30P^{+}*Snca-/-* line. A poor rate of transgenesis was obtained when using C57Bl6 embryos; therefore a switch to C57Bl6/CBA F1 hybrid embryos was required. The resulting three lines required further backcrossing for a minimum of six generations to obtain a *Snca-/-* 99.2% pure C57Bl6 background. Backcrossing was performed over the course of 18 months for three *SNCA*-WT lines, called *SNCA*-WT-

21, *SNCA*-WT-37 and *SNCA*-WT-39. A summary of the breeding strategy adopted is summarised in Figure 3.1.

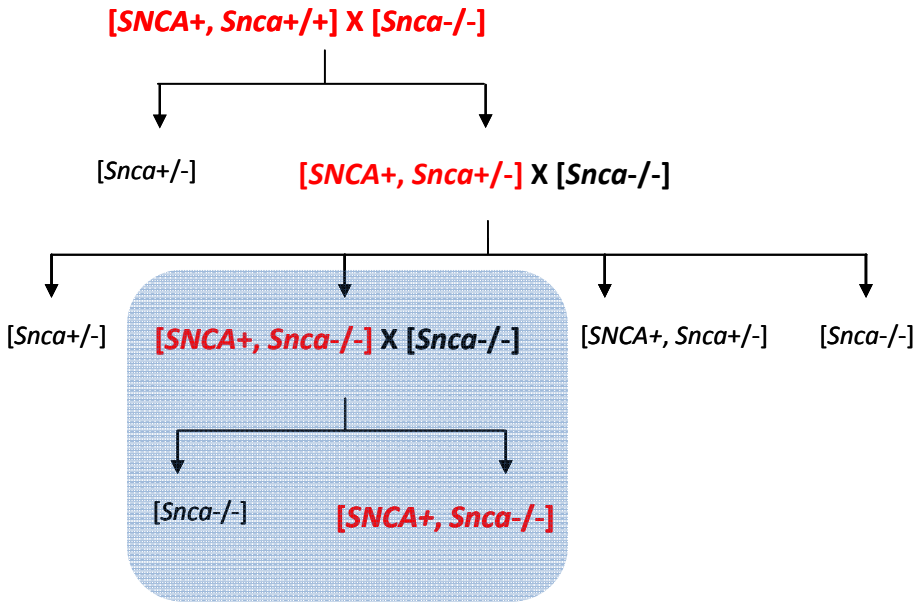


Figure 3.1. ***Summary of breeding strategy for the production and maintenance of SNCA-BAC transgenic mouse lines on a Snca-/- background.*** Offspring genotypes (shown in red) are crossed with *Snca-/-* pure C57Bl6 mice in the next round of breeding. The blue highlighted area of the diagram shows the stage of the breeding plan which is repeated to maintain hemizygous mouse lines on a *Snca-/-* background.

Breeding pairs produced litters of pups which were ear-clipped to obtain a DNA sample. A genotyping PCR assay was routinely used to screen for the presence of the transgene. The results from a typical genotyping assay are illustrated in Figure 3.2. Mice positive for the transgene were then mated with a mouse from an independent colony of *Snca-/-* pure C57Bl6 mice. All transgenic lines were maintained as hemizygotes. The *SNCA*-WT mice were not ready for functional characterisation in this thesis because the breeding programme was 18 months behind the *SNCA*-

A30P^{+}*Snca-/-* line. Therefore, the functional analysis described in this thesis pertains to the *SNCA*-A30P^{+}*Snca-/-* line to assess the potential effects of the A30P mutation, and non-transgenic *Snca-/-* littermates, are used as controls.

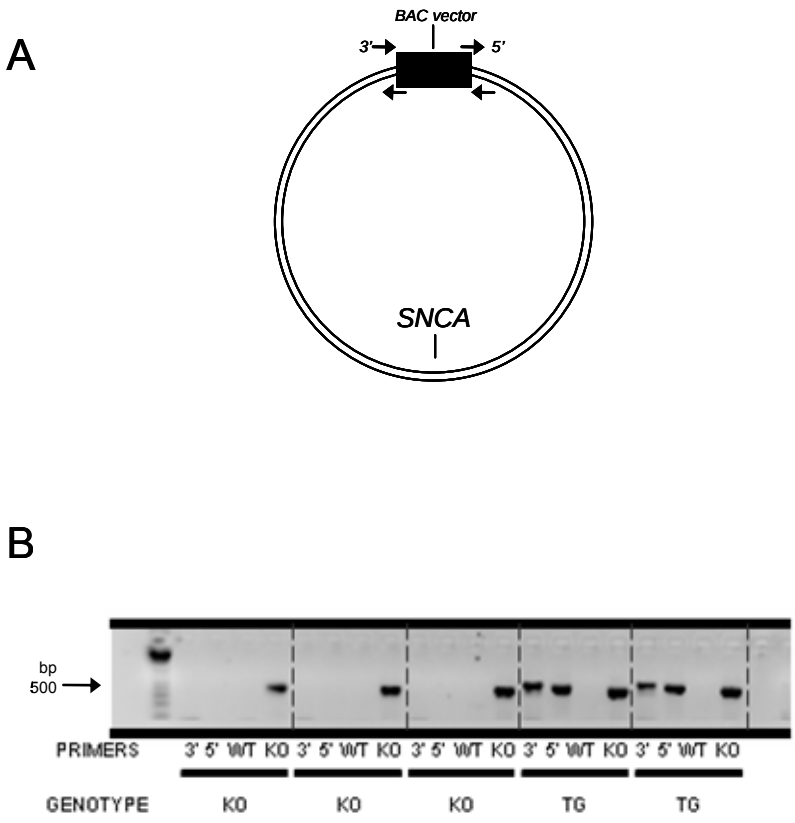


Figure 3.2. ***Typical results from PCR genotyping assay of SNCA^{+} transgenic mice.*** (A) Diagram showing the location of primers used to detect the BAC-SNCA transgene in transgenic mice. (B) These primers amplify BAC DNA to confirm the presence of the transgene. Primers for wild-type and knockout mouse Snca were also used to confirm a *Snca-/-* background.

Given that PD is a disorder associated with advancing age, it was desirable to perform studies in these novel transgenics in ageing cohorts of mice. This required careful colony planning and management from an early stage to ensure the correct gender, genotype and cohort size would be achieved for the maximum lifespan of the mice.

3.2.2. Human α-synuclein protein expression in SNCA-BAC lines

Western blot analysis was used to confirm expression of human α-synuclein protein in the *SNCA*-BAC lines. This confirmed the presence of the protein in the *SNCA*-A30P^{+}*Snca-/-* line (Figure 3.3A), and established the transgene expression in the *SNCA*-WT lines at the level of the protein (Figure 3.3B). Importantly, the *SNCA*-WT-39 line and *SNCA*-A30P^{+}*Snca-/-* line express α-synuclein protein in the brain at a similar level to each other. Since *SNCA*-A30P^{+}*Snca-/-* mice do not contain endogenous α-synuclein, it was not possible to quantitatively compare transgene expression to this protein. Therefore actin was used as the control protein for quantitative analysis, since it is a standard 'housekeeping' protein which is expressed at very similar levels in all lines of mice. Quantitative analysis indicates that when expressed as a percentage of actin expression, the average intensity of α-synuclein protein expression on the western blot in the *SNCA*-A30P^{+}*Snca-/-* line is 14.2% (±SD 4.6%) and in the *SNCA*-WT-39 line is 27.5% (±SD 8.8%). The *SNCA*-WT-39 line and *SNCA*-A30P^{+}*Snca-/-* line also express α-synuclein at a similar level to that seen in a human brain sample from a healthy control individual (kindly obtained from Dr T. Caffrey) (Figure 3.3C). Therefore, the *SNCA*-WT-39 line was established as the control line to be used in future experiments by the Wade-Martins group to study of the effects mutation carrying *SNCA*-A30P^{+}*Snca-/-* line. Furthermore, the one of the *SNCA*-WT transgenic mouse lines produced, *SNCA*-WT-37 expresses considerably more human wild-type α-synuclein protein than any of the other lines, demonstrated by western blot. Quantitative western blot analysis indicates that when expressed as a percentage of actin expression, the average intensity of α-synuclein protein expression

on the western blot in the *SNCA*-WT-37 line is 199.8% (±SD 102.4%). This α-synuclein overexpression line therefore represents a novel approach to studying *SNCA*-WT overexpression at a level similar to that seen in patients with gene duplications or multiplications in a transgenic mouse model carrying the complete *SNCA* locus. It should be noted that the quantification analysis described here was performed on a repeat series of western blot experiments by S. Janezic, Wade-Martins Group.

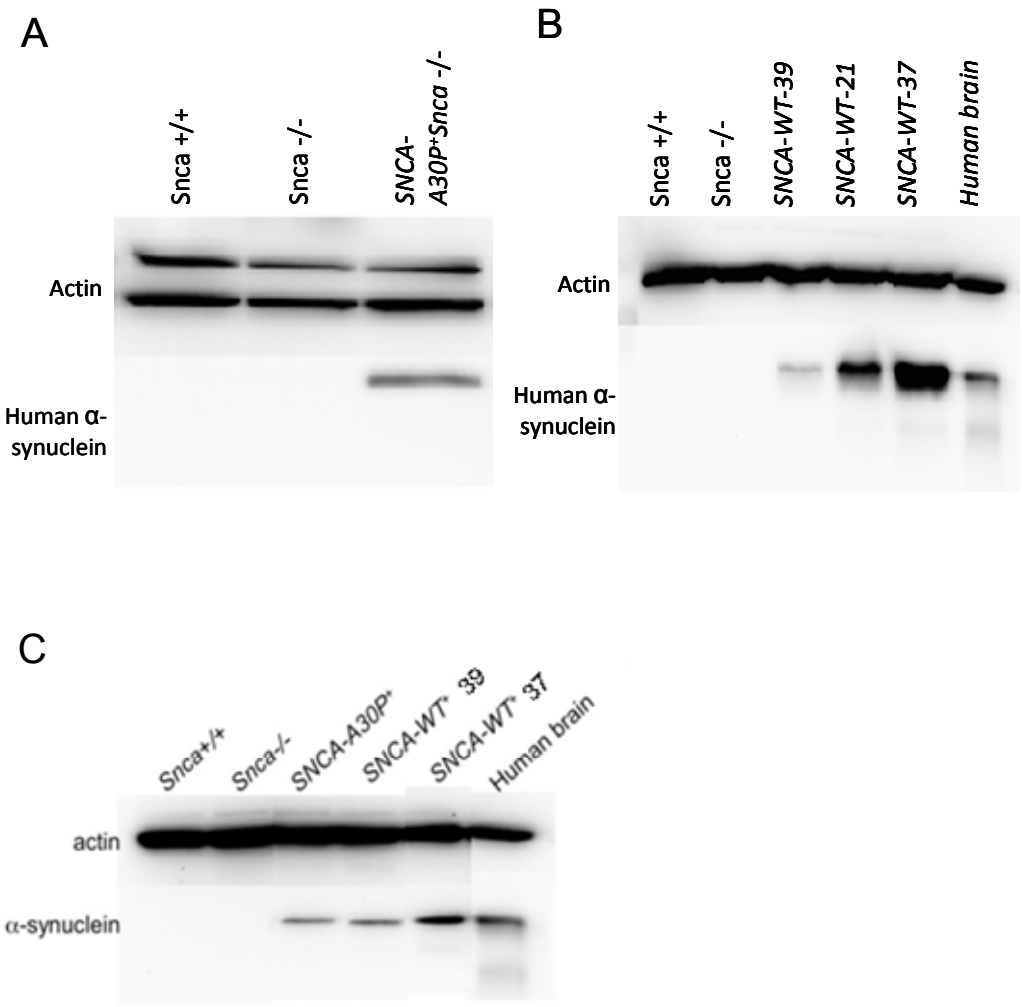


Figure 3.3. ***Expression of human α-synuclein in BAC-SNCA transgenic mice.***
Western blot analysis of BAC-*SNCA* mice (obtained using a human specific monoclonal antibody against α-synuclein) confirmed expression of the human protein in the *SNCA*-A30P+*Snca-/-* line (A); and established transgene expression in *SNCA*-WT lines at the level of the protein (B). (C) shows the *SNCA*-WT-39 line and *SNCA*-A30P+*Snca-/-* line also express α-synuclein at levels similar to each other and to that seen in human brain tissue.

3.2.3. Confirmation of transgene integrity in SNCA-A30P^{+}Snca-/- line

The BAC construct used to generate these mice is 142 kb in size, and the BAC ends are separated only by 7 kb of DNA. Therefore, it was important to check that the entire construct had been inserted into the genome without having undergone partial deletions or rearrangements during microinjection and integration. This had previously been checked by PCR (S.L Senior, *thesis*). However, the *SNCA*-A30P^{+}*Snca-/-* line was re-checked because of its extensive characterisation in this thesis. The presence of all *SNCA* exons in this transgenic mouse line was confirmed by PCR (Figure 3.4).

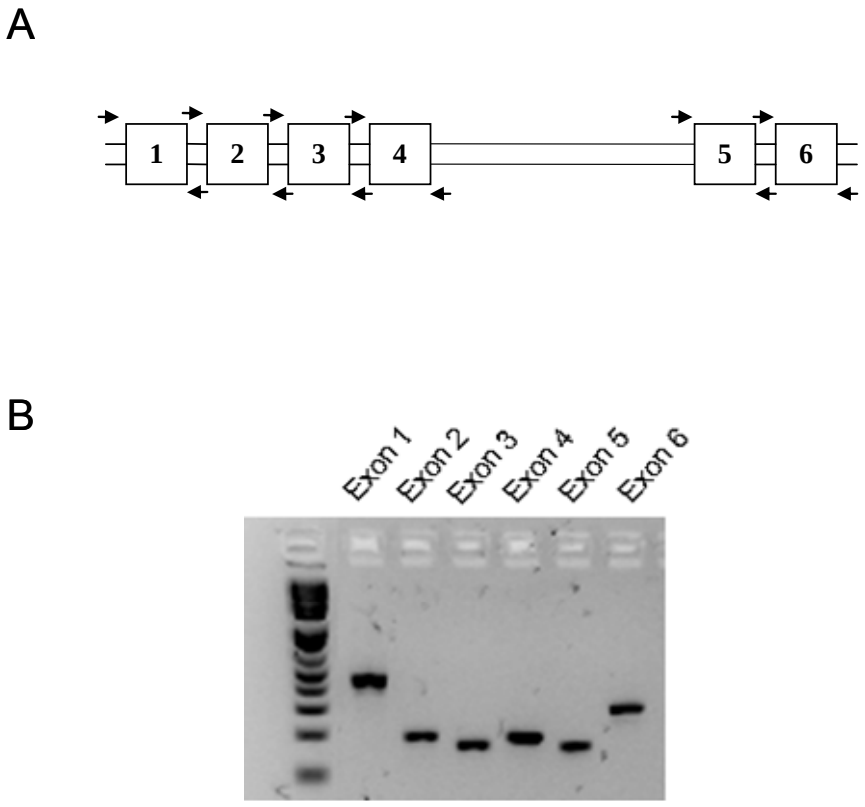


Figure 3.4. ***Confirmation of BAC transgene integrity by PCR in SNCA-A30P+ line.*** (A) PCR primers spanning each of the *SNCA* coding exons were used (exons 1-6). (B) Results from exon PCR screening confirms the BAC transgene has been successfully inserted into the genome without having undergone rearrangements during microinjection and integration.

3.2.4. Location of transgene integration in SNCA-BAC *lines*

FISH analysis was performed on all transgenic lines (with the exception of *SNCA*-WT-21) to determine the site of transgene integration, using the human *SNCA* BAC as the probe. Primary fibroblast cultures were grown from ear-clips to obtain metaphase spreads. FISH was then performed by Cytogenetics Core Services, Wellcome Trust

for Human Genetics, University of Oxford. A single integration site was confirmed for all transgenic lines (Figure 3.5). The *SNCA*-A30P^{+}*Snca-/-* transgene integration was observed near the telomere on one copy of chromosome 15, the *SNCA*-WT-39 integration occurred near the telomere on one copy of chromosome 2 and the *SNCA*-WT-37 integration was found near the centromere on one copy of chromosome 4.

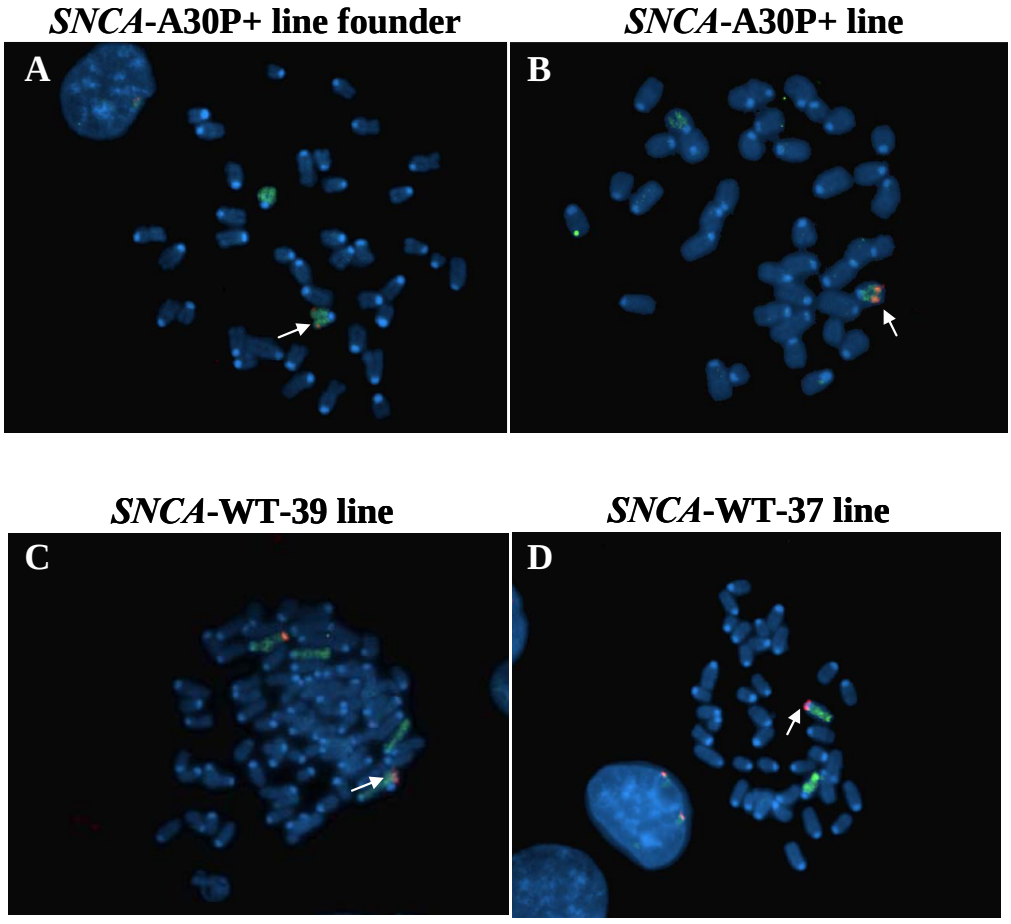


Figure 3.5. ***Location of transgene integration in SNCA*-BAC *lines.*** Fluorescent in-situ hybridisation (FISH) confirms a single integration site was for all transgenic lines using BAC probes in combination with chromosome painting. The *SNCA*-A30P^{+}*Snca-/-* transgene integration was observed near the telomere on one copy of chromosome 15(A, B); the *SNCA*-WT-39 integration occurred near the telomere on one copy of chromosome 2 (C); and the *SNCA*-WT-37 integration was found near the centromere on one copy of chromosome 4 (D). Human SNCA BAC probe (red), chromosome paint (green).

3.2.5. Characterisation of spatial transgene expression in SNCA-A30P^{+}Snca-/- line

Immunohistochemistry was performed on *SNCA*-A30P^{+}*Snca-/-* transgenic mice aged 3-4 months and young adult wild-type C57Bl6 mice which express endogenous α-synuclein protein. This was done to characterise the spatial localisation of transgene expression in the *SNCA*-A30P^{+}*Snca-/-* line. Analysis was performed on coronal and saggital sections, which spanned the entire mouse brain, in both genotypes. The α-synuclein transgene in *SNCA*-A30P^{+}*Snca-/-* mice was shown to have a spatial expression pattern that recapitulates the physiological localisation of endogenous α-synuclein protein in the wild-type C57Bl6 mouse brain (George et al., 1995). Regions showing α-synuclein protein expression include the cerebral cortex, hippocampus (CA1, CA3 and dentate gyrus), striatum, and importantly the SNpc and VTA (Figures 3.6 – 3.10).

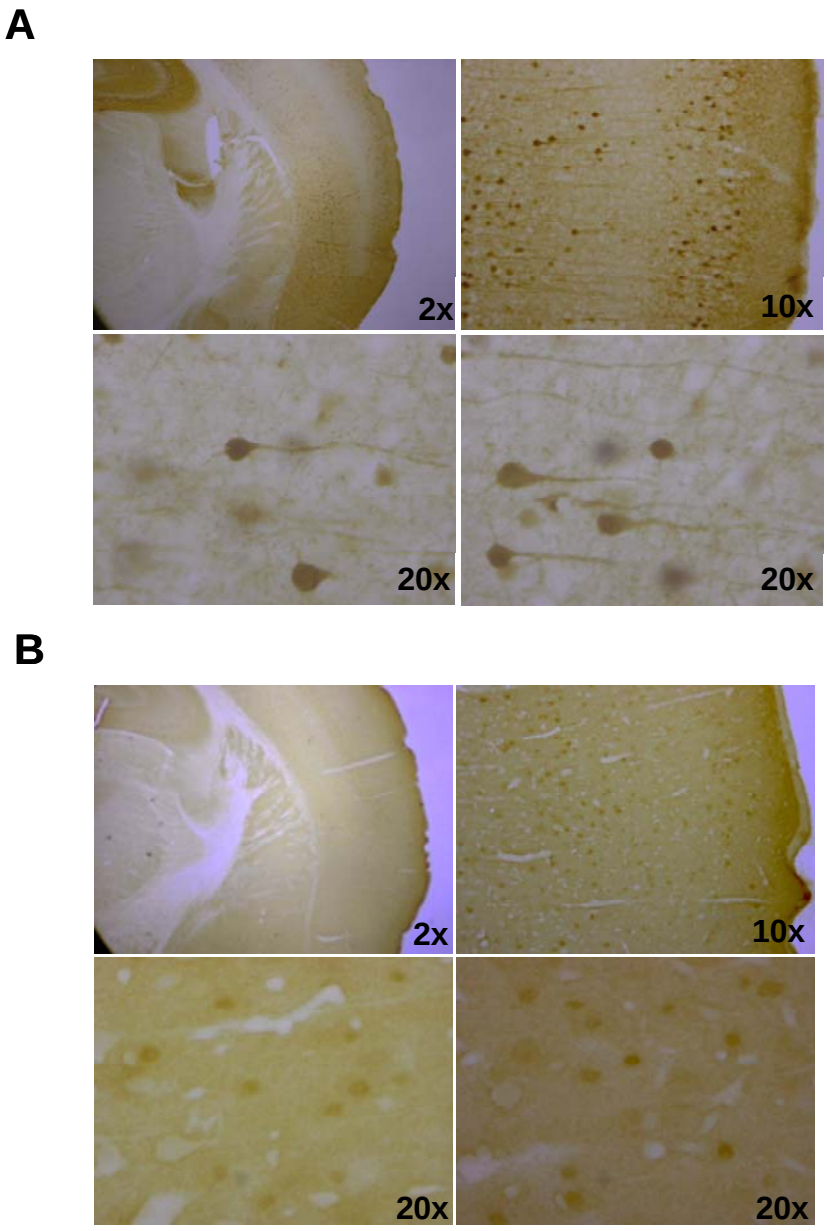


Figure 3.6. ***Characterisation of spatial transgene expression in cerebral cortex in SNCA*-A30P+ *line.*** Immunohistochemical analysis in coronal mouse sections reveals α-synuclein transgene expression in the cerebral cortex in *SNCA*-A30P^{+}*Snca-/-* mice (A) exhibits a spatial pattern of expression which recapitulates that of endogenous α-synuclein protein in wild-type C57Bl6 mice (B).

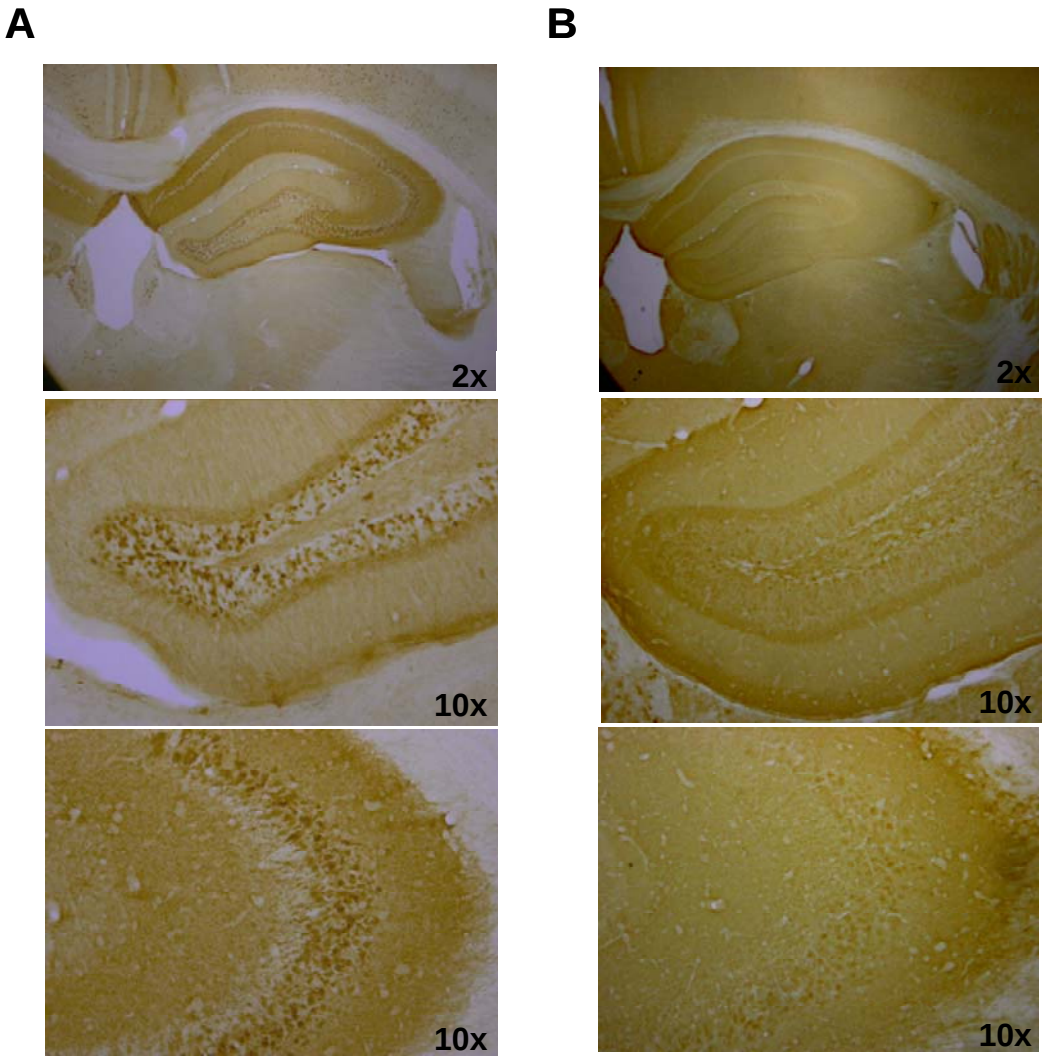


Figure 3.7. ***Characterisation of spatial transgene expression in the hippocampus in SNCA*-A30P+ *line.*** Immunohistochemical analysis in coronal mouse sections reveals α-synuclein transgene expression in the hippocampus in *SNCA*-A30P^{+}*Snca-/-* mice (A) exhibits a spatial pattern of expression which recapitulates that of endogenous α-synuclein protein in wild-type C57Bl6 mice (B).

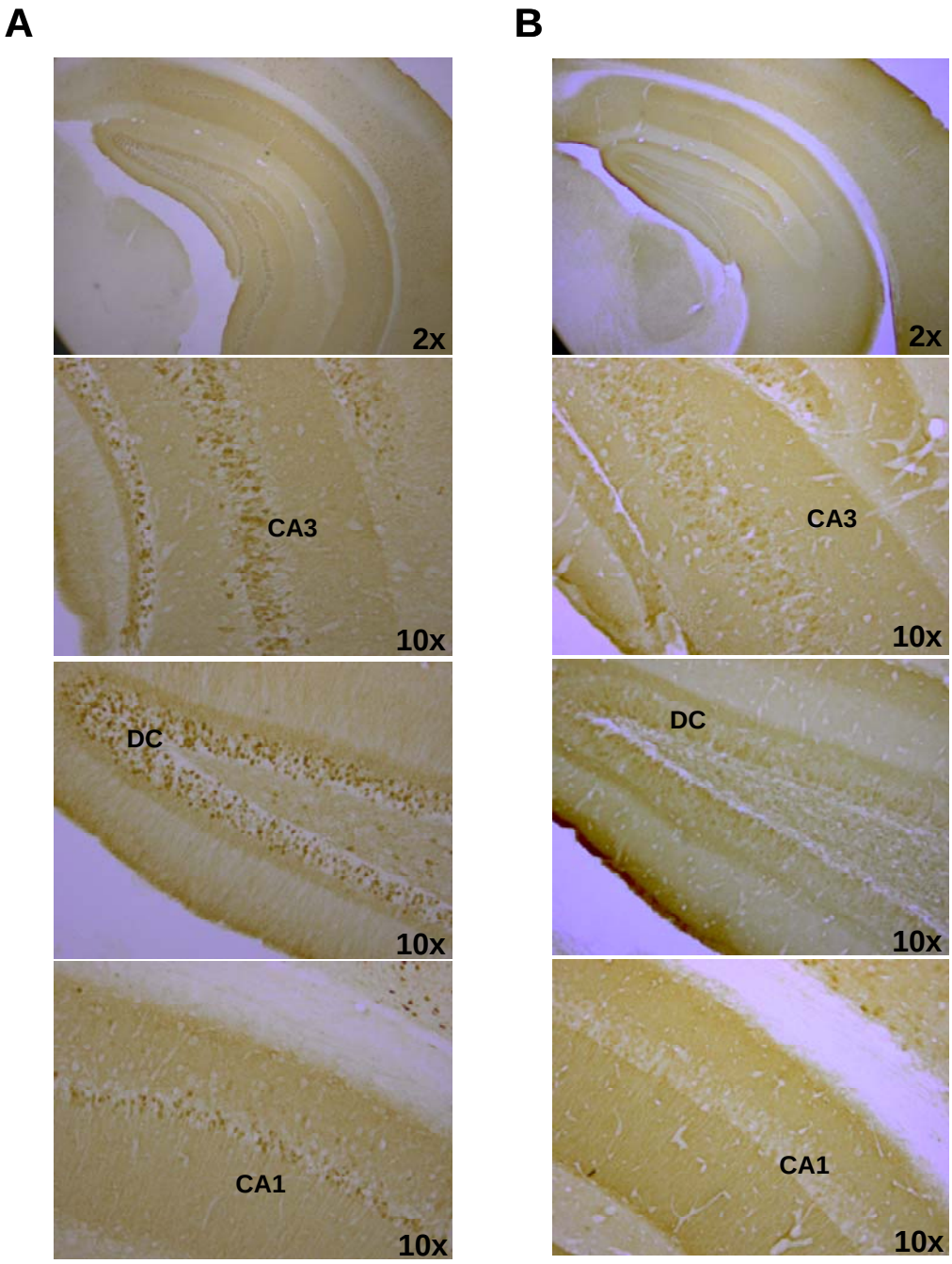


Figure 3.8. ***Characterisation of spatial transgene expression in the hippocampus in SNCA*-A30P+ *line.*** Immunohistochemical analysis in coronal mouse sections reveals α-synuclein transgene expression in the hippocampus in *SNCA*-A30P+*Snca-/-* mice (A) exhibits a spatial pattern of expression which recapitulates that of endogenous a-synuclein protein in wild-type C57Bl6 mice (B).

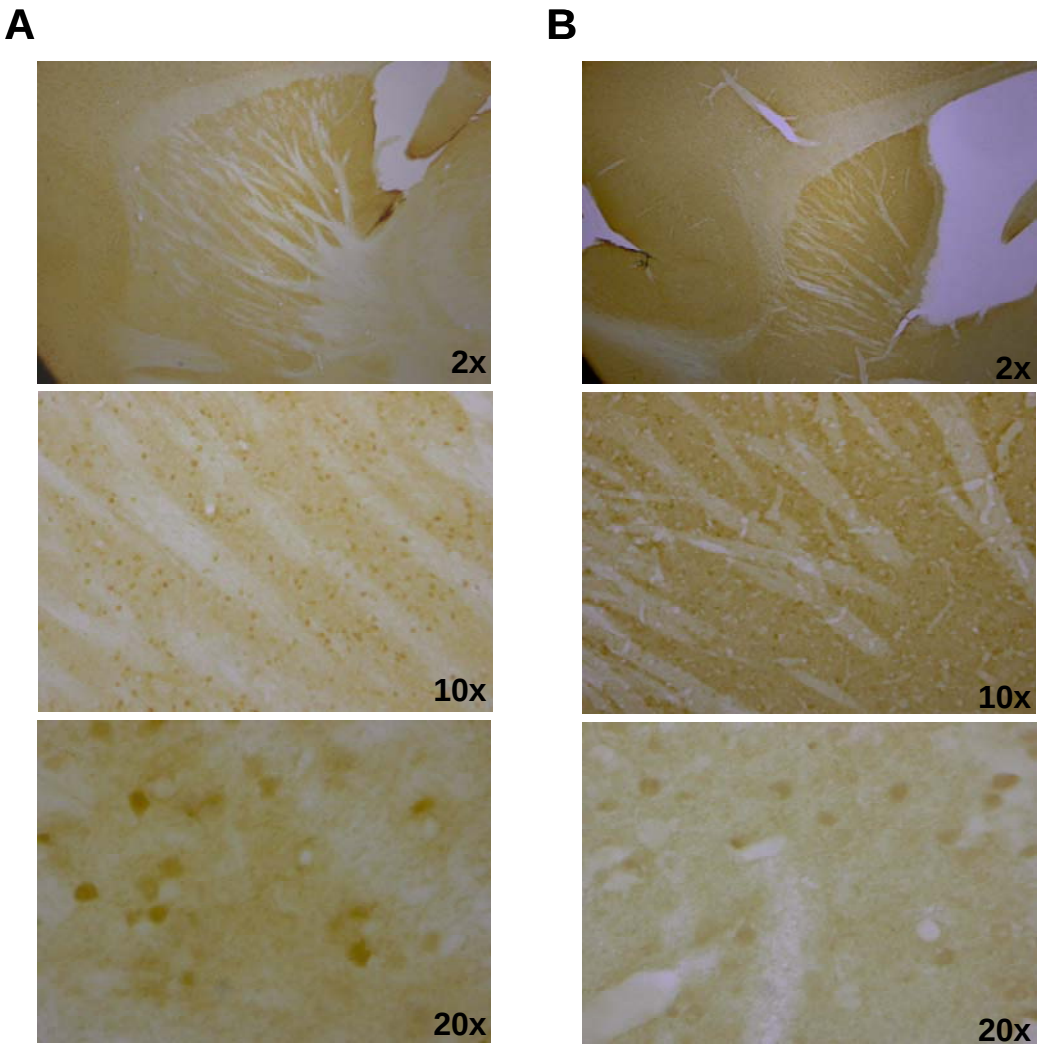


Figure 3.9. ***Characterisation of spatial transgene expression in the striatum in SNCA*-A30P+ *line.*** Immunohistochemical analysis in saggital mouse sections reveals α-synuclein transgene expression in the striatum in *SNCA*-A30P^{+}*Snca-/-* mice (A) exhibits a spatial pattern of expression which recapitulates that of endogenous α-synuclein protein in wild-type C57Bl6 mice (B).

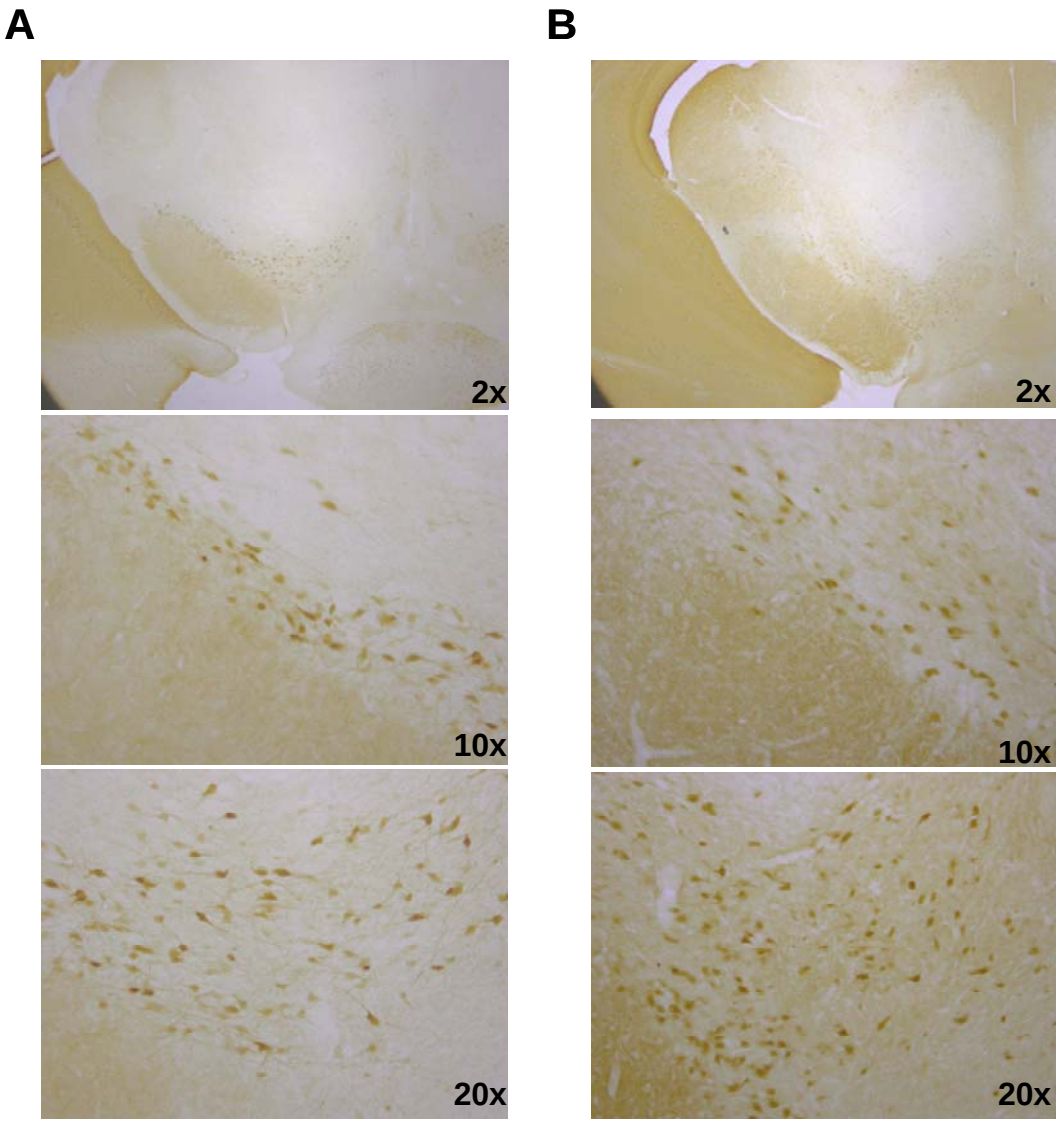


Figure 3.10. ***Characterisation of spatial transgene expression in the substantia nigra pars compacta and ventral tegmental area in SNCA*-A30P+ *line.*** Immunohistochemical analysis in coronal mouse sections reveals α-synuclein transgene expression in the SNpc and VTA in *SNCA*-A30P^{+}*Snca-/-* mice (A) exhibits a spatial pattern of expression which recapitulates that of endogenous α-synuclein protein in wild-type C57Bl6 mice (B).

As well as recapitulating the endogenous expression pattern of mouse α-synuclein, expression in these regions also corresponds to the normal pattern of α-synuclein expression observed in the human brain, according to the Allen Brain Atlas (http://human.brain-map.org/search.html?page=1&query=SNCA), although it should be noted the Allen Brain Atlas reports on gene expression at the RNA level by in situ hybridisation. On an intracellular level, α-synuclein protein was observed in the axons, cell bodies and in the nucleus in both *SNCA*-A30P^{+}*Snca-/-* mice and in wild-type mice. This indicates that in young adult mice, α-synuclein presence in the cell body and nucleus is a physiologically normal feature and is not a consequence of human A30P-mutated α-synuclein being present. In addition, double label immunofluoresence analysis confirms the presence of the α-synuclein transgene in TH^{+} cells in the SNpc, the region of preferential neuronal degeneration in PD. The double label immunofluoresence analysis was performed in the *SNCA*-A30P^{+}*Snca-/-* line (Figure 3.11) and the *SNCA*-WT-39 control transgenic line (not shown).

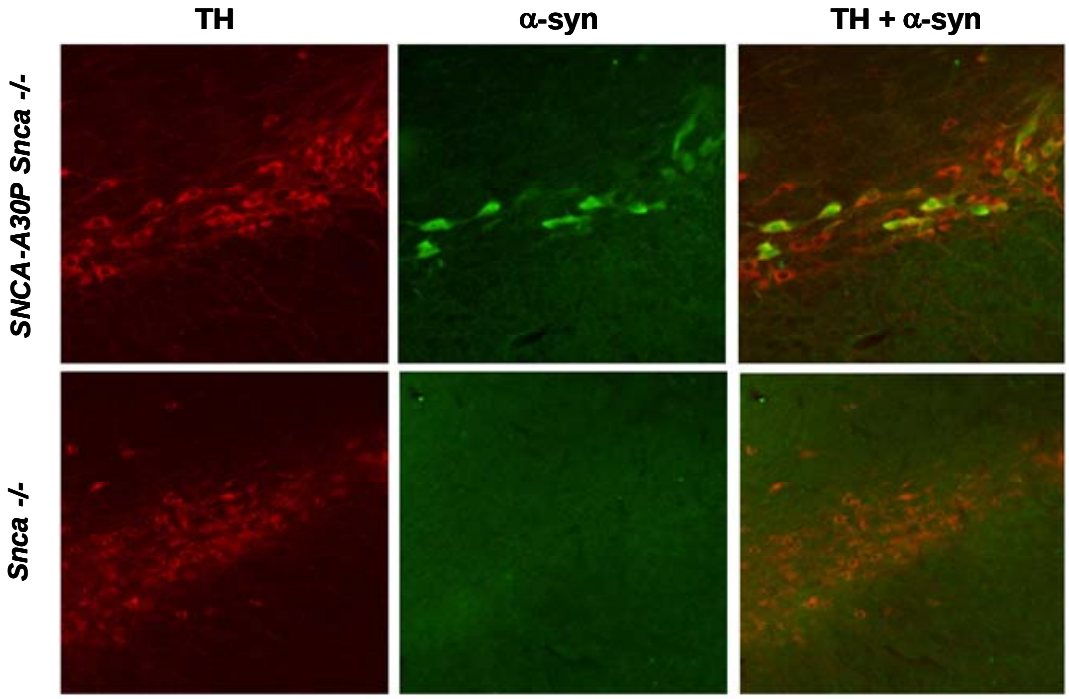
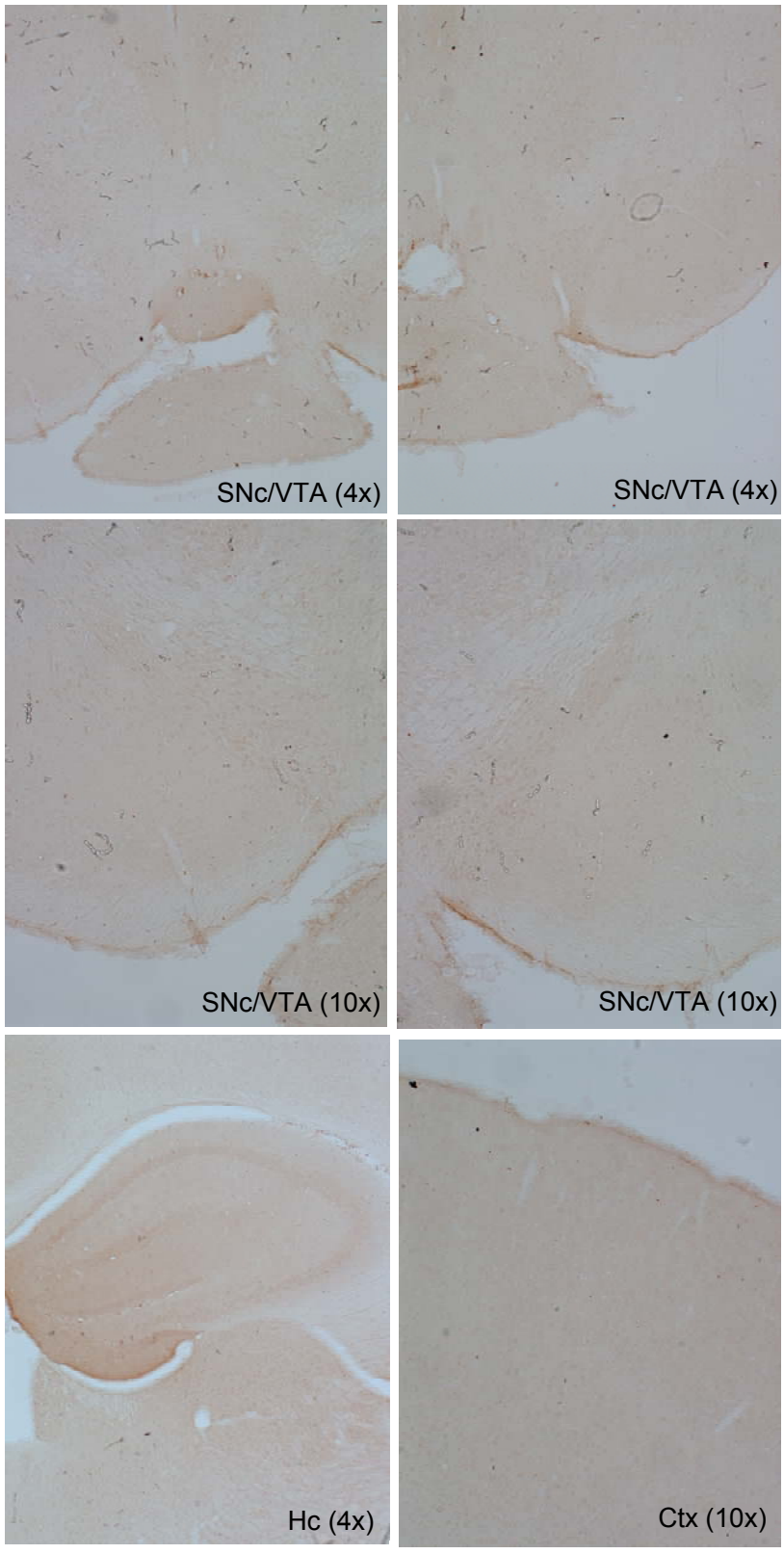


Figure 3.11 ***Human α-synuclein protein expression in BAC-SNCA transgenic mice by immunofluorescence (IF).*** Immunfluorescence staining reveals human α-synuclein protein expression in 3 month old *SNCA*-A30P^{+}Snca$^{-/-}$ mice recapitulates the endogenous pattern of α-synuclein in human and mouse brain in TH-positive neurons in the SNpc, seen as yellow cells in the merged frame (all images shown are 10x magnification).

Two antibodies which work well in immunohistochemistry were used for this analysis. A human-specific anti-α-synuclein antibody was used to detect transgene expression, and a mouse-specific anti-α-synuclein antibody was used for the detection of mouse α-synuclein protein. A comprehensive comparison of spatial expression pattern in the brains of *SNCA*-A30P^{+}*Snca-/-* and wild-type mice was conducted, but the levels of α-synuclein expression in specific brain regions have not been assessed here, since immunohistochemistry staining does not allow robust quantitative analysis of the **level** of protein present in cells. Representative images for negative control staining of *Snca-/-* mice for both antibodies used are shown in Figure 3.12.

A



B

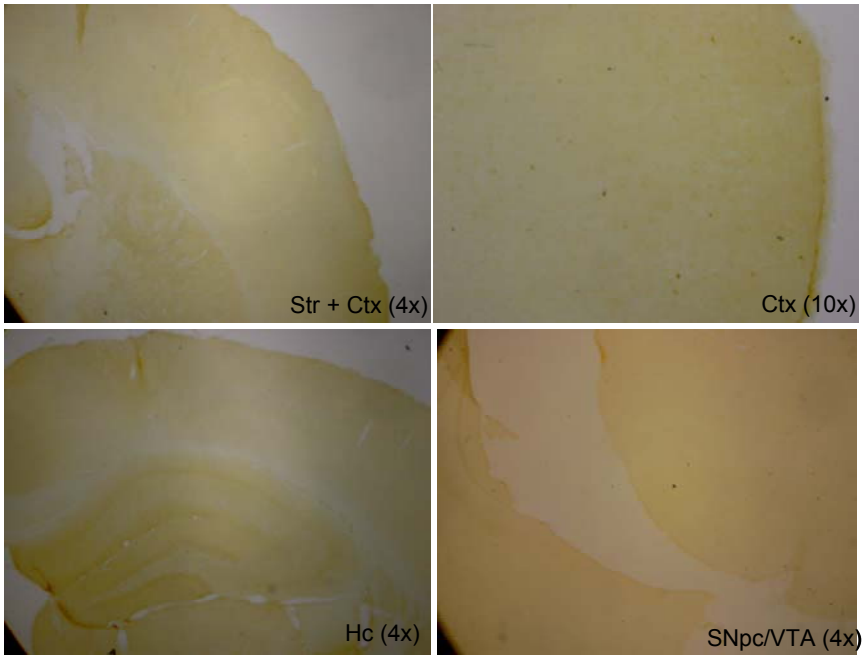


Figure 3.12. ***Characterisation of spatial transgene expression in non transgenic littermate control (Snca-/-) mice.*** Immunohistochemical analysis in coronal mouse sections reveals no α-synuclein transgene expression or endogenous α-synuclein is detected in *Snca-/-* non-transgenic littermate control mice, using the human-specific anti-α-synuclein antibody, used for the detection of transgene expression (A), and the mouse-specific anti-α-synuclein antibody used for detection of mouse α-synuclein protein (B) in Figures 3.6 to 3.10.

3.2.6. Characterisation of transgene expression over time in SNCA-A30P$^+$Snca-/- line

Double-label immunofluorescence and cell counting was performed to analyse and quantify the number of cells which express α-synuclein (not the level of transgene expressions) in the *SNCA*-A30P$^+$*Snca-/-* line with age. This analysis was carried out in male *SNCA*-A30P$^+$*Snca-/-* transgenic mice and *Snca-/-* littermate control mice aged 3-6 months, 12 months and 21 months, with an n=3 per age group for both genotypes. The number of TH-positive neurons, α-synuclein positive neurons and TH/α-synuclein double positive neurons in the SNpc and VTA was assessed. Low power images of TH$^+$ immunoreactivity were used to precisely map the SNpc and VTA regions for counting.

The bilateral number of TH$^+$ cells in *SNCA*-A30P$^+$*Snca-/-* transgenic mice and non-transgenic *Snca-/-* littermate control mice at 3-6 months, 12 months and 21 months for each genotype in the SNpc and VTA was assessed by one-way ANOVA and Bonferroni post-tests (Figure 3.13). In the SNpc, *SNCA*-A30P$^+$*Snca-/-* transgenic mice showed a significant decrease in TH$^+$ cell number across all three age groups (one-way ANOVA, n=3 animals per age group, Bonferroni post-tests, 3-6 months vs. 12 months, *P<0.01***; 3-6 months vs. 21 months, *P<0.001****). By contrast, in non-transgenic *Snca-/-* littermate control mice, no significant differences in TH$^+$ cell number were observed between any of the age groups (one-way ANOVA, n=3 animals per age group, P>0.05). In the VTA, no significant differences in TH$^+$ cell number were observed in *SNCA*-A30P$^+$*Snca-/-* transgenic mice or non-transgenic *Snca-/-* control mice (one-way ANOVA, n=3 animals per age group, P>0.05).

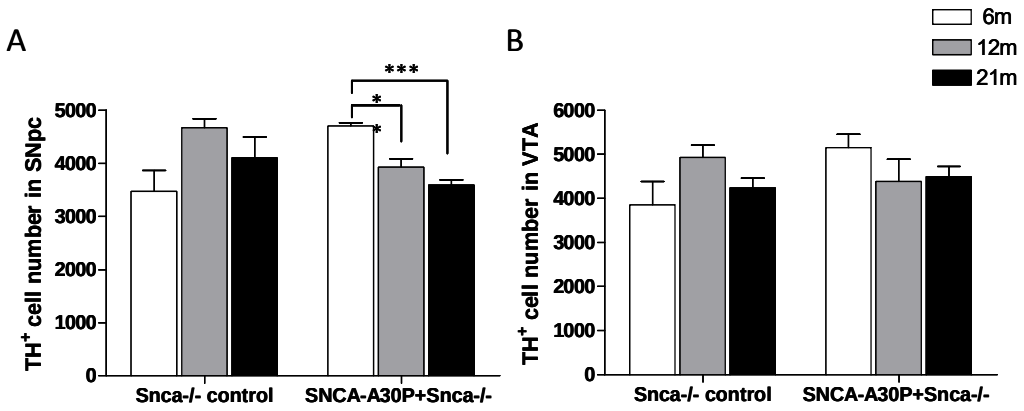


Figure 3.13. ***Comparison of TH+ cell number in the SNpc and VTA in SNCA-A30P+Snca-/- transgenic mice and non-transgenic Snca-/- littermate controls with age.*** The bilateral number of TH+ cells in SNCA-A30P+*Snca-/-* transgenic mice and non-transgenic *Snca-/-* littermate control mice at 3-6 mo, 12 mo and 21 mo for each genotype in the SNpc and VTA was assessed. (A) In the SNpc, SNCA-A30P+*Snca-/-* mice exhibited a significant decrease in TH+ cell number across all three age groups (one-way ANOVA, n=3 animals per age group, Bonferroni post-tests, 3-6 m vs. 12 m, *P<0.01***; 3-6 m vs. 21 m, *P<0.001****). By contrast, in non-transgenic *Snca-/-* littermate control mice, no significant differences in TH+ cell number were observed between any of the age groups (one-way ANOVA, n=3 animals per age group, P>0.05). (B) In the VTA, no significant differences in TH+ cell number were observed in SNCA-A30P+*Snca-/-* mice or non-transgenic *Snca-/-* control mice (one-way ANOVA, n=3 animals per age group, P>0.05).

Next, an analysis of the number of TH+/α-synuclein double positive neurons with age in *SNCA*-A30P+*Snca-/-* transgenic mice was performed using two-way ANOVA and Bonferroni post-tests. In the SNpc, as well as a significant reduction in TH+ cell number, described above, a significant reduction in TH+/α-synuclein double positive cell number occurs at 12 months and 21 months of age, both compared to 3-6 months of age (Fig 3.14A, two-way ANOVA, n=3 animals per age group, Bonferroni post-tests, TH+/α-synuclein double positive cell number, 3-6 months vs. 12 months, *P<0.001****; 3-6 months vs. 21 months, *P<0.001****). By comparison, in the VTA, a

small significant decrease in TH+/α-synuclein double positive cell number occurs only at 12 months, but not 21 months, when compared to 3-6 months (Figure 3.14B, two-way ANOVA, n=3 animals per age group, Bonferroni post-tests, TH+/α-synuclein double positive cell number, 3-6 months vs. 12 months, *P<0.05**; 3-6 months vs. 21 months, *P>0.05*). In *SNCA*-A30P^{+}*Snca-/-* transgenic mice, the decreased number of TH^{+} cells at 12 months and 21 months, compared to 3-6 months, in the SNpc is approximately similar to the decrease in the number of TH+/α-synuclein double positive cells, indicating the majority of TH^{+} cells that are lost are positive for the A30P mutated α-synuclein transgene. Representative immunofluorescence images of the SNpc and VTA at 3-6 months, 12 months and 21 months are shown in Figures 3.15 (SNpc) and Figure 3.16 (VTA).

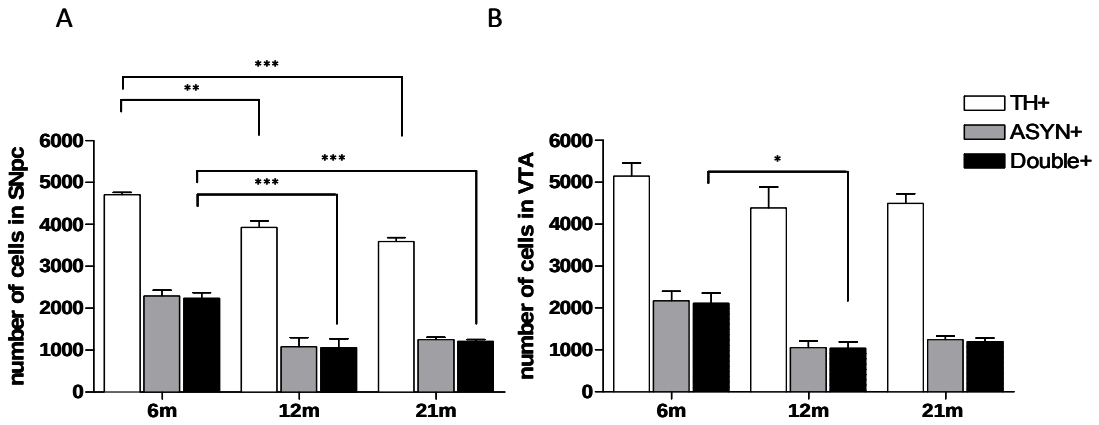


Figure 3.14. ***Comparison of TH+/α-synuclein double positive neuronal cell number in the SNpc and VTA of SNCA-A30P+Snca-/- transgenic mice with age.*** (A) In the SNpc, a significant reduction in TH+/α-synuclein double positive cell number occurs at 12 mo and 21 mo, when compared to 3-6 m (two-way ANOVA, n=3 animals per age group, Bonferroni post-tests, TH+/α-synuclein double positive cell number, 3-6 mo vs. 12 mo, *P<0.001****; 3-6 mo vs. 21 mo, *P<0.001****). (B) In the VTA, a small significant decrease in TH+/α-synuclein double positive cell number occurs at 12 mo, but not 21 mo, when compared to 3-6 mo (two-way ANOVA, n=3 animals per age group, Bonferroni post-tests, TH+/α-synuclein double positive cell number, 3-6 mo vs. 12 mo, *P<0.05**; 3-6 mo vs. 21 mo, *P>0.05*).

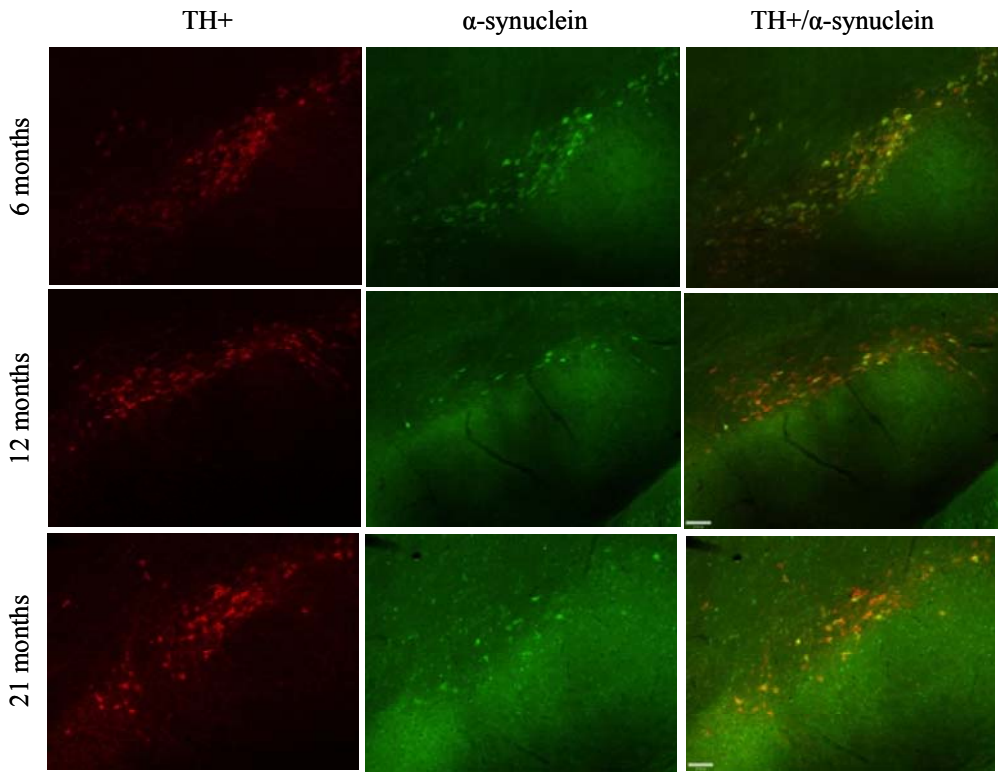


Figure 3.15. ***TH+ and α-synuclein protein expression in the SNpc in SNCA-A30P^{+}Snca-/- transgenic mice with age.*** Representative immunofluorescence images of the SNpc in SNCA-A30P^{+}*Snca-/-* transgenic mice aged 3-6 mo, 12 mo and 21 mo. A significant reduction in TH+ positive cells and TH+/α-synuclein double positive cells is observed at 12 mo and 21 mo, when compared to 3-6 mo (all images shown are 10x magnification).

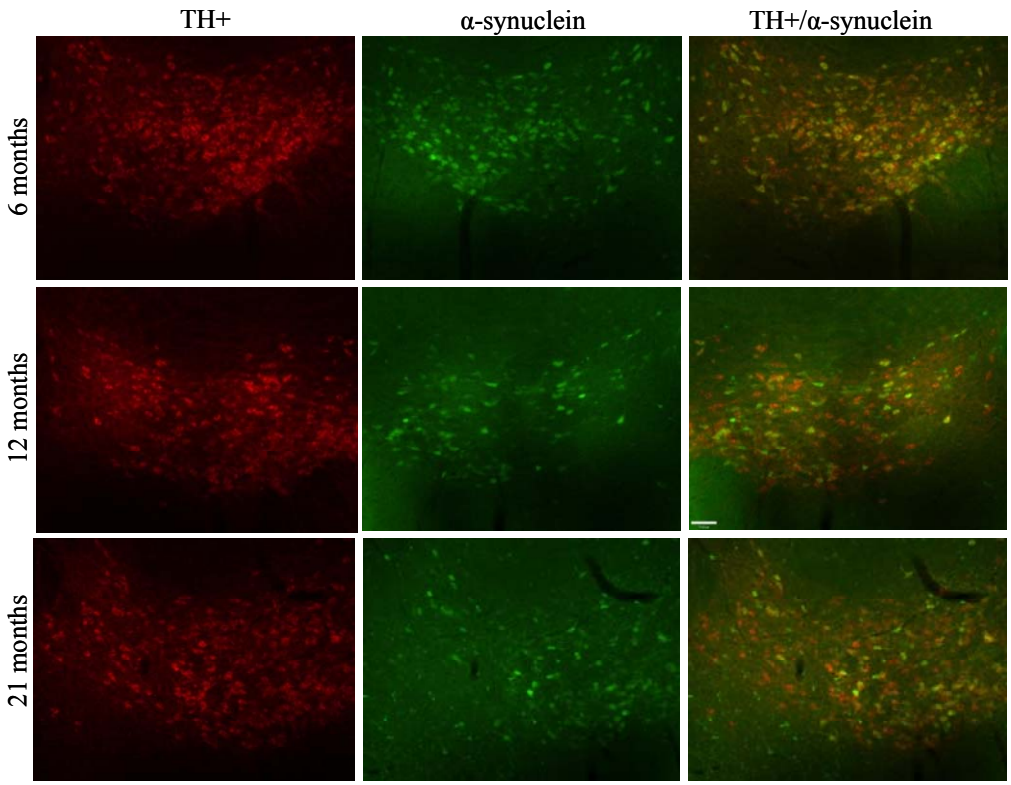


Figure 3.16. ***TH+ and α-synuclein protein expression in the VTA in SNCA-A30P^{+}Snca-/- transgenic mice with age.*** Representative immunofluorescence images of the VTA in SNCA-A30P^{+}*Snca-/-* transgenic mice aged 3-6 mo, 12 mo and 21 mo. A small reduction in TH+/α-synuclein double positive cell number occurs at 12 mo, but not 21 mo, when compared to 3-6 mo (all images shown are 10x magnification).

The average percentage of TH^+ cells which are also α-synuclein positive in the SNpc and VTA allows a comparison of the cell numbers between the three age groups, since each individual animal has then been normalised to TH^+ cell number (Figure 3.17). In the SNpc, percentage of TH^+ cells which are also α-synuclein positive at 3-6, 12 and 21 months are 48%, 27% and 33%, respectively, with a significant percentage reduction occurring at 12 months of age (one-way ANOVA, n=3 animals per age group, Bonferroni post-test; percentage of TH^+ cells which are also α-synuclein positive, 3-6 months vs. 12 months, *P<0.05**). In the VTA, percentage of TH^+ cells which are α-synuclein positive at 3-6, 12 and 21 months are 40%, 24% and 27%, respectively, with a significant percentage reduction again occurring at 12 months of age (one-way ANOVA, n=3 animals per age group, Bonferroni post-test; percentage of TH^+ cells which are α-synuclein positive, 3-6 months vs. 12 months, *P<0.05**).

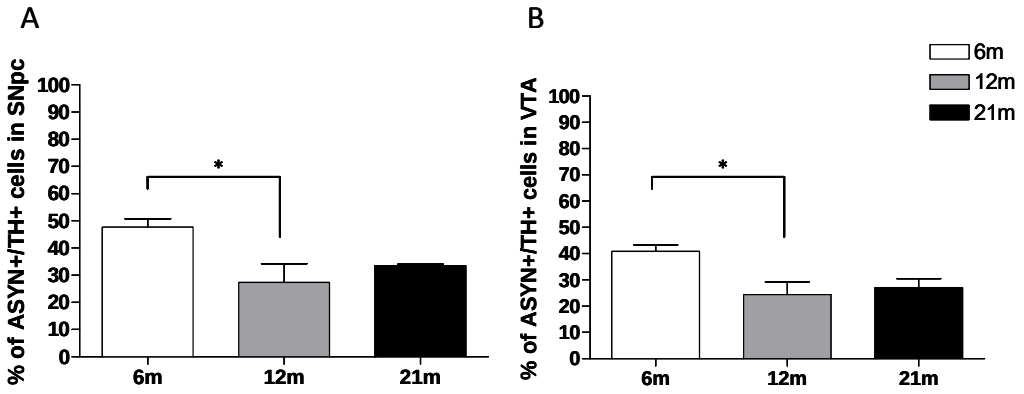


Figure 3.17. ***Comparison of percentage of TH+/α-synuclein double positive cells in the SNpc and VTA in SNCA-A30P+Snca-/- transgenic mice with age.*** (A) In the SNpc, a significant percentage reduction occurs at 12 months of age, when compared to 3-6 mo (one-way ANOVA, n=3 animals per age group, Bonferroni post-test; percentage of TH+ cells which are α-synuclein positive, 3-6 mo vs. 12 mo, P<0.05*). (B) In the VTA, a significant percentage reduction again occurs at 12 mo (one-way ANOVA, n=3 animals per age group, Bonferroni post-test; percentage of TH+ cells which are α-synuclein positive, 3-6 mo vs. 12 mo, P<0.05*).

3.2.7. Initial characterisation of pathology in the SNpc of ageing SNCA-A30P^{+}Snca-/- line

Small pilot experiments were performed to test antibodies for pathological PD markers using double-label immunofluorescence techniques in 18 month old *SNCA*-A30P^{+}*Snca-/-* transgenic mice and age-matched non-transgenic *Snca-/-* controls. This was done in order to analyse potential early-stage molecular pathology in the SNpc in this novel transgenic line. Double labelling studies were performed in the SNpc using antibodies for synuclein and either ubiquitin, a characteristic marker of PD pathology which typically co-localises with α-synuclein in LBs, or with an antibody to the phosphorylated form of α-synuclein, another PD associated marker.

Double-label immunofluorescence of the α-synuclein transgene and ubiquitin did not reveal any readily observable inclusion-like structures or abnormal accumulations of α-synuclein in neuronal cell bodies or axons in neurons in the SNpc, nor was there any obvious abnormal co-localisation of ubiquitin with α-synuclein into inclusion-like structures (Figure 3.18). However, the quality of the antibody stain was poor, with a widespread uniform staining hard to discern from background. In similar pilot investigations, double immunofluorescence labelling of the α-synuclein transgene with an anti-phosphorylated α-synuclein antibody in the SNpc yielded similar results in *SNCA*-A30P^{+}*Snca-/-* transgenic mice aged 18 months old (not shown). Furthermore, a neuronal expression profile of the α-synuclein transgene, similar to that seen in 3 month old transgenic mice, was observed in 21 month old *SNCA*-A30P^{+}*Snca-/-* transgenic mice, with α-synuclein present in the axons, cell bodies and the nucleus of TH+ cells in the SNpc.

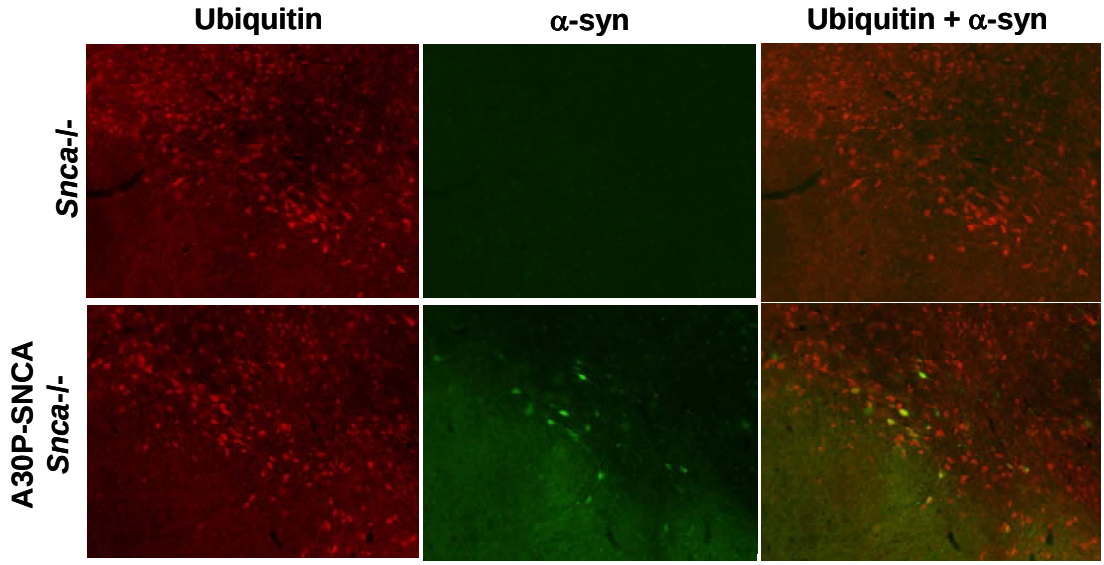


Figure 3.18. ***Human α-synuclein transgene and ubiquitin protein expression in BAC-SNCA transgenic mice by double label immunofluorescence (IF).*** Double-label immunofluorescence of the α-synuclein transgene and ubiquitin did not reveal any readily observable inclusion-like structures or abnormal accumulations of α-synuclein in neuronal cell bodies or axons in neurons in the SNpc, or any obvious abnormal co-localisation of ubiquitin with α-synuclein into inclusion-like structures (all images shown are 10x magnification).

Further experiments with the ubiquitin and phosphorylated α-synuclein antibodies were limited by the availability of aged *SNCA*-A30P^{+}*Snca-/-* transgenic mice and age-matched *Snca-/-* controls. The absence of a positive control for pathological ubiquitin and phosphorylated α-synuclein also presented a drawback for conducting these experiments. Therefore, the results described here represent only pilot data, and a thorough investigation of early-stage emerging pathology in *SNCA*-A30P^{+}*Snca-/-* transgenic mice is required.

3.3. Discussion

This chapter describes the molecular characterisation of a novel α-synuclein transgenic mouse model which carries the PD-associated A30P mutation. Transgenic lines of mice carrying insertions of a BAC containing the entire *SNCA* genomic locus with either the wild-type or A30P mutated form of α-synuclein, on a *Snca -/-* background were previously generated in the Wade-Martins group.

Expression of transgenic protein was confirmed in four *SNCA*-BAC lines by western blot. The *SNCA*-WT-39 line and *SNCA*-A30P^{+}*Snca-/-* transgenic line express the protein at a similar level to each other and at a similar level to that seen in a human brain sample from a healthy control individual. Transgene expression in these mice is driven and controlled by endogenous gene regulatory elements in the BAC. This means the transgene is expressed at levels similar to those endogenously expressed, making *SNCA* expression physiologically relevant. In a recent study by Kuo and colleagues, human wild-type α-synuclein transgenic mice, and mice expressing the PD-associated A30P and A53T mutated forms of the protein were created using P1 artificial chromosomes (PAC) RP-127M07 transgenic containing the entire human *SNCA* gene (Kuo et al., 2010). These authors observed a modest increase in the levels of transgenic protein (1.3 to 2-fold compared to endogenous mouse *Snca*) in the brain, further confirming the BAC transgenic approach does not lead to the considerable overexpression of α-synuclein transgene seen when using cDNA-based transgenic models. Whilst protein expression has been confirmed in all four *SNCA*-BAC lines described in this chapter, the number of inserted copies of the transgene remains to be

determined. These experiments, using qPCR, are currently being undertaken in the Wade-Martins group.

The immunohistochemical analysis in 3-4 month old mice confirmed the neuronal expression of the transgene in the *SNCA*-A30P^{+}*Snca-/-* line. The results also demonstrate the transgene has a regional pattern of α-synuclein expression which is very close to that of endogenous mouse α-synuclein in wild-type mice, with expression in key brain regions, including the SNpc. The intracellular pattern of α-synuclein transgene expression in neurons in *SNCA*-A30P^{+}*Snca-/-* transgenic mice also closely matches that of α-synuclein expression seen endogenously in wild-type mice. The α-synuclein transgene is present in the cell bodies of neurons, and also co-localises with DAPI staining in double-label immunoflourescence images, indicating there is some nuclear expression of the transgene. Another human-specific anti-α-synuclein antibody (Zymed) was used to confirm cell body staining of α-synuclein in *SNCA*-A30P^{+}*Snca-/-* transgenic mice (data not shown). Cell body staining of endogenous α-synuclein was similarly observed throughout the brain of the wild-type C57Bl6 mice when a mouse-specific anti-α-synuclein antibody was used. These results demonstrate α-synuclein is present in neuronal cell bodies in normal young wild-type mice. Therefore, the cell body staining of *SNCA*-A30P^{+}*Snca-/-* transgenic mice represents a normal, physiological α-synuclein localisation similar to that seen in wild-type mice. There is considerable controversy in the literature regarding the pattern of intracellular localisation of α-synuclein. According to much published literature, α-synuclein is normally expressed in presynaptic nerve terminals, whilst

pathological α-synuclein aggregations are located in the cytoplasm and neurites (Forno, 1987; Galvin et al., 2001; George et al., 1995; Iwai et al., 1995).

Whilst the results presented in this chapter have clearly demonstrated α-synuclein is present in the cell body of healthy wild-type mice, the presence of α-synuclein in the cell bodies and the nucleus in several lines of transgenic mice has been interpreted as pathological. Transgenic mice overexpressing wild-type human α-synuclein under the control of the PDGF promoter have reported the presence of α-synuclein-positive intranuclear and cytoplasmic inclusions, which were most frequently observed in the deep layers of the neocortex and the hippocampus (Masliah et al., 2000). Furthermore, findings in mutant A53T α-synuclein transgenic mice under the control of the murine Thy-1 promoter have shown that, in addition to the synapses, α-synuclein accumulates in cell bodies of neurones in the neocortex and limbic system (van der Putten et al., 2000); although these authors reported low or no expression of the transgene in the SNpc and the extensive pathology is associated with motor neuron and spinal cord pathology. Transgenic mice expressing mutant A53T α-synuclein under the regulation of the PrP promoter showed the transgenic mutant protein, but not wild-type protein was abnormally distributed, with many regions throughout the brain showing diffuse α-synuclein staining in many neuronal cell bodies, as well as in axons, dendrites and the nucleus (Gispert et al., 2003). This abnormal distribution was striking because it occurred without the presence of obvious α-synuclein aggregation.

The accumulation of α-synuclein the cell body in transgenic mice remains controversial. Whilst some studies report α-synuclein accumulates in the cell body of wild-type human α-synuclein transgenic mice, including in SNpc neurons (Kahle et

al., 2000b; Richfield et al., 2002), somal accumulation only occurred with the mutated form of α-synuclein in other studies (Giasson et al., 2002; Lee et al., 2002). The results in this chapter demonstrate that endogenous α-synuclein is present in the cell body in wild-type mice and *SNCA*-A30P^{+}*Snca-/-* transgenic mice. Future studies planned in the Wade-Martins group will include a comprehensive histological characterisation of the control *SNCA*-WT-39 transgenic mouse line, to confirm a similar expression pattern to *SNCA*-A30P^{+}*Snca-/-* transgenic mice and wild-type endogenous mouse α-synuclein, throughout the key brain regions. Whilst a similar intraceullar pattern of expression has been confirmed in the SNpc, confirmation of a similar intracellular localisation of α-synuclein throughout the brain in the control *SNCA*-WT-39 transgenic mouse line is required.

A more comprehensive characterisation of emerging neuropathology in these *SNCA*-BAC transgenic lines is required, and is currently being planned in the Wade-Martins group. A readily available positive control would be an important advantage for these experiments, although it is not clear what the most suitable control would be. Human brain tissue sections from PD patients do not undergo the same fixation procedures when preparing the sections for immunohistochemistry, therefore results of staining protocols for human and mouse tissue may vary. Therefore, the most suitable positive control may be previously published α-synuclein transgenic mice which reportedly have α-synuclein and ubiquitin positive inclusions (Masliah et al., 2000; van der Putten et al., 2000). The application of immunohistochemical techniques routinely used to assess LB pathology could be employed, using antibodies which are specific to insoluble and aggregated forms of α-synuclein. Of note, in a study by Giasson and colleagues, α-synuclein inclusions were detected with several antibodies raised to

preferentially detect pathological α-synuclein in human brain tissue (Duda et al., 2002). Furthermore, when conventional α-synuclein antibodies were used by Giasson and colleagues, which were not necessarily specific for pathological α-synuclein, the staining was variable, ranging from weak to robust staining in the same transgenic mouse model (Duda et al., 2002). Therefore, a careful consideration of which α-synuclein antibodies to use for the *SNCA*-BAC transgenic mice will be required. In addition, histological analysis throughout the brain in *SNCA*-BAC transgenic mice using antibodies for PD-specific neuropathological markers such as ubiquitin, phosphorylated neurofilament protein, phosphorylated forms of α-synuclein and glial fibrillary acidic protein (GFAP) will be required. Western blot analysis will also be useful in determining whether aggregates of α-synuclein are present. This can be done by detecting high molecular weight, oligomeric forms of α-synuclein which reportedly represent aggregated forms of the protein, rather than monomeric, non-aggregated forms. In addition, a number of cDNA construct based transgenic mice overexpressing wild-type or mutant human α-synuclein have reported widespread pathology in the brain stem and spinal cord, without any modifications in the nigrostriatal system (Giasson et al., 2002; van der Putten et al., 2000). Therefore, it would be interesting to assess protein expression and potential brain stem and spinal cord pathology in the *SNCA*-A30P^{+}*Snca-/-* line or the *SNCA*-WT-37 line which overexpresses human wild-type α-synuclein protein.

Kuo and colleagues recently characterised PAC transgenic mice expressing wild-type and mutant A30P and A53T α-synuclein from insertions of an entire human *SNCA* gene (Kuo et al., 2010). This study reported the histological characterisation of one transgenic mouse line which had increased levels of A53T mutated α-synuclein. Apart from a few rare dystrophic synapses in the hippocampus observed in this line in

the mice at 12 and 22 months of age, no characteristic Lewy body pathology or α-synuclein aggregation was observed in the brain or spinal cord. The absence of aggregates was confirmed by western blot analysis. Investigation of the CNS pathology of the transgenic line carrying A30P α-synuclein was not reported in this study. Therefore, investigating potential neuropathology in the transgenic *SNCA*-BAC lines described here remains an important goal.

Quantitiative analysis of TH^+ neurons in the SNpc and the VTA in *SNCA*-A30P$^+$*Snca-/-* transgenic mice and non-transgenic *Snca-/-* littermate controls at 3-6 months, 12 months and 21 months of age in this chapter shows that there is a significant loss of TH^+ cells in the SNpc of *SNCA*-A30P$^+$*Snca-/-* transgenic mice, but not in *Snca-/-* control mice. Furthermore, this loss of TH^+ cells appears to be specific to the SNpc, since no significant differences in TH^+ cell number with age in either genotype occurring in the VTA. This replicates the phenotype seen in PD patients, where a preferential loss of dopaminergic neurons is seen in the SNpc with age, but not in dopaminerigc neurons in the VTA which are relatively spared in PD. Future planned work in our laboratory will now quantify TH^+ neurons in the SNpc and the VTA in the *SNCA*-WT-39 control line at all three age points. This will confirm whether the significant decrease in TH+ cell number seen in the SNpc in *SNCA*-A30P$^+$*Snca-/-* transgenic mice can be attributed to the presence of A30P mutated form of α-synuclein, or whether the presence of human α-synuclein alone accounts for the TH^+ cell loss. Further, it is not possible to discount a potential effect on TH^+ expression occurring in *Snca-/-* mice, or an effect caused by the presence of the BAC construct. Finally, it is also possible that changes in TH^+ immunoreactivity may reflect altered TH^+ expression rather than actual cell loss.

Analysis of the number of cells expressing the α-synuclein transgene in the *SNCA*-A30P^{+}*Snca-/-* transgenic mice at 3-6 months, 12 months and 21 months of age using double-immunoflourescence staining of TH+ and α-synuclein positive neurons in the SNpc and VTA is also reported in this chapter. Once more, TH+/α-synuclein double positive cell loss appears to preferentially target SNpc dopaminergic neurones, which are most vulnerable in PD. Further, the actual number of TH+ cells lost in the SNpc at 12 and 21 months is approximately similar to the loss of TH+/α-synuclein double positive cells, indicating the majority of TH^{+} neurons lost in ageing mice contain the A30P mutated form of α-synuclein. This observation suggests the mutated protein may be exerting a toxic cellular effect on TH^{+} neurons, preferentially targeting the SNpc, though at present a toxic effect attributed simply to the presence of human α-synuclein cannot be ruled out. Closer analysis of the data also indicates that whilst α-synuclein dysfunction is a likely explanation for TH^{+} cell loss, it cannot account fully for the reduction in of α-synuclein transgene expression in TH^{+} cells. This is because, for example between 3-6 and 12 months, there is a loss of approximately 800 TH+ cells in the SNpc and approximately 1200 α-synuclein positive cells, 99% of which are also TH^{+}. Therefore, while the presence of mutant α-synuclein can account for the majority of TH+ neurons lost in ageing *SNCA*-A30P^{+}*Snca-/-* transgenic mice, there is there is a population of α-synuclein positive cells lost that cannot be accounted for by TH+ cell loss. Future experiments in our laboratory which quantify α-synuclein transgene expression in *SNCA*-WT-39 transgenic mice at all three age points will therefore provide important insights, including a firmer conclusion for whether the presence of A30P α-synuclein, or simply the presence of the human transgene.

The method of serial reconstruction and direct cell counting of alternating coronal mouse sections employed in this study represents an accurate and valid method of determining neuron cell number in these brain regions. Published estimates of bilateral numbers of TH-positive neurons in the SNpc in the C57Bl6 mouse have varied considerably, from approximately 5200 (Coban and Filipov, 2007) to over 23000 (Jakowec et al., 2004). Most studies have however reported a more limited range, from approximately 8000 to 12000 (Baquet et al., 2005; Cao et al., 2006; Liberatore et al., 1999; McCormack et al., 2005; McCormack et al., 2006; Smeyne et al., 2005; Sugama et al., 2003; Thiruchelvam et al., 2003; Vijitruth et al., 2006; Walters et al., 1999; Zou et al., 2000). The average numbers per hemisphere (±SEM) in this study for alternating sections (every other section, n=10) are approximately 4085±328 for the 3-6 month age group, 4298±195 for the 12 month age group and 3847±213 for the 21 month age group, which when doubled (to give an estimate of total cell number in the SNpc), lie within or very close to this reported range, and the slightly lower number of TH-positive cells in the 21 month age group could be accounted for by cell loss due to age. Furthermore, the cell counts for all 18 mice tested lie consistently within this range, indicating the accuracy and reproducibility of this method.

In further support of this, a recent study by Baquet and colleagues directly counted the number of dopaminergic neurons, using serial sections to reconstruct the SNpc from C57Bl6 mice (Baquet et al., 2009). Using this method, four C57BL6 mouse brains were examined, using the same landmarks for determining the anterior to posterior extent of the SNpc as used in the present study (Paxinos and Franklin, 2001). The use

of alternating sections in the present study, which were 35 µm in thickness, prevented the possibility of cells being counted twice. Therefore aligning and overlaying adjacent sections to prevent duplicate counting, as done by Baquet and colleagues, was not required. The total bilateral number of dopaminergic neurons (±SEM) was reported to be 8305±539 (n=4, range 7464-9763). They then compared this empirically derived number to model-based (2D) and design-based (3D) stereological estimates and found no significantly statistical differences in cell number between the three methods. The model-based estimates (±SEM) gave a value of 8002±91 (n=10, range 7563-8415) while design-based estimates (±SEM) were 8716±338 (n=10 range 7546-9869). All three values are similar to the estimated values of total number of SNpc neurons in our study, which, when the numbers per hemisphere are multiplied by two to give an estimate of the total number of SNpc neurons, are approximately 8070 for the 3-6 month age group, 8596 for the 12 month age group and 7694 for the 21 month age group. This indicates the accuracy, precision and reliability of design-based methods are equal to that of method-based analysis when strict rules are observed. The characterisation of transgene expression over time in the SNpc and VTA will need to be completed in the *SNCA*-WT-39 transgenic control line to determine whether the decreased transgene expression observed at 12 and 21 months in the *SNCA*-A30P^{+}*Snca-/-* transgenic line is attributable to mutated A30P α-synuclein.

Behavioural characterisation of the *SNCA*-BAC transgenic mice is also required. A broad-based behavioural screening of motor and cognitive behaviour would permit the identification of subtle changes in phenotype in these mice. Further, given that PD is associated with advancing age, long term behavioural assessment of age-matched

cohorts of the *SNCA*-A30P^{+}*Snca-/-*, the *SNCA*-WT-39 and *Snca-/-* should be performed. Specific behavioural assays include: the open-field and home-cage tests, and the rotarod and multiple static rods test for assessing activity and motor performance; the hanging wire test and lifting weights for grip strength and the automated Catwalk system for posture and gait observations, including fore-paw placements and footprint pattern measures. Cognitive tests and assessment of short-term spatial working memory using spontaneous and rewarded alternation would also be useful. A recent study has assessed motor function in SNCA-BAC transgenic mice (Kuo et al., 2010). These authors reported that in all lines of mice studied, wild-type, A30P and A53T α-synuclein, no gross motor phenotype such as tremor or ataxia was observed. The open-field and rotarod tests were employed to assess motor function in these mice and revealed that in mice which had increased levels of A53T mutated α-synuclein, but not A30P mice, a consistently reduced motor activity in the open-field and abnormal endurance and coordination on the rotarod test compared with wild-type controls. A more thorough investigation of motor performance and cognition was not reported. Studies in the *SNCA*-BAC transgenic mice described here are required to enable a comparison between the two separate lines of BAC transgenic mice.

Future characterisation of potential enteric nervous system (ENS) abnormalities of the *SNCA*-BAC transgenic mice described in this chapter is also warranted. In the same recent study on *SNCA*-BAC transgenic mice (Kuo et al., 2010), profound, early ENS abnormalities, including reduced colonic motility and expulsion time and a prolonged whole-gut transit time, were observed in both the mutant A30P and A53T α-synuclein lines, but not in the wild-type control lines. This indicates that the phenotype observed is not due to an effect of transgene, but rather an effect of the mutated protein. This

dysfunction was coupled with synuclein-immunoreactive aggregates in ENS ganglia by 3 months of age in the A53T α-synuclein line (which also exhibited motor abnormalities, discussed above). These findings are particularly interesting given that PD patients often suffer ENS symptoms early on in the disease process, before the manifestation of CNS symptoms. Therefore, analysis of ENS function in *SNCA*-A30P^{+}*Snca-/-*, the *SNCA*-WT-39 and *Snca-/-* mice described is this chapter would be useful.

A number of broader future steps should also be considered. Firstly, the second *SNCA*-A30P^{+}*Snca-/-* transgenic line generated did not demonstrate protein expression by western blot analysis. This line was desirable to confidently attribute phenotypic changes to the presence of the A30P mutation. Although a potential deleterious site-of-integration effect of the transgene has been overcome by maintaining the transgenic lines as hemizygotes, there still remains a very small possibility of a site of integration effect. The generation of a second *SNCA*-A30P^{+}*Snca-/-* transgenic line is therefore a future consideration. The generation of *SNCA*-BAC transgenic mice carrying the A53T mutation on a *Snca-/-* background would also be interesting. This is because in both the cDNA-based transgenic mice and the very recently characterised *SNCA*-BAC transgenic mice, the phenotype observed seems to be more prominent in the presence of the A53T mutation than with the A30P mutation (Giasson et al., 2002; Kuo et al., 2010; Lee et al., 2002; van der Putten et al., 2000). However, these results would be difficult to interpret, given the endogenous mouse *Snca* gene sequence already has a threonine at position 53. Finally, the *SNCA*-WT-37 transgenic mouse line represents a potentially interesting and novel overexpression *SNCA*-BAC model to study the effects of physiological overexpression of α-

synuclein. This is of particular interest given the known gene-dose toxicity effects of PD-associated duplication and multiplication mutations. Experiments focussing on the molecular and neurochemical characterisation of this line are currently underway in the Wade-Martins group.

4. CHAPTER 4: Neurochemical characterisation of DA synapse function in a novel transgenic mouse model of Parkinson's disease carrying the α-synuclein A30P mutation

4.1. Introduction

The intracellular location of α-synuclein in presynaptic nerve terminals and its close association with neurotransmitter-containing synaptic vesicles suggests the protein has a role in synaptic physiology (Iwai et al., 1995; Maroteaux et al., 1988). α-synuclein has been shown to have effects on numerous aspects of vesicle function and has lipid binding and protein chaperone-like properties which suggest the protein can directly bind to or interact with synaptic vesicle membranes to regulate vesicle recycling (Sharon et al., 2001; Ueda et al., 1993). In addition, the intimate regulation of various components of DA homeostasis by α-synuclein, including DA synthesis, release and uptake indicates the protein has an important role in DA synapse function. Despite this, the role of α-synuclein in shaping synaptic transmission remains unclear. There are surprisingly few published reports on the effects of α-synuclein on neurotransmitter release, or more specifically DA release, at synapses. Furthermore, there are even fewer reports of the effects of α-synuclein on DA signalling in the striatum, the region where DA levels are decreased in PD due to loss of nerve terminals projecting from degenerating DA neurons in the SNpc.

Studies in α-synuclein knockout mice have reportedly shown either no or very subtle effects on transmitter release and the regulation of synaptic vesicle pools (Abeliovich et al., 2000; Cabin et al., 2002; Chandra et al., 2004; Yavich et al., 2004). One group

demonstrated subtle changes in DA release in α-synuclein knockout mice, reporting an enhanced rate of recovery from paired pulse depression (PPD) at DA terminals in the striatum (Abeliovich et al., 2000). Another report demonstrated a decreased post-synaptic response after repetitive stimulation in non-DA synapses in the hippocampus in α-synuclein knockout mice (Cabin et al., 2002). Overexpression of α-synuclein reportedly inhibits catecholamine release from adrenal chromaffin cells and granule release from platelets (Larsen et al., 2006; Park et al., 2002). Most recently, a study by Nemani and colleagues, using both *in vitro* and *in vivo* approaches, have demonstrated that a modest (2- to 3-fold) increase in wild-type α-synuclein expression inhibits glutamate release in hippocampal pyramidal neurons and DA release in mesencephalic DA neurons in culture (Nemani et al., 2010). This impairment in neurotransmitter release is not coupled with obvious α-synuclein toxicity or inclusion formation, but is reportedly due to a failure of synaptic vesicle reclustering after endocytosis (Nemani et al., 2010). There is evidence that mutant forms of α-synuclein impair the ability of the protein to bind to vesicle membranes, which may disrupt vesicular recycling and DA storage in vesicles (Jensen et al., 1998; Lotharius and Brundin, 2002a). The PD-associated A30P mutation significantly alters the tertiary conformation of α-synuclein and reportedly causes reduced α-synuclein binding to small phospholipids vesicles (Jensen et al., 1998; Jo et al., 2002). Studies of DA function in transgenic mice with A30P mutant α-synuclein did not observe deficits in DA synthesis or metabolism (Gomez-Isla et al., 2003; Neumann et al., 2002). However, α-synuclein positive inclusions and motor deficits in these mice have been observed, though this is not a consistent finding and may depend on the levels of α-synuclein expression and the promoters used to drive the transgene (Gomez-Isla et al., 2003; Kahle et al., 2000a). Yavich and colleagues have shown that transgenic mice

overexpressing A30P mutant α-synuclein exhibit reduced DA release from striatal synapses under conditions of heavy vesicle loading of presynaptic terminals during intensive, repeated stimulation protocols (Yavich et al., 2005). These authors propose this phenotype is due to an inability of the A30P mutant α-synuclein to traffic DA vesicles between the readily releasable and reserve pools in striatal terminals, or because of a low capacity of the reserve pool.

To date, there are no studies directly examining the effects of the PD-associated A30P mutant form of α-synuclein on DA neurotransmission in the striatum under physiologically relevant neuronal firing conditions. Moreover, this has not been examined in novel *SNCA*-BAC transgenic mice in which the transgene expression is regulated, tissue-specific and expressed at physiologically relevant levels. Further, given that PD is a disease associated with advancing age, there are no studies which compare changes in DA neurotransmission with age in the presence of this mutation. This chapter describes the neurochemical characterisation of DA synapse function with age in a *SNCA*-BAC transgenic mouse line which carries the PD-associated A30P mutation. In this model, the effects of the transgene may be more subtle than those observed in transgenic mice which overexpress forms of α-synuclein. Therefore, measurements of DA release and reuptake kinetics from striatal DA axons has been achieved using fast-scan cyclic voltammetry (FCV) at carbon-fibre microelectrodes, a powerful technique to detect and quantify release of endogenous DA with subsecond resolution on a real-time scale (Cragg, 2003; Cragg and Greenfield, 1997; Cragg et al., 1997; Garris et al., 1994; Garris and Wightman, 1994; Millar et al., 1985; Stamford, 1985; Stamford et al., 1986a; Stamford et al., 1986b, c; Stamford et al., 1984). Furthermore, FCV permits an examination of potential changes in the dynamic

interactions between dopaminergic and cholinergic systems in the striatum, which are pivotal to normal striatal DA signalling (Cragg, 2006; Cragg and Rice, 2004; Zhang and Sulzer, 2004; Zhou et al., 2001). Real time kinetics of DA signalling in the striatum in BAC-*SNCA* transgenic mice which express physiological levels of α-synuclein has never been assessed.

Specifically, the aims of this chapter are:

1. To assess differences in the levels of electrically evoked DA in the dorsal striatum (caudate putamen (CPu)) and ventral striatum (nucleus accumbens (NAc)) of *SNCA*-BAC transgenic mice carrying the A30P mutation (*SNCA*-A30P^{+}*Snca-/-*) compared to non-transgenic littermate control mice (*Snca-/-*) at 5 weeks, 3-4 months, 6-7 months, 12 months and 18 months of age, using FCV at carbon fibre microelectrodes
2. To assess whether specific regional differences in electrically evoked DA occur within the CPu in *SNCA*-A30P^{+}*Snca-/-* transgenic mice compared to *Snca-/-* control mice at 5 weeks, 3-4 months, 6-7 months, 12 months and 18 months of age
3. To investigate whether differences in DA uptake occur in CPu in *SNCA*-A30P^{+}*Snca-/-* transgenic mice compared to *Snca-/-* control mice at 5 weeks, 3-4 months, 6-7 months, 12 months and 18 months of age
4. To assess differences DA content in the CPu and NAc in *SNCA*-A30P^{+}*Snca-/-* transgenic mice compared to non-transgenic littermate control mice (*Snca-/-*) at 5 weeks, 3-4 months, 6-7 months, 12 months and 18 months on age, using high-performance liquid chromatography (HPLC)

5. To conduct preliminary investigations into whether differences in frequency sensitivity and nicotinic receptor function occur in young adult (3-6 months of age) *SNCA*-A30P^{+}*Snca-/-*transgenic mice compared to *Snca-/-* control mice

4.2. Results

4.2.1. Decreased levels of electrically evoked DA in the CPu of SNCA-A30P^{+}Snca-/- transgenic mice at ages 5 weeks, 3-4 months and 6-7 months

The release of DA in the CPu and NAc was assessed using FCV at carbon-fibre microelectrodes in the *SNCA-A30P^{+}Snca-/-* transgenic mouse line and non-transgenic *Snca-/-* littermate control mice. DA release was assessed in five ageing cohorts of mice, at ages 5 weeks, 3-4 months, 6-7 months, 12 months and 18 months. In the CPu, DA release data was collected from five different subdivisions, encompassing the dorsal and ventral CPu. Specifically, recordings were obtained from dorso-medial, dorso-central, dorso-lateral, central, ventro-medial and ventro-lateral regions. In the NAc, recordings were taken from two sites below the anterior commissure. These recording sites are depicted in Figure 4.1. For each age group, four or five mice from each genotype were assessed. $[DA]_o$ evoked by single stimulus pulses (1p) and by burst stimuli (4 pulses, 100 Hz) was assessed. All data was analysed by one or two way ANOVA and post-hoc Bonferroni tests.

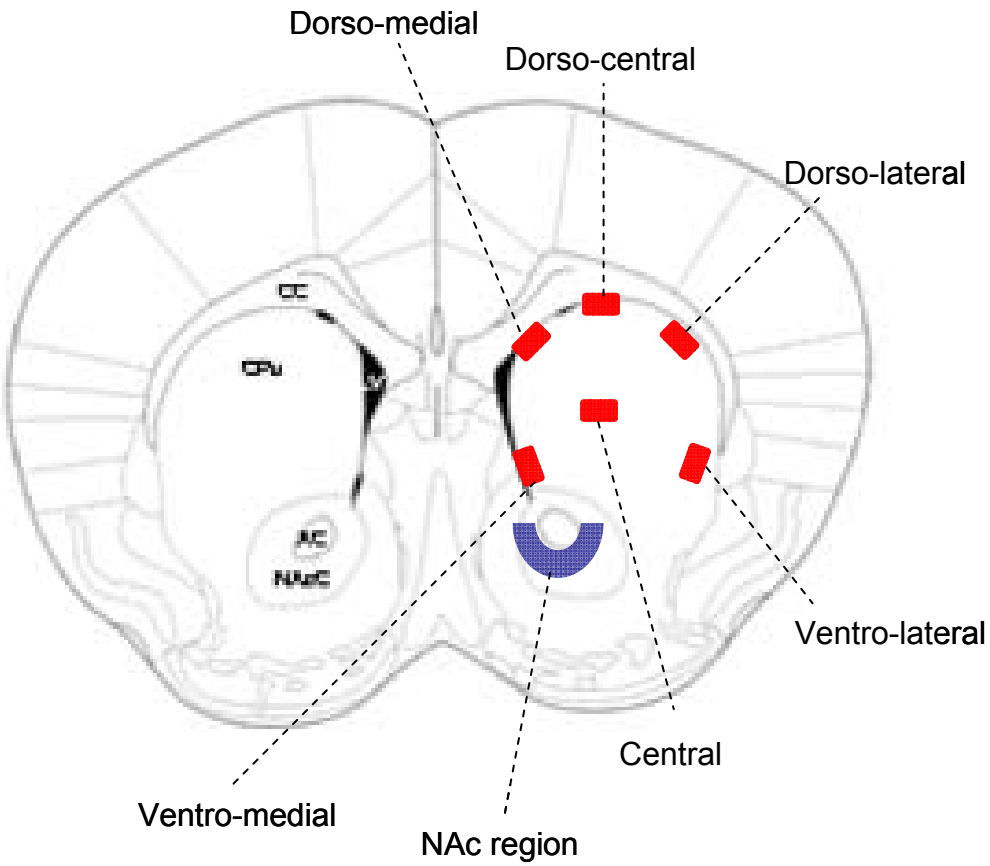
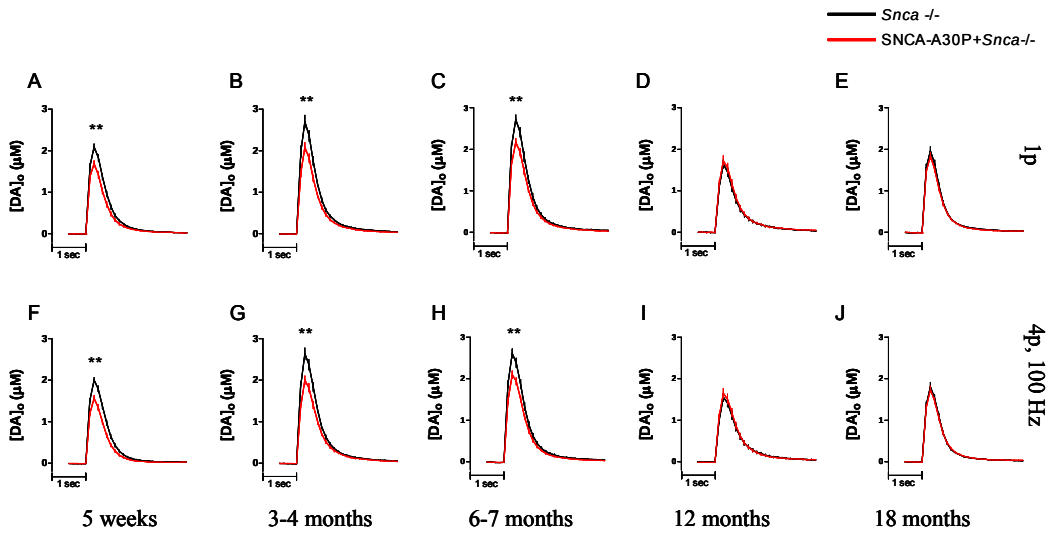


Figure 4.1. ***Recording regions in striatal sections for assessment of electrically evoked DA by FCV.***

In the CPu, mean peak $[DA]_o$ evoked by either stimulus was approximately twenty percent lower in *SNCA*-A30P$^+$*Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate controls (Figure 4.2, Table 4.1) for mice aged 5 weeks, 3-4 months, and 6-7 months, but was not different in mice aged 12 months or 18 months.



Single pulse (1p) data CPu										
Age	5 weeks		3-4 months		6-7 months		12 months		18 months	
Genotype	KO	TG	KO	TG	KO	TG	KO	TG	KO	TG
Mean peak $[DA]_o$ (μM)	2.10	1.69	2.70	2.09	2.72	2.17	1.65	1.73	1.97	1.85
SEM	0.08	0.07	0.15	0.11	0.11	0.09	0.10	0.12	0.11	0.09
N	60	60	50	49	60	60	40	40	54	54
% of control	100	81	100	78	100	80	100	100	100	99
Post-test *p*-value	<0.01		<0.01		<0.01		>0.05		>0.05	

Burst pulse (4p, 100 Hz) data CPu										
Age	5 weeks		3-4 months		6-7 months		12 months		18 months	
Genotype	KO	TG	KO	TG	KO	TG	KO	TG	KO	TG
Mean peak $[DA]_o$ (μM)	2.00	1.56	2.64	2.00	2.62	2.10	1.55	1.65	1.81	1.75
SEM	0.06	0.06	0.14	0.11	0.10	0.09	0.10	0.12	0.10	0.09
n	60	59	47	49	60	60	41	39	54	53
% of control	100	79	100	76	100	81	100	101	100	100
Post-test *p*-value	<0.01		<0.01		<0.01		>0.05		>0.05	

Figure 4.2. ***Electrically evoked DA transients in CPu of SNCA-A30P+Snca-/- transgenic mice compared to Snca-/- controls with age.*** Mean peak $[DA]_o$ evoked by either a single pulse (1p) or a burst (4p, 100 Hz) was approximately twenty percent lower in *SNCA*-A30P+*Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate controls for mice aged 5 weeks, 3-4 months, and 6-7 months (Figures A-C 1p; F-H 4p, 100 Hz), but was not different in mice aged 12 months or 18 months (Figures D-E for 1p; I-J for 4p, 100 Hz). *N*=number of recording sites. All data was analysed by one or two way ANOVA and post-hoc Bonferroni tests. (**Table 4.1**).

A plot of mean peak evoked $[DA]_o$ between genotypes versus age (Figure 4.3; Figure 4.3A; single pulse data, two-way ANOVA, significant differences between genotypes as follows;*$P<0.05$* [five weeks of age], ***$P<0.001$* [3-4 m and 6-7 m], no significant differences between genotype at 12 m or 18 m; Figure 4.3B; burst stimuli data, two-way ANOVA, significant differences between genotypes as follows; **$P<0.01$* [five weeks of age], ***$P<0.001$* [3-4 m and 6-7 m], no significant differences between genotype at 12 m or 18 m) indicates the differences in evoked $[DA]_o$ observed between genotypes diminishes with age, since release in non transgenic *Snca-/-* control mice declines with time to levels seen in *SNCA*-A30P$^+$*Snca-/-* transgenic mice, whilst evoked $[DA]_o$ in *SNCA*-A30P$^+$*Snca-/-* transgenic mice remains broadly constant with age.

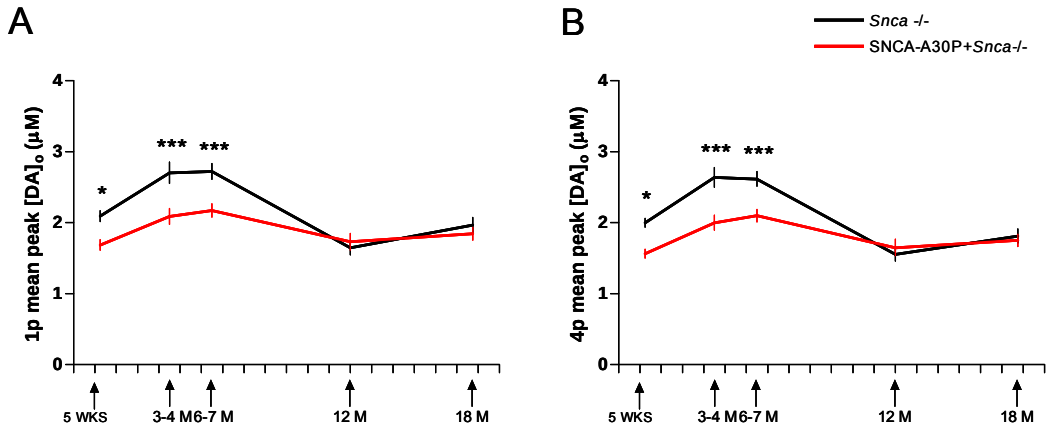
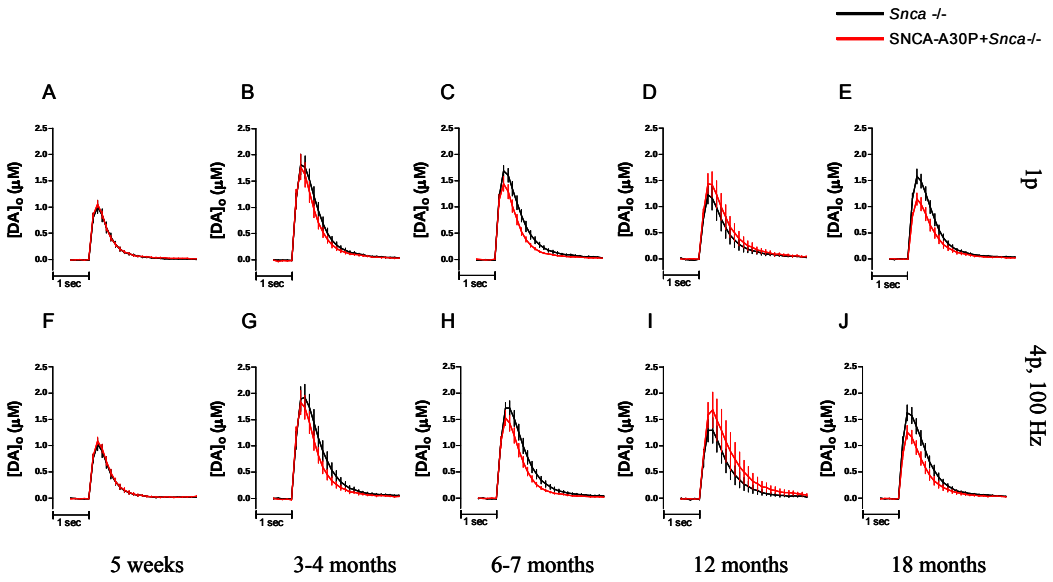


Figure 4.3. ***Mean peak electrically evoked [DA]$_o$ in CPu of SNCA-A30P$^+$Snca-/- transgenic mice compared to Snca-/- controls versus age.*** A plot of mean peak [DA]$_o$ evoked by either 1p (Fig A) or 4p, 100 Hz (Fig B), versus age indicates the differences in evoked [DA]$_o$ between SNCA-A30P$^+$*Snca*-/- transgenic mice compared to *Snca*-/- controls diminishes with age, since release in non transgenic *Snca*-/- control mice declines with time to levels seen in SNCA-A30P$^+$Snca-/- transgenic mice, whilst evoked [DA]$_o$ in SNCA-A30P$^+$Snca-/- transgenic mice remains broadly constant with age. (Figure A; 1p data, two-way ANOVA, significant differences between genotypes as follows; *P<0.05 [five weeks of age], ***P<0.001 [3-4 mo and 6-7 mo], no significant differences between genotype at 12 mo or 18 mo; Figure B, 4p, 100 Hz data, two-way ANOVA, significant differences between genotypes as follows; **P<0.01 [five weeks of age], ***P<0.001 [3-4 mo and 6-7 mo], no significant differences between genotype at 12 m or 18 m).

By contrast, in the NAc no significant differences in evoked $[DA]_o$ were detected between the two genotypes at ages 5 weeks, 3-4 months, 6-7 months, and 12 months. The results for the single stimulus and burst stimulus data are shown in Figure 4.4 and summarised in Table 4.2. Although a small (but non significant) difference in evoked $[DA]_o$ in *SNCA*-A30P$^+$*Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate control mice was observed at 18 months, these data represent only a pilot analysis in the NAc, since the data sets can be considered to be underpowered. This is due to a small number of recording sites in each animal, which are restricted by the small size of the NAc. Additional repeat experiments are required before firm conclusions can be drawn regarding this data.



Single pulse (1p) data NAc										
Age	5 weeks		3-4 months		6-7 months		12 months		18 months	
Genotype	KO	TG	KO	TG	KO	TG	KO	TG	KO	TG
Mean peak $[DA]_o$ (μM)	1.01	1.06	1.81	1.77	1.69	1.44	1.23	1.44	1.58	1.15
SEM	0.127	0.071	0.196	0.242	0.102	0.140	0.172	0.196	0.139	0.114
n	20	20	16	16	20	20	10	10	16	16
% of control	100	100	100	98	100	86	100	116	100	73
Post-test *p*-value	>0.05		>0.05		>0.05		>0.05		>0.05	

Burst pulse (4p, 100 Hz) data NAc										
Age	5 weeks		3-4 months		6-7 months		12 months		18 months	
Genotype	KO	TG	KO	TG	KO	TG	KO	TG	KO	TG
Mean peak $[DA]_o$ (μM)	1.024	1.089	1.928	1.826	1.717	1.527	1.292	1.679	1.622	1.256
SEM	0.120	0.076	0.245	0.229	0.137	0.147	0.240	0.340	0.149	0.127
n	19	19	14	15	20	20	11	10	16	16
% of control	100	100	100	95	100	89	100	129	100	78
Post-test *p*-value	>0.05		>0.05		>0.05		>0.05		>0.05	

Figure 4.4. ***Electrically evoked DA transients in NAc of SNCA-A30P$^+$Snca-/- transgenic mice compared to Snca-/- controls with age.*** No significant differences seen in mean peak $[DA]_o$ evoked by either a single pulse (1p) or a burst (4p, 100 Hz) in *SNCA*-A30P$^+$*Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate controls for mice at 5 weeks, 3-4 months, 6-7 months, 12 months or 18 months (Figures A-E 1p; F-J 4p, 100 Hz). All data was analysed by one or two way ANOVA and post-hoc Bonferroni tests (**Table 4.2**).

In the CPu, an in-depth analysis of the data was performed to confirm the difference in evoked $[DA]_o$ between the two genotypes observed at 5 weeks, 3-4 months and 6-7 months was not due to a subset of anomalous data which influenced or skewed the rest of the data set. Therefore, the spread of evoked $[DA]_o$ peaks from individual recording sites across experimental days for these age groups was assessed (Figures 4.5A; 4.6A and 4.7A). This demonstrated that, between experimental days for each genotype, the *SNCA-A30P*$^+$*Snca-/-* transgenic mice showed consistently reduced evoked $[DA]_o$ compared to non-transgenic *Snca-/-* littermate controls, and that the phenotype observed was not a consequence of a small sub-set of outlying recording sites, for example obtained from only a single animal.

Sub-regional analysis of evoked $[DA]_o$ between the two genotypes at 5 weeks, 3-4 months and 6-7 months was performed in the CPu to investigate whether the observed differences could be attributed to any specific region of the CPu, for example whether one region significantly influenced the overall observed differences in evoked $[DA]_o$. Therefore, evoked $[DA]_o$ peaks from individual recording sites for each sub-region for these age groups was analysed by two-way ANOVA and Bonferroni post-tests. For the 5 week age group, all regions of the CPu exhibited lower evoked $[DA]_o$ in *SNCA-A30P*$^+$ *Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate controls, with statistically significant lower evoked $[DA]_o$ observed in the central and ventromedial regions (Figure 4.5B). At 3-4 months and at 6-7 months, all CPu regions once more displayed lower evoked $[DA]_o$ in *SNCA-A30P*$^+$*Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate controls (Figures 4.6B and 4.7B). Although statistical analysis is here included for reference, and can indicate some sub regions affected by genotype, the small numbers of data points for each subregion

make it difficult to identify whether differences in one region or CPu generally are responsible consistently for the effect of genotype on evoked $[DA]_o$. In particular, the data set for each age group and subregion may be too underpowered (n=7-11 per region, 5 animals per genotype) to be able to *exclude* any regions that contribute to the effects of genotype. In summary, all CPu regions exhibit lower evoked $[DA]_o$ in *SNCA-A30P*$^+$*Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate controls.

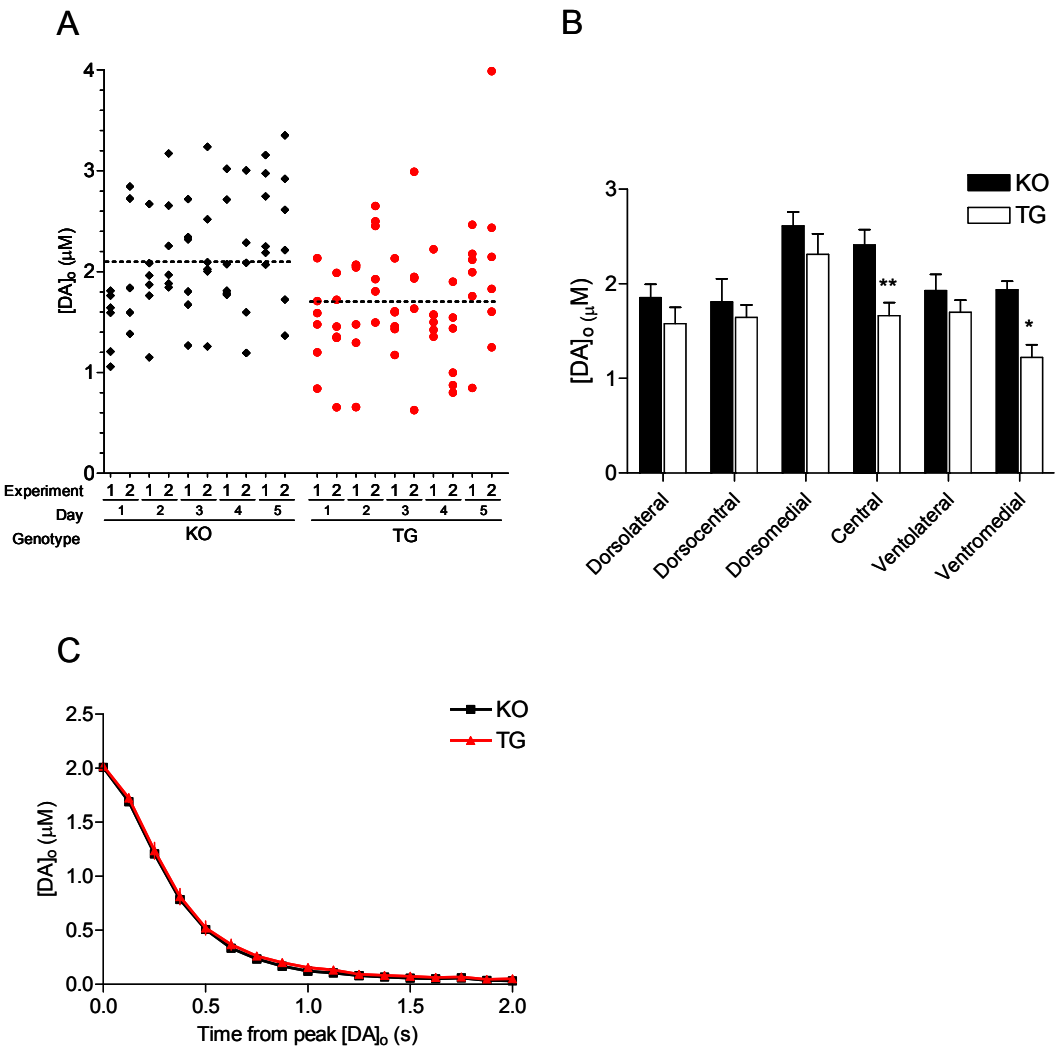


Figure 4.5. ***Spread of evoked [DA]$_o$ peaks from individual recording sites across experimental days, sub-regional analysis of evoked [DA]$_o$ and rates of uptake of evoked DA transients of SNCA-A30P$^+$Snca-/- transgenic mice compared to Snca-/- controls at 5 weeks of age in CPu.*** (A) The spread of evoked [DA]$_o$ peaks from individual recording sites across experimental days, demonstrate that, between experimental days for each genotype, *SNCA-A30P$^+$Snca-/-* transgenic mice showed consistently decreased evoked [DA]$_o$ compared to non-transgenic *Snca-/-* littermate controls at 5 weeks of age in CPu. (B) Sub-regional analysis of evoked [DA]$_o$ at 5 weeks indicate all regions of the CPu exhibited lower evoked [DA]$_o$ in SNCA-A30P$^+$*Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate controls, with statistically significant lower evoked [DA]$_o$ observed in the central and ventromedial regions (two-way ANOVA, *SNCA-A30P$^+$Snca-/-* n=10 per region, *Snca-/-* controls n=10 per region [5 animals per genotype]; significant differences in central CPu region, $^{**}P<0.01$; and ventromedial CPu region, $^{*}P<0.05$). (C) Comparison of the rates of decay of concentration-matched DA transients suggests that DA uptake rates are not significantly different between the two genotypes (two-way ANOVA, *SNCA-A30P$^+$Snca-/-* n=8, *Snca-/-* controls n=8 [5 animals per genotype], $P>0.05$).

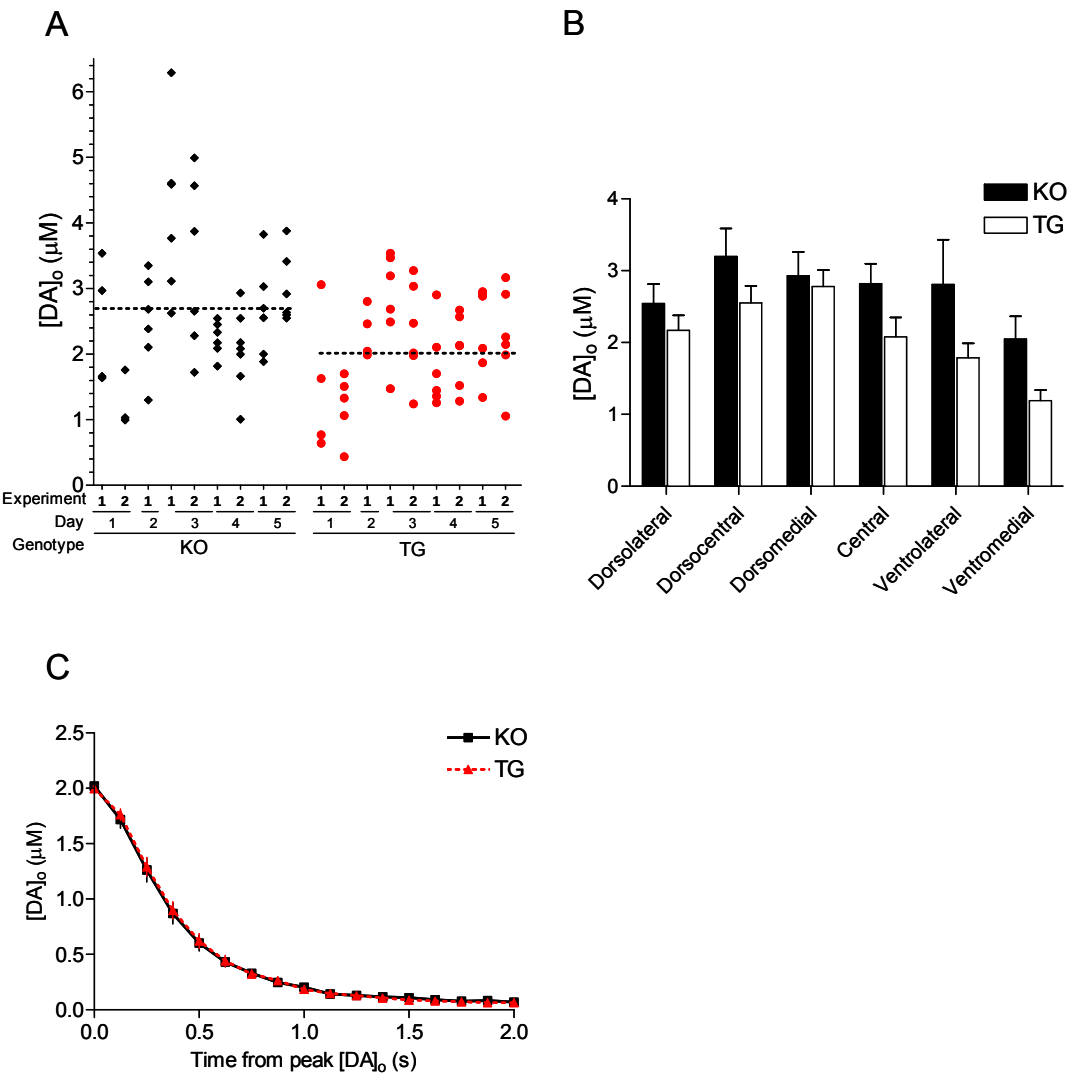


Figure 4.6. ***Spread of evoked [DA]$_o$ peaks from individual recording sites across experimental days, sub-regional analysis of evoked [DA]$_o$ and rates of uptake of evoked DA transients of SNCA-A30P$^+$Snca-/- transgenic mice compared to Snca-/- controls at 3-4 months of age in CPu.*** (A) The spread of evoked [DA]$_o$ peaks from individual recording sites across experimental days, demonstrate that, between experimental days for each genotype, *SNCA-A30P$^+$Snca-/-* transgenic mice showed consistently decreased evoked [DA]$_o$ compared to non-transgenic *Snca-/-* littermate controls at 3-4 months of age in CPu. (B) Sub-regional analysis of evoked [DA]$_o$ at 3-4 months indicate all regions of the CPu exhibited lower evoked [DA]$_o$ in SNCA-A30P$^+$*Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate controls, however no statistical significance is attributed to individual CPu regions (two-way ANOVA, *SNCA-A30P$^+$Snca-/-* n=8-9 per region, *Snca-/-* controls n=7-10 per region [5 animals per genotype]; P>0.05 for all CPu regions). (C) Comparison of the rates of decay of concentration-matched DA transients suggests that DA uptake rates are not significantly different between the two genotypes (two-way ANOVA, *SNCA-A30P$^+$Snca-/-* n=8, *Snca-/-* controls n=8 [5 animals per genotype], *P>0.05*).

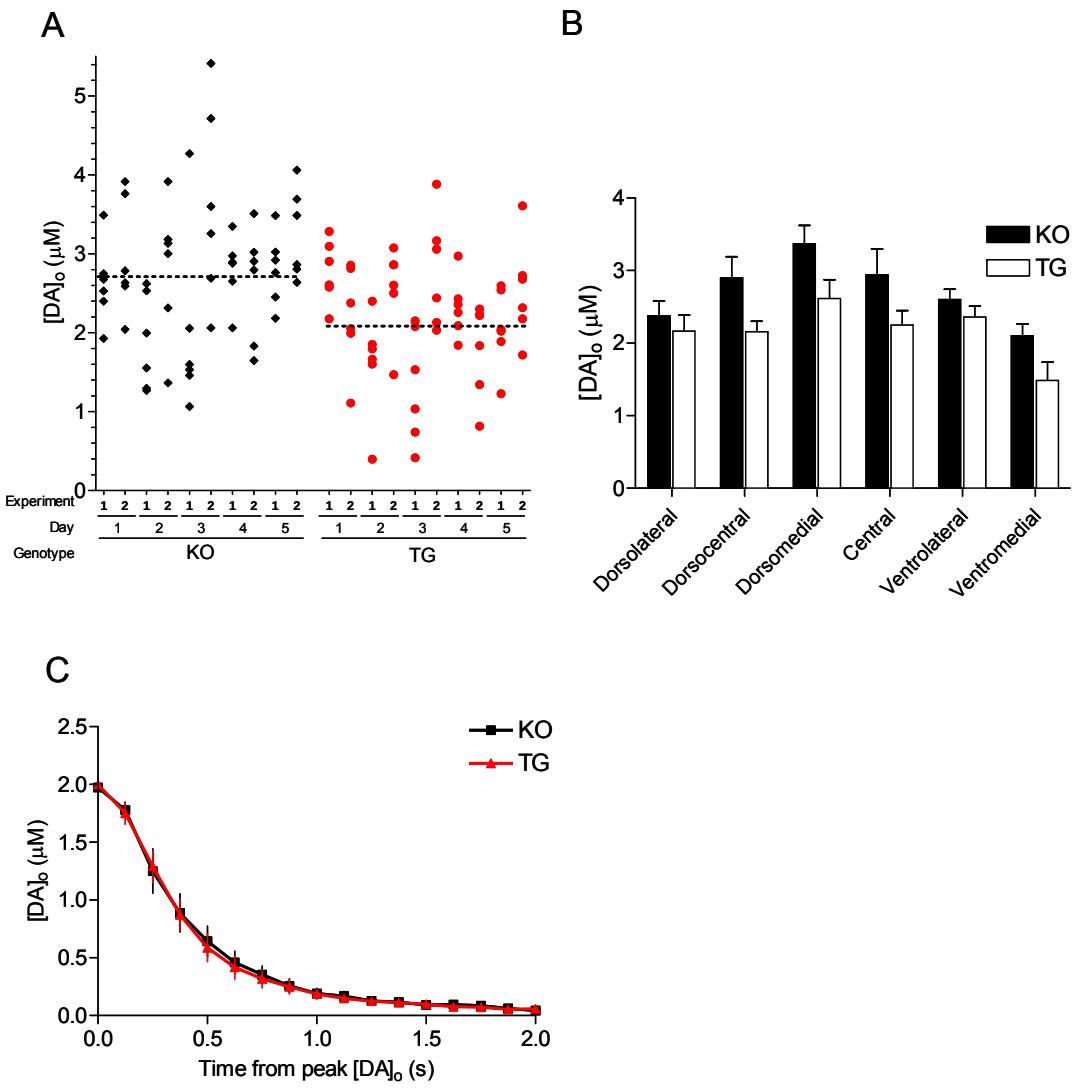


Figure 4.7. ***Spread of evoked [DA]$_o$ peaks from individual recording sites across experimental days, sub-regional analysis of evoked [DA]$_o$ and rates of uptake of evoked DA transients of SNCA-A30P$^+$Snca-/- transgenic mice compared to Snca-/- controls at 6-7 months of age in CPu.*** (A) The spread of evoked [DA]$_o$ peaks from individual recording sites across experimental days, demonstrate that, between experimental days for each genotype, *SNCA-A30P$^+$Snca-/-* transgenic mice showed consistently decreased evoked [DA]$_o$ compared to non-transgenic *Snca-/-* littermate controls at 6-7 months of age in CPu. (B) Sub-regional analysis of evoked [DA]$_o$ at 6-7 months indicate all regions of the CPu exhibited lower evoked [DA]$_o$ in SNCA-A30P$^+$*Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate controls, however no statistical significance is attributed to individual CPu regions (two-way ANOVA, *SNCA-A30P$^+$Snca-/-* n=9-11 per region, *Snca-/-* controls n=9-11 per region [5animals per genotype]; $P>0.05$ for all CPu regions). (C) Comparison of the rates of decay of concentration-matched DA transients suggests that DA uptake rates are not significantly different between the two genotypes (two-way ANOVA, *SNCA-A30P$^+$Snca-/-* n=7, *Snca-/-* controls n=7 [4 animals per genotype], *$P>0.05$*).

Mean peak $[DA]_o$ evoked by burst stimuli varies between the two genotypes with age in a similar manner to that seen for single pulse stimuli. The relative release probability of DA by bursts versus non-burst stimuli is not significantly different between genotypes in any of the age groups studied (Figure 4.8). This indicates that a simple change in release probability at striatal DA synapses cannot account for the observed difference in evoked $[DA]_o$.

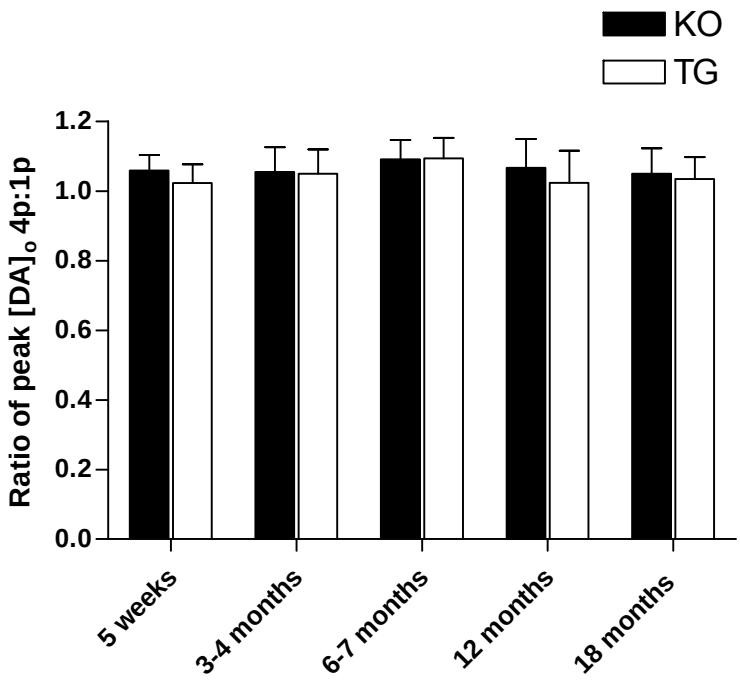


Figure 4.8. ***The relative release probability of DA by bursts versus non-burst stimuli of SNCA-A30P+Snca-/- transgenic mice compared to Snca-/- controls in CPu.*** The low ratios of DA evoked by a burst stimuli (4p, 100 Hz) compared to a single pulse (4p:1p ratio) show that the limited release by a burst after prior release by a single pulse is typical of dorsal striatal synapses (Cragg, 2003) and occur at all ages in a manner that did not differ between genotypes.

4.2.2. Lower evoked $[DA]_o$ in the CPu in SNCA-A30P$^+$Snca-/- transgenic mice is not due to changes in striatal DA content, which is not significantly changed with age

Differences in DA content in the CPu and NAc between *SNCA-A30P$^+$Snca-/-* transgenic mice and non-transgenic *Snca-/-* littermate control mice were assessed using HPLC. This was done firstly to explore whether any overall changes in DA content at different age points were seen in this novel BAC-*SNCA* transgenic mouse line which carry the A30P mutation, and secondly to investigate whether the lower evoked $[DA]_o$ observed in the CPu at 5 weeks, 3-4 months and 6-7 months corresponded to a reduced DA content. In both the CPu and NAc, an overall effect of age within a genotype was observed, but statistically significant differences in DA content between the two genotypes were not observed for any of the age groups analysed (Figure 4.9). This indicates that the observed lower evoked $[DA]_o$ cannot to be attributed to an overall reduction in DA content in the CPu in *SNCA-A30P$^+$Snca-/-* transgenic mice.

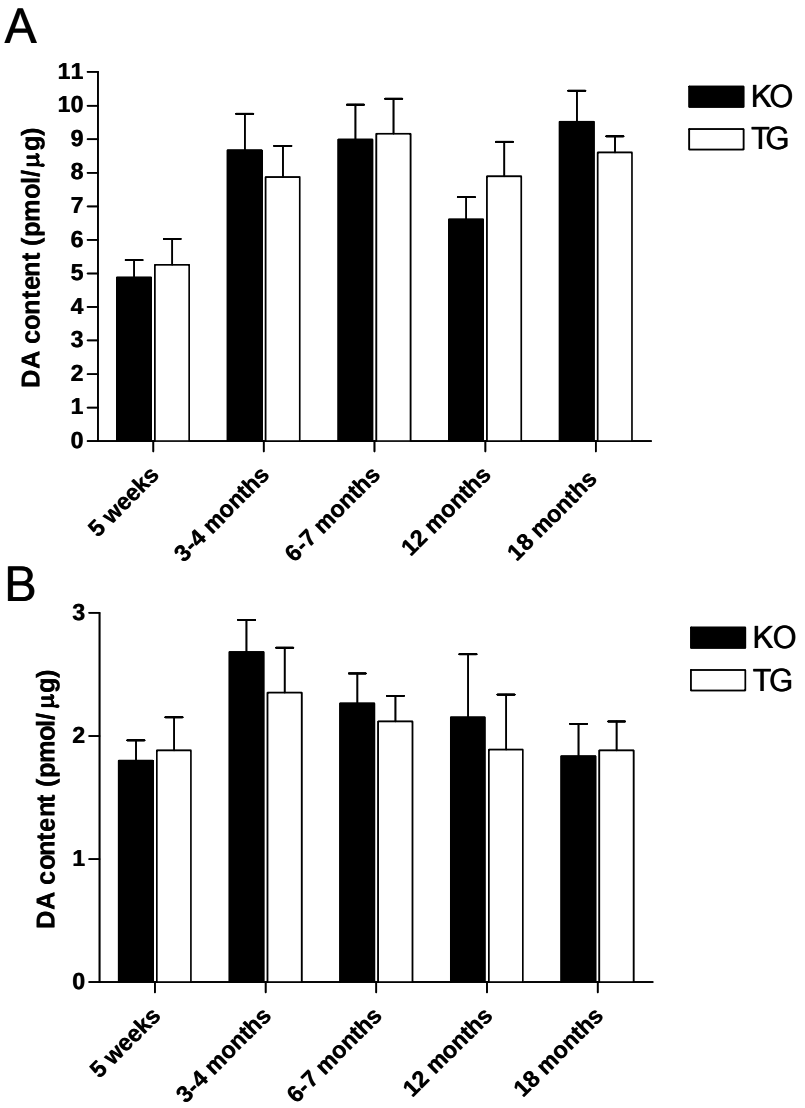


Figure 4.9. ***Striatal dopamine (DA) content in SNCA-A30P^{+}Snca-/- transgenic mice and Snca-/- littermate control mice measured by HPLC.*** In both the CPu and NAc, statistically significant differences in DA content between the two genotypes was not observed for any of the age groups analysed (Figure A; CPu data, two-way ANOVA, *SNCA-A30P^{+}Snca-/-* n=6-10, *Snca-/-* controls n=6-10, [4-5 animals per genotype]; P>0.05 for all age groups analysed in CPu; Figure B; NAc data, two-way ANOVA, *SNCA-A30P^{+}Snca-/-* n=6-10, *Snca-/-* controls n=5-10, [4-5 animals per genotype]; P>0.05 for all age groups analysed in NAc).

4.2.3. Lower evoked $[DA]_o$ in the CPu SNCA-A30P$^+$Snca-/- transgenic mice is not due to changes in DA uptake at striatal synapses, which does not significantly change with age

Functional interactions between α-synuclein and the DA transporter (DAT) have been reported (Fountaine et al., 2008; Fountaine and Wade-Martins, 2007; Lee et al., 2001; Wersinger et al., 2003; Wersinger and Sidhu, 2003). Therefore, differences in DA uptake from the synaptic cleft were assessed for two reasons. The first was to see whether any overall changes in DA uptake in *SNCA-A30P$^+$Snca-/-* transgenic mice occurred. This would implicate an altered DAT function as a consequence of A30P mutated α-synuclein. The second reason was to investigate whether the observed decrease in evoked $[DA]_o$ in *SNCA-A30P$^+$Snca-/-* transgenic mice at 5 weeks, 3-4 months and 6-7 months could be attributed to an increased level of DA uptake. This was undertaken by a comparing the $[DA]_o$ transients from both genotypes, which had been matched for peak $[DA]_o$ (2.0 ± 0.01 μM). The decay phase of the transients is a function of DA uptake by DAT (Giros et al., 1996) and therefore allows ready comparison of any alterations in uptake between the two genotypes. The rate of decay was similar for both genotypes in all age groups (Figures 4.5C, 4.6C and 4.7C). This suggests the rate of reuptake is not significantly different in the CPu in *SNCA-A30P$^+$Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate control mice.

4.2.4. Assessment of frequency sensitivity of DA release and nicotine receptor function in SNCA-A30P$^+$Snca-/- transgenic mice

In the CPu, evoked $[DA]_o$ is approximately twenty percent lower in *SNCA-A30P*$^+$*Snca-/-* transgenic mice compared to non-transgenic *Snca-/-* littermate control mice. However, DA content and DA uptake in the CPu are unchanged which indicates the releasability of available DA is decreased. Therefore, initial experiments to explore potential differences in the frequency sensitivity of DA release and nicotinic receptor function between the two genotypes was assessed, using mice aged 3-6 months.

Firstly, the frequency sensitivity of DA release in *SNCA-A30P*$^+$*Snca-/-* transgenic mice and non-transgenic *Snca-/-* control mice was assessed in CPu using 5-pulse trains at frequencies ranging from 1 to 100 Hz. These stimulation frequencies represent the full physiological range of presynaptic DA neuron firing frequencies during tonic and burst-like, or phasic, activities. Although $[DA]_o$ varied with frequency according to a slight inverted U-relationship in the CPu in both genotypes as previously published in wild-type mice (Exley et al., 2008), a greater frequency sensitivity was observed in *SNCA-A30P*$^+$*Snca-/-* transgenic mice (Figure 4.10). This enhanced reporting of frequency suggest that short-term depression of release is less in SNCA-*A30P*$^+$*Snca-/-* transgenic mice which indirectly suggest a potential decrease in DA release probability, but one not identified by the previously described experiments comparing release by single pulses and bursts at higher frequencies of 100 Hz.

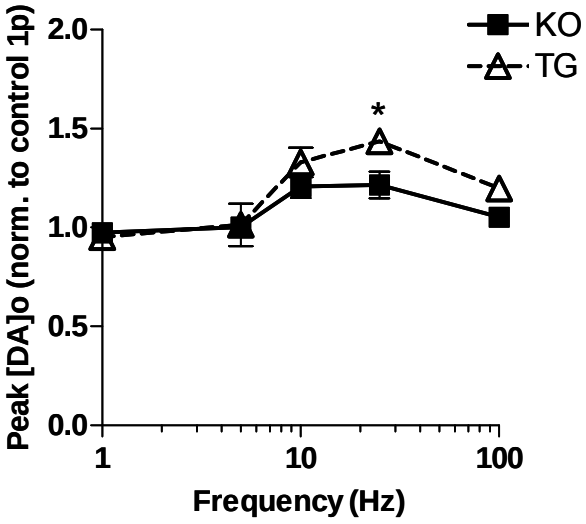


Figure 4.10. ***Regulation of DA signals by firing frequency in the CPu of SNCA-A30P^{+}Snca-/- transgenic mice and Snca-/- littermate control mice measured by FCV.*** Mean peak $[DA]_o$ SEM± vs. frequency during 5 pulse trains (1-100 Hz) in the CPu varied with frequency according to a slight inverted U-relationship in the CPu in both genotypes in control conditions, with a greater frequency sensitivity observed in *SNCA-A30P^{+}Snca-/-* transgenic mice, significantly at 25 Hz (two-way ANOVA, 3 animals per genotype; **P<0.05* at 25 Hz).

Secondly, to explore whether any underlying difference in cholinergic regulation of DA release might be responsible for effects of genotype on evoked $[DA]_o$, nicotinic receptor function in the CPu was assessed. nAChR's play a critical role in the dynamic regulation of DA release probability during different patterns of presynaptic activity (Rice and Cragg, 2004; Zhang and Sulzer, 2004; Zhou et al., 2001). The regulation of DA release in *SNCA-A30P^{+}Snca-/-* transgenic mice and non-transgenic *Snca-/-* control mice across a range of stimulation frequencies was assessed. In both genotypes, nAChR antagonist dihydro-β-erythroidine hydrobromide (DHβE, 1μM) decreased $[DA]_o$ evoked by low stimulation frequencies (<25 Hz) but enhanced $[DA]_o$ evoked by higher frequencies (>25Hz) in the CPu in a manner to that previously

reported (Figure 4.11). However, the expected decrease of DA release by a single pulse is less pronounced in *SNCA-A30P*$^{+}$*Snca-/-* transgenic mice when compared to non transgenic *Snca-/-* littermate controls (66% reduction of DA release in non transgenic *Snca-/-* control mice compared with only 36% reduction in *SNCA-A30P*$^{+}$*Snca-/-* transgenic mice). This is in keeping with a potential reduction in cholinergic regulation, and may be evidence that the lower evoked $[DA]_o$ phenotype observed in *SNCA-A30P*$^{+}$*Snca-/-* transgenic mice is due in part to a decreased cholinergic tone. These results constitute only preliminary analysis as the data is limited by the restricted number of observations possible in the mice available (single recording site, n=3 animals). Additional repeat experiments and an assessment of the effects of partial loss of cholinergic tone across the full frequency range (mimicked by lower DHβE concentrations) are required before firm conclusions can be drawn regarding these data.

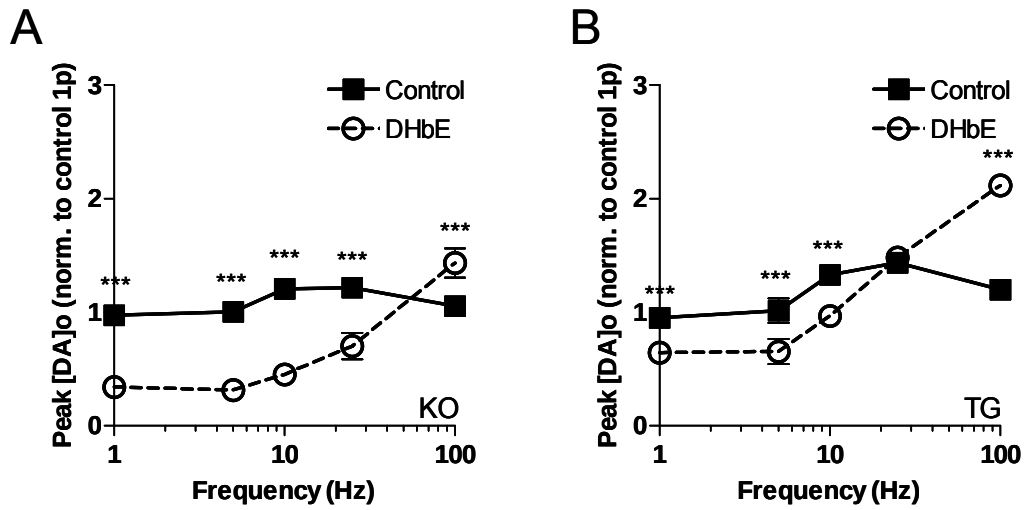


Figure 4.11. ***Cholinergic regulation of DA release in the CPu of SNCA-A30P^{+}Snca-/- transgenic mice and Snca-/- littermate control mice measured by FCV. (***A) Mean peak $[DA]_o$ SEM± vs. frequency during 5 pulse trains (1-100 Hz) in the CPu of *Snca-/-* control mice in the presence of DHβE normalised to $[DA]_o$ under control conditions (two-way ANOVA, 3 animals per genotype; ****P<0.001* at all frequencies); (B) Mean peak $[DA]_o$ SEM± vs. frequency during 5 pulse trains (1-100 Hz) in the CPu *SNCA-A30P^{+}Snca-/-* transgenic mice in the presence of DHβE normalised to $[DA]_o$ under control conditions (two-way ANOVA, 3 animals per genotype; ****P<0.001* at all frequencies except 25 Hz, at which there is no significant difference between the two genotypes).

4.3. Discussion

This chapter describes the neurochemical characterisation of a novel α-synuclein transgenic mouse model which carries the PD-associated A30P mutation. *SNCA-A30P*$^{+}$*Snca-/-* transgenic mice display approximately 20 percent lower evoked $[DA]_o$ in the CPu at ages 5 weeks, 3-4 months and 6-7 months compared to non-transgenic *Snca -/-* littermate controls. This difference diminishes with age, due to a decline in evoked $[DA]_o$ in non transgenic *Snca -/-* littermate controls. The reduction in DA releasability in the CPu of *SNCA-A30P*$^{+}$*Snca-/-* transgenic mice cannot be attributed to changes in DA uptake or DA content, however there is some mixed and limited evidence for a reduction in DA release probability. Enhanced frequency sensitivity seen in these mice indicates a short-term depression of release may be occurring. This in turn indirectly indicates either a reduced initial DA release probability, or a mechanism that limits re-release during repeated neuronal firing, such as D2-autoreceptor control may occur. Therefore, even though it is not supported by the observed 4p:1p ratios, it is not possible to discount a change in DA release probability as an explanation for the reduction in DA releasability. Future studies must focus on elucidating potential reasons for these internally inconsistent results. For example the data described here represent a limited set of observations; therefore additional repeat experiments to obtain a wider set of observations would be useful. Alternative explanations for ehanced frequency sensitivity should also be tested in future studies. Although preliminary data suggest a reduction in cholinergic tone may in part account for the lower evoked $[DA]_{o,}$ additional repeat experiments in more mice are required to draw firmer conclusions about these results.

Ongoing work in the Cragg group has investigated the release of DA in the CPu in *SNCA*-WT-39 transgenic mice, described in Chapter 3. This transgenic line serves as an important control for the α-synuclein transgenic mouse line which carries the A30P mutation. In mice aged 3 months, there is no significant difference in evoked $[DA]_o$ in the *SNCA*-WT-39 mice, compared to non-transgenic *Snca-/-* littermate controls. This is shown in Figure 4.12. These experiments were performed by Dr Sarah Threlfell.

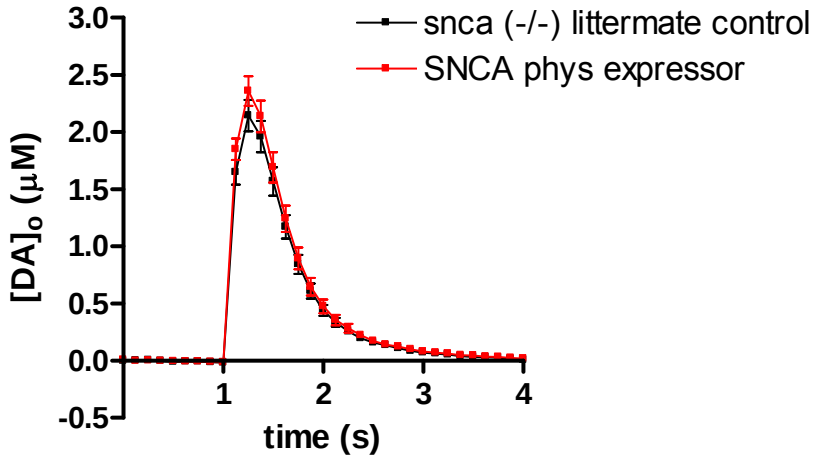


Figure 4.12. ***Electrically evoked DA transients in CPu of SNCA-WT39+Snca-/- mice compared to Snca-/- controls at 4-6 months of age.*** No significant differences seen in mean peak $[DA]_o$ evoked by either a single pulse (1p) in *SNCA*-WT39$^+$*Snca-/-* mice compared to non-transgenic *Snca-/-* littermate controls for mice at 4-6 months of age (Figure provided by Dr Sarah Threlfell).

Therefore, these studies have confirmed that that phenotype observed in *SNCA-A30P*$^+$*Snca-/-* transgenic mice, at 3 months of age at least, can be attributed to the presence of the mutated A30P form of α-synuclein, and is not simply due to the presence of the human transgene or an alteration introduced by the use of BACs. The

effects on evoked $[DA]_o$ with age in this genotype are currently not known and these experiments are currently being planned in the Cragg group.

Most recently, Nemani and colleagues (Nemani et al., 2010) demonstrated that in α-synuclein transgenic mice, modest overexpression of wild-type human α-synuclein markedly inhibited glutamate release in hippocampal pyramidal neurons, and in mesencephalic DA neurons in cell culture. Furthermore, the group reported that in cultured hippocampal neurons, the A30P mutation abolished the ability of overexpressed α-synuclein to inhibit neurotransmitter release. This does not readily agree with the results described here, which indicate decreased DA transmitter release in the CPu in young *SNCA-A30P*$^{+}$*Snca-/-* transgenic mice.

The inhibition of neurotransmitter release caused by wild-type α-synuclein, but not the A30P-mutated form, observed by Nemani and colleagues was not coupled with obvious α-synuclein toxicity or inclusion formation, and is reportedly due to a failure of synaptic vesicles to recluster at presynaptic release sites after endocytosis.

There are a number of possible explanations for the contrasting results between the study by Nemani and colleagues and the findings described in this chapter, namely the ability of the A30P mutated form of α-synuclein to inhibit transmitter release. Firstly, the α-synuclein transgenic mouse model used in the two studies differs. Nemani and colleagues use a cDNA-based transgenic model in which α-synuclein expression is driven by the prion promoter. As described in the Introduction and Chapter 3, these models are unlikely to provide temporally regulated, tissue-specific expression of the transgene. This could give rise to findings which may not be seen when α-synuclein is

expressed in a stable, regulated and tissue specific manner, as seen with the *SNCA*-BAC transgenic model used in this chapter. Secondly, the experiments described by Nemani et al. were conducted in pyramidal hippocampal neurons whereas the study described here characterises DA neurones in the striatum, which are preferentially targeted in PD. It is likely that, although exocytotic mechanisms of neurotransmitter release are similar in presynaptic neurons, the difference in findings may be due to potential differences in the brain regions and neuron types studied. For the purposes of studying α-synuclein function in PD, the study of DA axons in the striatum described in this chapter represents the most physiologically relevant approach. Although Nemani et al. demonstrated similar inhibition of transmitter release in mesencephalic DA neurons by overexpression of α-synuclein; these experiments were done *in vitro,* which may not be representative of the behaviour of DA neurons *in vivo.* In addition, A30P mutated α-synuclein failed to inhibit transmitter release was shown in non-dopaminergic neurons. Studies of striatal DA synapse function in the α-synuclein transgenic mice used by Nemani et al. will enable a more direct and precise comparison of the effects of A30P mutated α-synuclein. In addition, it would be interesting to investigate the relationship between wild-type and A30P α-synuclein and CSPα using *SNCA*-BAC transgenic mice.

The work described in this chapter reveals for the first time that *SNCA-A30P*$^{+}$*Snca-/-* transgenic mice, which express α-synuclein in spatially and temporally regulated manner, exhibit altered DA dynamics in the striatum, namely a decrease in evoked $[DA]_o$ in the CPu, under conditions of physiologically relevant neuronal firing conditions. Studies in α-synuclein transgenic mice which overexpress A30P-mutated human α-synuclein under the control of the prion promoter have also reported altered

dynamics in DA release in the striatum (Yavich et al., 2005). Yavich and colleagues demonstrated, using constant potential amperometry, that these mice exhibit reduced DA release from striatal synapses under conditions of heavy vesicle loading of presynaptic terminals during intensive, prolonged stimulations. This decrease in DA release was not attributable to differences in uptake between A30P and control mice. Rather, the authors propose it is due to less DA being available for release upon repeated stimulation, caused by the inability of mutated A30P α-synuclein to traffic DA vesicles between the readily releasable pool and reserve pools in striatal terminals, or because of a lowered reserve pool capacity. A lowered reserve pool capacity has previously been suggested in α-synuclein knockout mice, where prolonged, repeated stimulations significantly decreased neuronal responses, albeit in non-dopaminergic neurons (Cabin et al., 2002). Similar results were observed under similar stimulation conditions in hippocampal neurons in mice overexpressing A30P mutated α-synuclein (Steidl et al., 2003). The present study reveals this DA release deficit in A30P mice is also present using physiologically relevant stimulation protocols which examine the immediate releasability of DA, namely single stimulations and high-frequency stimulation 'bursts' (4p, 100 Hz). Further experiments planned in our laboratory will now examine further candidate mechanisms for this phenotype using FCV, including using high-frequency, prolonged and repeated stimulation protocols to dissect the role of A30P-mutated α-synuclein on vesicular trafficking and reserve and readily releasable pool capacity.

There are a number of potential explanations for the decrease in evoked $[DA]_o$ observed in *SNCA-A30P*$^+$*Snca-/-* transgenic mice. *SNCA* point mutations in dominant forms of PD are believed to cause neurodegeneration through a toxic 'gain of

function', due to the increased propensity of mutated α-synuclein to aggregate in PD (Gasser, 2009). It is possible that an earlier, more subtle gain of function of mutated α-synuclein protein occurs via its postulated role as a negative regulator of DA release, as detailed in the Introduction. Notably, double (α- and γ-) synuclein knockout mice (Senior et al., 2008), and triple (α-, β-, γ-) synuclein knockout mice, which are characterised in Chapter 5, demonstrate a two-fold increase in evoked $[DA]_o$ from DA striatal synapses, strongly supporting the notion that α-synuclein has an important presynaptic function in negatively regulating DA release. It is possible then that A30P mutated α-synuclein acts, through a gain of function, in much the same way as overexpressed wild-type α-synuclein, inhibiting transmitter release as described by Nemani and colleagues (Nemani et al., 2010). In both studies, deficits in transmitter release have been observed in the absence of obvious α-synuclein inclusions or LB-like pathology. How this early-stage deficit in transmitter release leads to extensive α-synuclein aggregation and LB pathology is unresolved. However, there are published speculations that neurons attempt to compensate for the reduced or inhibited transmitter release by elevating or upregulating other aspects of synaptic transmission (Surmeier, 2010). This in turn places an increased burden on these neurons and leads to a slow accumulation of cellular deficits, resulting in LB pathology and a more global dysfunction in brain function, which is only seen in later life as motor and non-motor symptoms of PD. Surmeier further postulates that the transmitter deficits seen at an early stage impact the neurons which are most vulnerable in PD more than others (Surmeier, 2010). This maybe because these neurons, which include DA neurons in the SNpc, noradrenergic neurons in the locus coerulus and cholinergic neurons in the basal forebrain nuclei, have extremely large and extensive axonal fields with orders of magnitude more synaptic terminals than

most neurons (Matsuda et al., 2009). Furthermore, these neurons are spontaneously active *in vivo*, continually releasing neurotransmitter in target structures, e.g. DA in the striatum, which is crucial to their correct functioning. The axonal and LB pathology seen later in life might be the consequence of compromising these neurons early on by overloading them.

To further investigate the presynaptic function of A30P mutated α-synuclein in the *SNCA*-BAC transgenic mice in future studies, it would be useful to investigate the interactions of the mutated protein with other elements of presynaptic machinery. These include SNARE proteins, synaptobrevin, syntaxin and SNAP-25, which crucially involved in membrane fusion during synaptic exocytosis. Furthermore, the relationship between α-synuclein and complexin proteins, which act as SNARE complex regulators, and synapsin protein, which are believed to be required for regulating and maintaining synaptic vesicle pools would be of interest. Nemani et al. reported a decrease in the levels of complexin and synapsin proteins, whilst changes in the levels of complexins have been reported in double (α- β-) synuclein knockout mice. Therefore, it would be useful in future studies to see whether similar changes in these proteins levels occur in the mice described in this chapter.

Another reason for the observed phenotype may be a reduction in transmitter-containing vesicle number due to disrupted vesicle genesis, disrupted trafficking mechanisms which lead to changes in reserve and readily releasable pool sizes or changes in the number of docked vesicles at active zones. Notably, Nemani and colleagues elucidated, through imaging and ultrastructural analysis, that the reported inhibition of transmitter release involved a specific reduction in the size of the

synaptic vesicle recycling pool, reduced synaptic vesicle density at the active zone and a defect in the reclustering of synaptic vesicles after endocytosis. Yavich and colleagues also suggest from their studies in A30P in α-synuclein transgenic mice, that the decreased DA release is due to deficits in trafficking DA vesicles between the readily releasable and reserve pools, or due to a lowered reserve pool capacity (Yavich et al., 2005). Investigating whether similar early defects in vesicular recycling occurs in SNCA-A30P^{+} transgenic mice, through FCV, imaging and electron microscopy of striatal DA terminals represents an important goal for future work.

Another aspect of the results presented in this chapter is that difference between genotypes in evoked $[DA]_o$ does not occur at 12 or 18 months of age. This difference emerges due to an apparent decline in evoked $[DA]_o$ in non transgenic *Snca-/-* littermate controls with age. There are several potential reasons for the diminished difference in evoked $[DA]_o$ between the two genotypes with age. It is possible that the already low evoked $[DA]_o$ in *SNCA-A30P*$^{+}$*Snca-/-* transgenic mice limits subsequent decline with age, or that these mice do not show an age-dependent decline in neurotransmitter release. It is also possible that the non transgenic *Snca-/-* control mice show a greater than normal decline in transmitter release with age. Indeed, modest reductions in striatal DA levels (~18%) have been previously reported in α-synuclein knockout mice (Abeliovich et al., 2000; Cabin et al., 2002), whilst significant reductions in striatal DA levels (~36%) were found in ageing mice, aged 24-26 months (Al-Wandi et al., 2010). It is possible that the loss of α-synuclein in ageing mice begins to disrupt transmitter release processes in a similar way to the A30P mutant form of the protein does at early stages. It is also possible that A30P

mutated α-synuclein, while compromising release from an early age, leads to better maintained DA release with age, which may actually be the abnormal effect of the mutated protein. To more accurately assess why differences in evoked $[DA]_o$ do not occur at 12 or 18 months of age, longitudinal analysis of evoked $[DA]_o$ with age in the CPu in normal wild-type mice represents an important goal for future studies. These experiments will better inform of the potential pathological consequences of the presence of A30P mutated α-synuclein.

Further neurochemical analysis of these novel *SNCA-A30P*$^+$*Snca-/-* transgenic mice, using a combination of FCV and pharmacological techniques is currently underway in the Cragg group. These include experiments using high-frequency, repeated stimulation protocols to explore vesicle mobilisation and trafficking, and exploration of candidate mechanisms which may be responsible for the observed decrease in evoked $[DA]_o$, such as potential calcium-dependent changes in exocytosis, Most recently, experiments in *SNCA*-BAC mice which overexpress wild-type human α-synuclein (line SNCA-WT-37, described in Chapter 3) have shown an approximate 30% reduction in evoked $[DA]_o$ compared to non-transgenic *Snca-/-* littermate controls, at 3 months of age. This is in agreement with the findings of Nemani and colleagues, as discussed. Of particular interest, whilst in *SNCA-A30P*$^+$*Snca-/-* transgenic mice, the evoked $[DA]_o$ reduction occurred uniformly throughout the CPu, in the wild-type overexpressing line, significant reductions in were observed in the dorsal portion of the CPu, the region closely associated with the control of motor commands. Further experiments exploring the phenotype seen in the SNCA-WT-37 transgenic line with age are currently being planned.

Broader neurochemical analysis of *SNCA*-BAC mice is also warranted. A number of studies in α-synuclein knockout mice and transgenic mice which overexpress or have mutated forms of the protein have investigated the effects upon synaptic transmission in other brain regions, particularly the hippocampus (Cabin et al., 2002; Nemani et al., 2010). It would be interesting to see whether the observed defect in evoked $[DA]_o$ in the CPu extends to other central neurotransmitters, causing a more global negative impact of the functioning of several circuits in the brain.

5. CHAPTER 5: Functional alterations to the nigrostriatal system in mice lacking all three members of the synuclein family

5.1. Introduction

The synucleins (α-, β-, and γ-synuclein) are a family of small, highly conserved proteins whose physiological functions remain poorly understood. All three proteins are highly expressed throughout the mammalian central nervous system where they localise to synaptic nerve terminals (Clayton and George, 1998; Maroteaux et al., 1988). Elucidating the functional roles of synuclein proteins has become increasing important to researchers over the past decade, specifically given that for one family member, α-synuclein, there is well-documented evidence of links between changes in α-synuclein structure or expression and neurodegenerative disorders, including PD (Goedert, 2001a, b).

Although the synuclein proteins have been intimately linked to almost every process governing synaptic transmission, understanding the precise synaptic functions of synucleins and identifying their molecular targets is confounded by the degree of functional redundancy within the synuclein gene family. A number of reports have characterised the effects of deleting one synuclein, or two synucleins in combination. Mice lacking a single synuclein protein, and mice lacking both α- and β- or α- and γ-synuclein have been reported to have either no, or only mild, behavioural and/or neurochemical phenotype in the absence of a drug challenge (Abeliovich et al., 2000; Cabin et al., 2002; Dauer et al., 2002; Schluter et al., 2003; Yavich et al., 2004). A degree of functional overlap is suggested by compensatory

changes in the levels of synuclein proteins in the absence of others. For example, increased expression of the remaining family member was observed in the nervous system of mice lacking α- and β- or α- and γ-synuclein (Chandra et al., 2004; Robertson et al., 2004).

Knockout mouse models have been useful in demonstrating the importance of synuclein proteins in regulating several aspects of synaptic physiology, including regulating neurotransmitter synthesis and content. For example, the levels of DA content in single synuclein-null mice or α- and γ-synuclein null mice are not significantly changed, at least until 24-26 months of age, when significant decreases in DA levels in α-synuclein-null mice have been reported (Al-Wandi et al., 2010; Chandra et al., 2004; Senior et al., 2008). Furthermore, it has been reported that mice lacking both α- and β-synuclein exhibit an approximately 20% decrease in DA levels (Chandra et al., 2004). In addition, several groups have demonstrated α-synuclein directly inhibits DA biosynthesis through regulatory effects on tyrosine hydroxlase, protein phosphatase 2A and aromatic amino acid decarboxylase (Peng et al., 2005; Perez et al., 2002; Tehranian et al., 2006).

As detailed in the Introduction section of this thesis, the use of synuclein knockout mice has also been important in determining α-synuclein's role in synaptic vesicle regulation and the role of synuclein proteins in regulating neurotransmitter release at dopaminergic synapses. For example, α-synuclein-null mice are reported to have increased recovery from PPD in striatal DA synapses (Abeliovich et al., 2000), and have also been shown to have an increased rate of filling of the DA vesicle pool (Yavich et al., 2004). Further, mice lacking both α- and γ-synuclein exhibit a two-

fold increase in DA release in the dorsal striatum, attributed to an increase in the release probability at dopaminergic synapses (Senior et al., 2008). α-synuclein, is also believed to regulate the rate of re-uptake of DA at synapses, having been shown to facilitate the localisation of the DAT to the cell surface (Fountaine et al., 2008; Fountaine and Wade-Martins, 2007). Further, mice singly lacking α-synuclein or γ-synuclein, and α-, γ-synuclein double-null mice are resistant to MPP+, a neurotoxin taken up by DAT (Abeliovich et al., 2000; Dauer et al., 2002; Robertson et al., 2004). Taken together, this evidence supports the hypothesis that α-synuclein, alone or together with its closely related family members, act as a negative regulator of DA release at synapses.

Another aspect of single synuclein knockout mouse models is that either no, or only a mild behavioural phenotype is observed. Indeed, several lines of α-synuclein-null mice have been generated, none of which exhibit overt behavioural abnormalities in the absence of a drug challenge (Abeliovich et al., 2000; Cabin et al., 2002; Dauer et al., 2002; Schluter et al., 2003; Yavich et al., 2004). Furthermore, some reports of behavioural abnormalities in α-synuclein-null mice have been conflicting. For example, Abeliovich and colleagues have reported a decrease in locomotor response in response to amphetamine in α-synuclein-null mice, whilst Schulter and colleagues reported an increase in response to methamphetamine. Conflicting reports again arise in experiments by Specht and Schoepfer, who report no changes in sensitivity of α-synuclein-null mice to amphetamines (Specht and Schoepfer, 2001). Further behavioural analysis indicates that deleting α-, β-, or γ-synuclein proteins singly, or doubly in combination does not cause obvious deficits in motor performance (Abeliovich et al., 2000; Senior et al., 2008). Notably also, mice

which lack both α- and γ-synuclein, but not single α- or γ-synuclein knockout mice, are significantly hyperactive in a novel environment, but not in the home cage, and reportedly alternate at a lower rate in a test of spontaneous alternation. Taken together, these behavioural reports further indicate that considerable functional redundancy exists within the synuclein gene family, and that functional complementation may occur when one or more synuclein proteins are deleted.

Indeed, the high sequence homology and functional overlap between the synucleins makes studying the effects of single synuclein proteins on changes in behaviour and alterations in DA transmission more difficult. To overcome this, mice which lacked all three members of the synuclein family were obtained from Professor Vladimir Buchman (University of Cardiff) for the functional characterisation of the nigrostriatal system in these animals.

This chapter investigated behaviour and DA neurotransmission in a novel mouse model lacking all three members of the synuclein gene family.

Specifically, the aims of this chapter are:

1. To assess the behavioural phenotype of synuclein triple-null mice compared to age-matched, wild-type control mice; specifically assessing whether abnormalities in activity-, motor- or anxiety-related behaviours are exhibited.
2. To assess differences in the levels of electrically evoked DA in the dorsal striatum (caudate putamen; CPu) and ventral (nucleus accumbens; NAc) of synuclein triple-null mice compared to age-matched, wild-type control mice using FCV at carbon fibre microelectrodes.

3. To assess differences in DA content in the CPu and NAc in synuclein triple-null mice compared to age-matched, wild-type control mice, using high-performance liquid chromatography (HPLC).
4. To test candidate mechanisms for potential differences in DA neurotransmission and DA content in the CPu in synuclein triple-null mice compared to age-matched wild-type control mice, using FCV at carbon fibre microelectrodes. Specifically, any differences in frequency sensitivity and nicotinic receptor function, DA uptake, or calcium dependent changes in exocytosis will be assessed.

5.2. Results

5.2.1. Synuclein triple-null mice exhibit a hyperdopaminergic-like behavioural phenotype

A broad behavioural screen was performed on synuclein triple-null mice to assess the effects of the absence of all three synuclein proteins. Performance was assessed on the accelerating rotor rod and multiple static rods. Both are tests of motor co-ordination, with the rotor rod test additionally assessing motor learning skills. An elevated plus maze test, and a bright open field test were performed to assess anxiogenic behaviour. Furthermore, activity in a non-anxiogenic open-field, activity in a holeboard test, and home-cage activity were assessed. Finally, performance on a T-maze test of spontaneous alternation was assessed to test short term spatial working memory in synuclein triple null mice compared to wild-type control mice. These behavioural tests were chosen so that direct comparisons between synuclein triple null mice and single and double synuclein null mice were possible (Abeliovich et al., 2000; Senior et al., 2008).

A significant main effect of genotype was observed on activity in the non-anxiogenic open-field. Synuclein triple-null mice were significantly hyperactive compared with wild-type control mice (squares entered; Figure 5.1A, unpaired t-test, wild-type n=14, synuclein triple-null n=18 $P<0.0001$). Synuclein triple-null mice also reared significantly more often in the open-field (Figure 5.1B, unpaired t-test, wild-type n=14, synuclein triple-null n=18 $P<0.001$).

Analysis of activity during the first hour after introduction of both genotypes into the home-cage revealed synuclein triple-null mice were significantly more active (Figure 5.1C, unpaired t-test, wild-type n=14, synuclein triple-null n=18 $P<0.01$). However, total activity of synuclein triple-null mice over the 15 hour period did not differ significantly (Figure 5.1D, unpaired t-test, wild-type n=14, synuclein triple-null n=18 $P=0.1001$). These results indicate that synuclein triple-null mice respond to changes in the environment with a substantially greater increase in locomotion than wild-type mice. However, after adaptation to the new surroundings, activity levels of synuclein triple-null mice are similar for both genotypes.

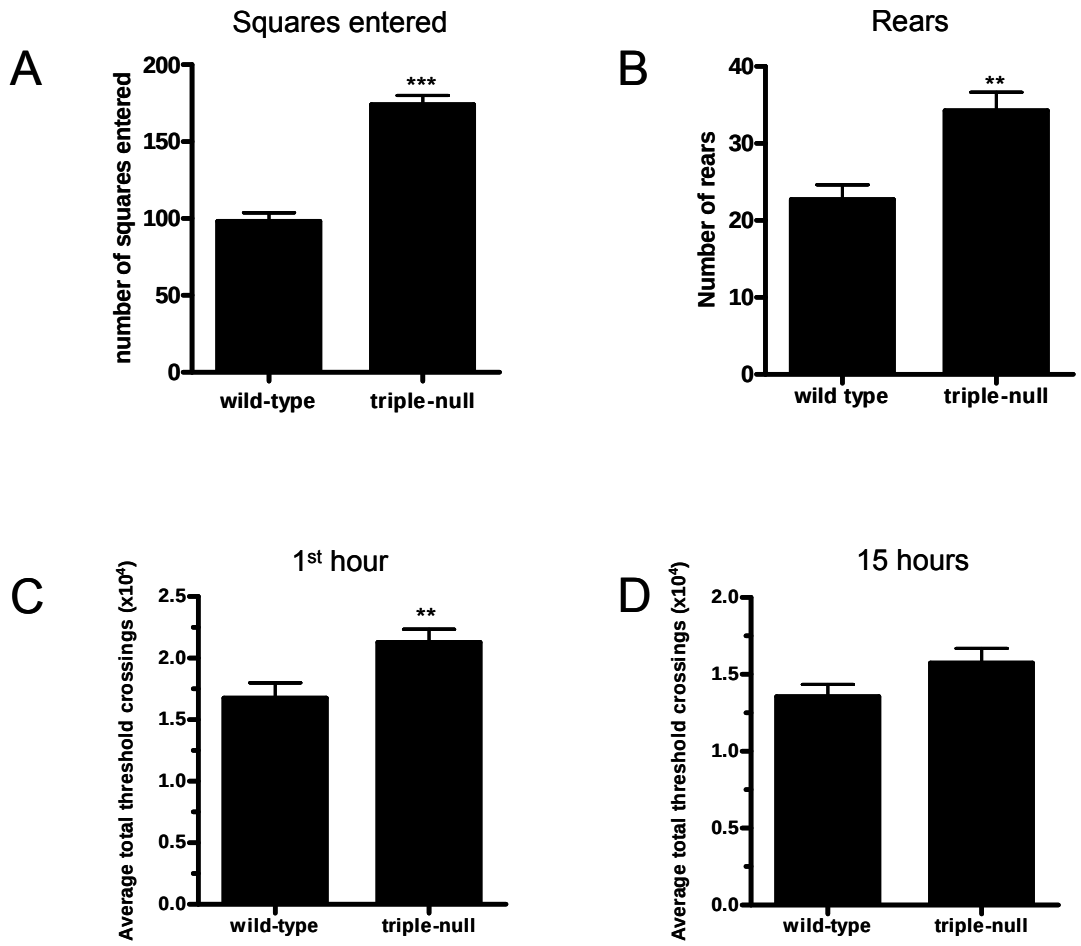


Figure 5.1. ***Activity of synuclein triple-null and wild-type mice in the non-anxiogenic open-field and home cage.*** (A) Triple-null mice were significantly hyperactive in the non-anxiogenic open-field compared with wild-type controls (unpaired t-test, wild-type n=14, triple-null n=18, ****P*<0.0001). (B) Triple-null mice exhibited a significantly increased rate of rearing in the non-anxiogenic open field compared with wild-type controls (unpaired t-test wild-type n=14, triple-null n=18, ***P*<0.001) (C) Triple-null mice were significantly hyperactive during the first hour upon introduction into the home cage (unpaired t-test, wild-type n=14, triple-null n=18, ***P*<0.01). (D) There were no significant differences with genotype in activity in the home cage measured over a 15 hour period (unpaired t-test, wild-type n=14, triple null n=18, *P*=0.1001).

A significant effect of genotype was also found in the holeboard test. Synuclein triple-null mice exhibited increased overall exploratory behaviour compared to wild-type controls, as was measured by the number nose pokes by each genotype group into both peripherally and centrally arranged holes (Figure 5.2A, unpaired t-

test, wild-type n=14, synuclein triple-null n=18 $P<0.0001$; Figure 5.2B unpaired t-test, wild-type n=14, synuclein triple-null n=18 $P<0.0001$).

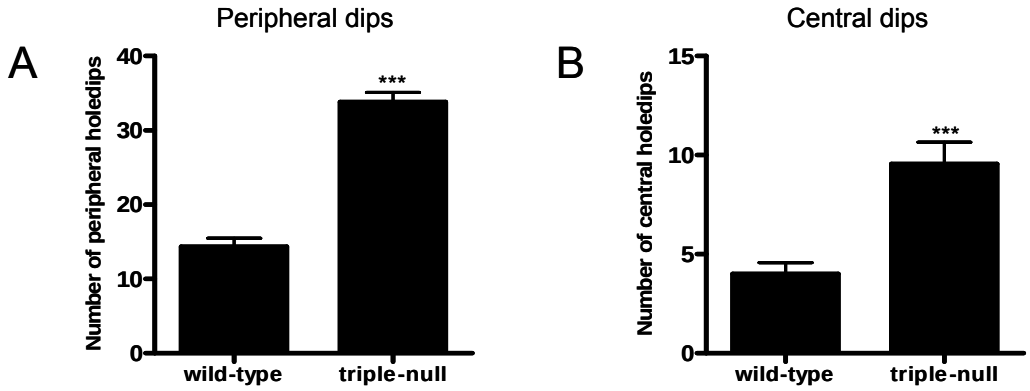


Figure 5.2. ***Exploratory behaviour of synuclein triple-null and wild-type mice in the holeboard.*** Triple-null mice exhibited significantly increased exploratory behaviour in the holeboard test compared with wild-type controls, indicated by an increased number of nose pokes into peripheral holes (A) (unpaired t-test, wild-type n=14, triple null n=18, ***$P<0.0001$) and central holes (B) (unpaired t-test, wild-type n=14, triple null n=18, ***$P<0.0001$).

On the basis of the previous study published by Senior *et al*., we predicted that synuclein-null mice would exhibit a deficit in spontaneous alternation. Our results confirmed this hypothesis; synuclein triple-null mice showed decreased spontaneous alternation (Figure 5.3, unpaired t-test, wild-type n=14, synuclein triple-null n=18, **P*=0.03, one-tailed).

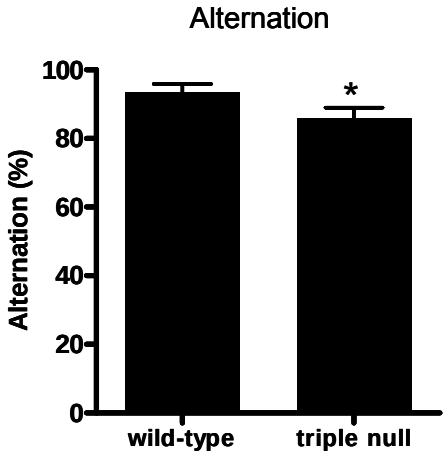


Figure 5.3. ***Performance of synuclein triple-null and wild-type mice on spontaneous alternation in a T-maze.*** Synuclein triple-null mice alternated at a significantly lower rate in a test of spontaneous alternation in a T-maze (unpaired t-test, wild-type n=14, synuclein triple-null n=18, **P*=0.03, one-tailed).

No statistically significant differences were observed between the two genotypes on the accelerating rotor rod (Figure 5.4; performance analysed by day and by trial using SPSS 17.0 software; repeated measures ANOVA, wild-type n=14, triple null n=18, *P>0.05* for both parameters), the multiple static rods (Figure 5.5; parameters analysed were the time taken to turn through 180º and the time taken to traverse the rods; two-way ANOVA, wild-type n=14, triple null n=18, *P>0.05* for both parameters analysed), the bright open field (Figure 5.6; parameters analysed were

the total distance moved, the duration spent in the inner zone and the mean frequency of inner zone entries; unpaired t-test, wild-type n=14, triple-null n=18, *P*>0.05 in all three parameters) or the elevated plus maze (Figure 5.7; parameters analysed were the total distance moved, the duration spent in the open arms and the mean frequency of open arm entries; unpaired t-test, wild-type n=14, triple-null n=18, *P*>0.05 in all three parameters). This suggests that synuclein triple-null mice do not exhibit any overt deficits in motor performance or differences in anxiety.

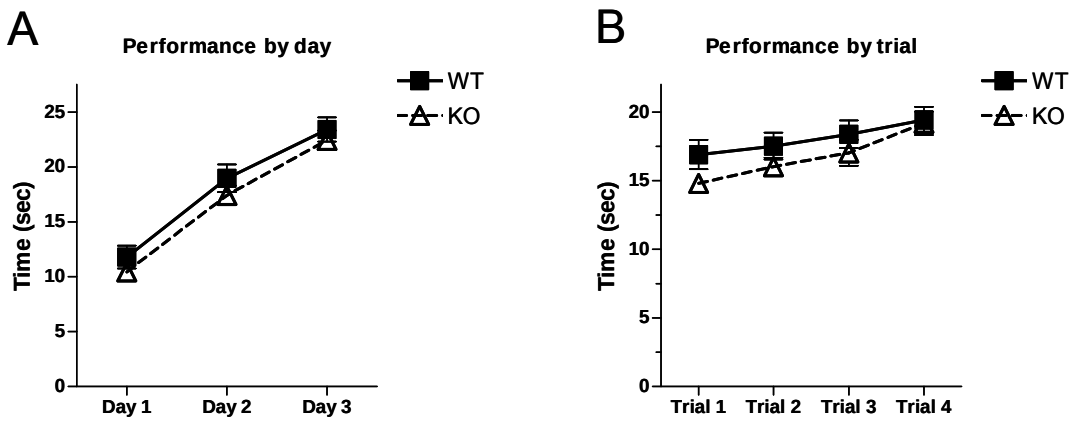


Figure 5.4. ***Performance of synuclein triple-null mice on rotating rod test*** (A) There were no significant differences with genotype on performance on the rotating rod test when analysed (A) by day (repeated measures ANOVA, wild-type n=14, triple null n=18, *P>0.05*), or (B) by trial (repeated measures ANOVA, wild-type n=14, triple null n=18, *P>0.05).*

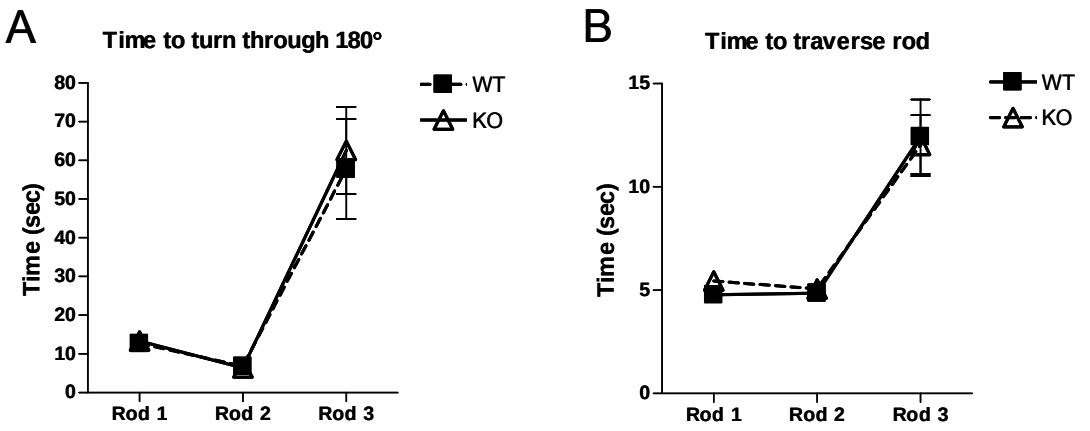


Figure 5.5. ***Performance of synuclein triple-null mice on the multiple static rods test*** (A) There were no significant differences with genotype in the time taken to turn through 180º (two-way ANOVA, wild-type n=14, triple null n=18, *P>0.05*), or (B) in the time taken to traverse the rods in the multiple static rods test (two-way ANOVA, wild-type n=14, triple null n=18, *P>0.05).*

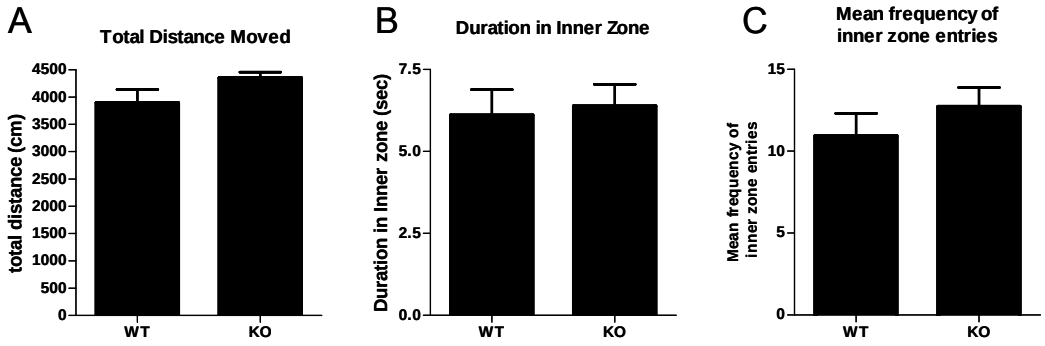


Figure 5.6. ***Activity of synuclein triple-null mice in the bright-open field test*** (A) There were no significant differences with genotype in the total distance moved in the bright open field test (unpaired t-test, wild-type n=14, triple-null n=18, *P*>0.05). (B) There were no significant differences with genotype in the duration spent in the inner zone in the bright open-field test (unpaired t-test, wild-type n=14, triple-null n=18, *P*>0.05). (C) There were no significant differences with genotype in the mean frequency of inner zone entries in the bright open-field test (unpaired t-test, wild-type n=14, triple-null n=18, *P*>0.05).

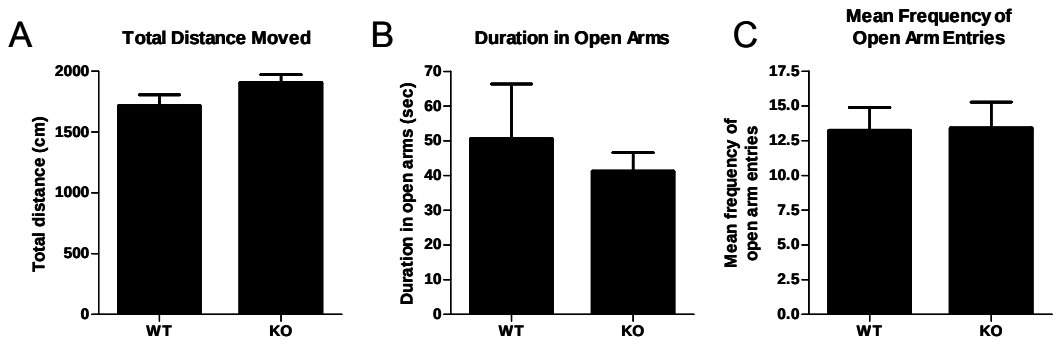


Figure 5.7. ***Activity of synuclein triple-null mice in the elevated plus maze test***
(A) There were no significant differences with genotype in the total distance moved in the elevated plus maze test (unpaired t-test, wild-type n=14, triple-null n=18, $P>0.05$). (B) There were no significant differences with genotype in the duration spent in the open arms in the elevated plus maze test (unpaired t-test, wild-type n=14, triple-null n=18, $P>0.05$). (C) There were no significant differences with genotype in the mean frequency of open arm entries in the elevated plus maze test (unpaired t-test, wild-type n=14, triple-null n=18, $P>0.05$).

These behavioural results are similar to previous reports on mice lacking both α- and γ-synuclein, or DAT knockout mice, that are significantly hyperactive in a novel environment, but not in the home cage (Senior et al., 2008; Zhuang et al., 2001). In both studies, the mice also exhibited elevated levels of extracellular DA. Therefore, to investigate the similarly observed behavioural phenotype, potential changes in DA transmission in synuclein triple null mice were assessed.

5.2.2. Increased levels of electrically evoked dopamine in the dorsal striatum of synuclein triple-null mice

To investigate this hyperdopaminergic-like behaviour, we explored the release of DA in the dorsal (caudate putamen; CPu) and ventral (nucleus accumbens; NAc) striatum using carbon-fibre microelectrodes in synuclein triple-null mice and wild-type control mice. Firstly, we identified $[DA]_o$ evoked by single stimulus pulses

and by burst stimuli (4 pulses, 100 Hz). In the CPu, mean peak $[DA]_o$ evoked by either stimulus were ~1.5-fold greater in synuclein triple-null mice compared to wild-type control mice across a large number of recording sites (Figure 5.8A; single stimulus data in CPu; one-way ANOVA, wild-type *n*=81, triple-null *n*=86, 5 animals for each genotype, *P<0.001*; Figure 5.8B, burst stimuli data in CPu; one-way ANOVA, wild-type *n*=33, triple-null *n*=30, 5 animals for each genotype, *P<0.001*). The modal value and range of evoked $[DA]_o$ is also greater (Figure 5.8C).

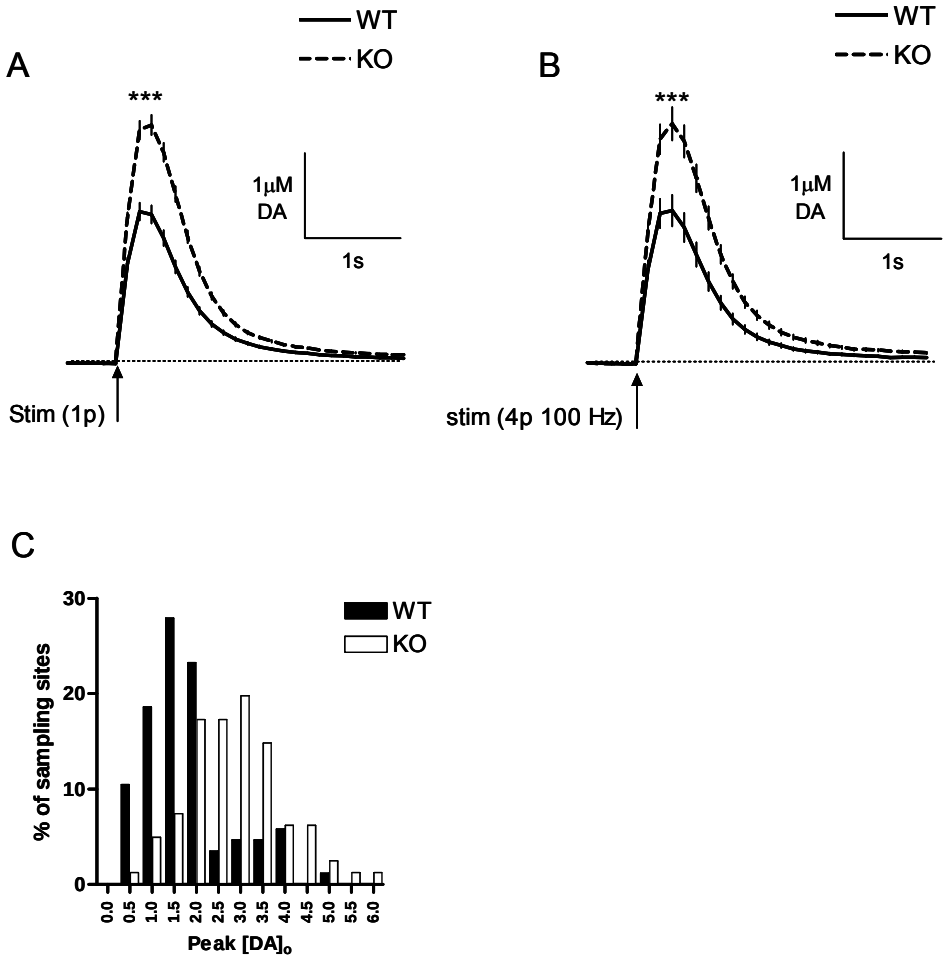


Figure 5.8. ***Electrically evoked DA transients in CPu in synuclein triple-null and wild-type mice measured by FCV.*** (A) Mean profiles of [DA]o vs. time (mean ± SEM) after a single pulse (0.2μs, arrow) in the CPu. Synuclein triple-null mice show increased levels of electrically evoked dopamine transients at synapses in the CPu relative to wild-type control mice (one-way ANOVA, wild-type n=81, triple-null n=86 [5 animals for each genotype] ***P<0.001); (B) Mean profiles of [DA]o vs. time (mean ± SEM) after 4 pulses at 100 Hz in the CPu. Synuclein triple-null mice show increased levels of electrically evoked dopamine transients at synapses in the CPu relative to wild-type control mice (one-way ANOVA, wild-type n=33, triple-null n=30 [5 animals for each genotype] ***P<0.001); (C) Distribution of peak evoked DA transients in the CPu in wild-type and triple-null mice for individual sampling sites.

By contrast, parallel experiments in the Cragg lab showed that in the NAc no difference in evoked $[DA]_o$ was detected between the two genotypes (Figure 5.9A; single pulse data in NAc; one-way ANOVA, wild-type *n*=26, triple-null *n*=26, 4

animals per genotype, *P>0.05*); Figure 5.9B; burst pulse data in NAc; one-way ANOVA wild-type *n*=13, triple-null *n*=13, 4 animals per genotype, *P>0.05*). The experiments for Figure 5.9 were performed by Dr Sarah Threlfell, and are included in this section to complement the results reported in the CPu.

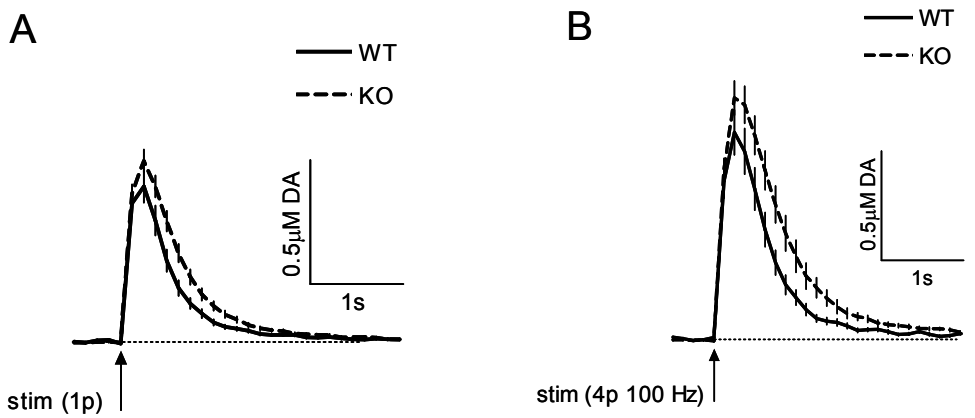


Figure 5.9. ***Electrically evoked DA transients in NAc in synuclein triple-null and wild-type mice measured by FCV.*** (A) Mean profiles of [DA]o vs. time (mean ± SEM) after a single pulse (0.2µs) in the NAc. There is no significant differences between genotype in levels of electrically evoked dopamine transients at synapses in the NAc (one-way ANOVA, wild-type n=26, triple-null n=26 [4 animals per genotype] P>0.05); (B) Mean profiles of [DA]o vs. time (mean ± SEM) after 4 pulses at 100 Hz in the NAc. There is no significant differences between genotype in levels of electrically evoked dopamine transients at synapses in the NAc (one-way ANOVA, wild-type n=13, triple-null n=13 [4 animals per genotype] P>0.05).

Secondly, we explored the frequency sensitivity of DA release, using 5-pulse trains at frequencies ranging from 1 to 100 Hz. $[DA]_o$ varied with frequency according to a slight inverted U-relationship in both the CPu and NAc similarly in both genotypes as previously published in wild-type mice (Exley et al., 2008) (Figure 5.10A (CPu); Figure 5.10B (NAc)).

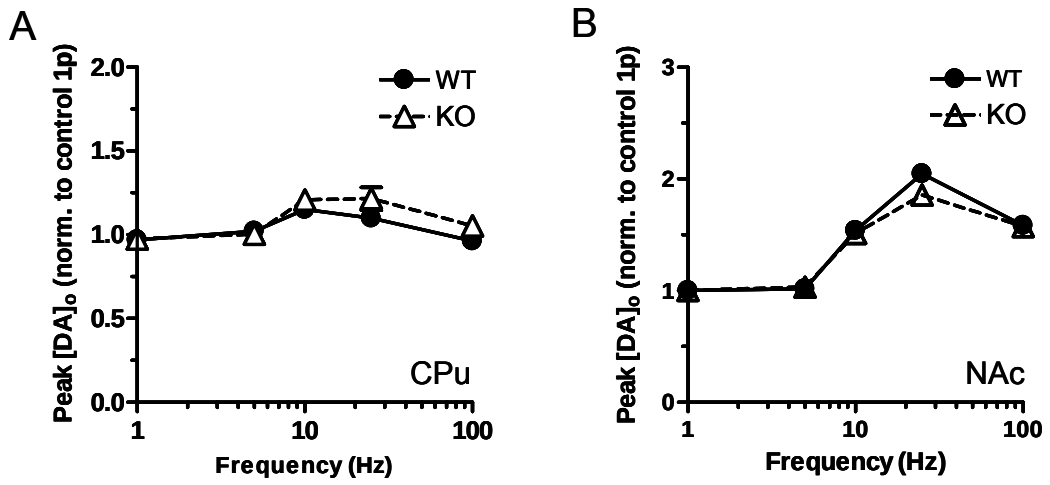


Figure 5.10. ***Regulation of DA signals by firing frequency in synuclein triple-null and wild-type mice measured by FCV.*** Mean peak [DA]o SEM± vs. frequency during 5 pulse trains (1-100 Hz) in the dorsal (CPu) striatum (A), and the ventral (NAc) (B), in both genotypes in control conditions (two-way ANOVA, 3 animals per genotype for both regions, P>0.05).

5.2.3. Decreased dopamine content in the dorsal striatum of synuclein triple-null mice

To check whether observed differences in evoked dopamine concentration in two striatal regions correlate with different steady-state dopamine content we dissected CPu and NAc from the striatal slides used for FCV and analysed extracted neurochemicals by HLPC. In the CPu, DA content was ~30% significantly lower in synuclein triple-null mice compared to the wild-type controls (Figure 5.11A, unpaired t-test, wild-type n=7, synuclein triple-null n=7 P<0.05). In the NAc however, there were no statistically significant differences in DA content between the two genotypes (Figure 5.11B, unpaired t-test, wild-type n=4, synuclein triple-null n=4 P=0.83). For the CPu, these data are paradoxical. Despite the hyperdopaminergic-like phenotype observed in synuclein triple null mice, CPu DA

content is lower. The dopamine content data are consistent with parallel results from the Buchman laboratory, in which analysis of samples of dorsal striatum dissected directly from the mouse brain demonstrated an approximate 40% lower DA content in the CPu in synuclein triple null mice compared with wild-type control mice.

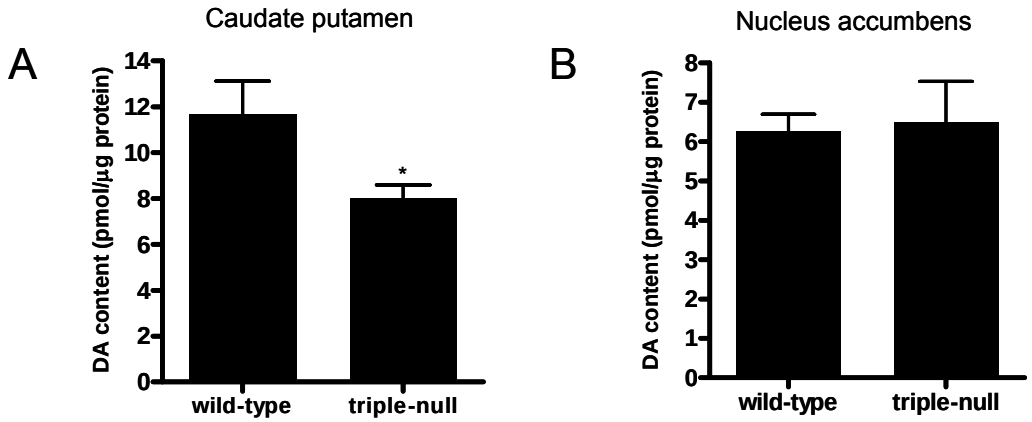


Figure 5.11. ***Striatal dopamine (DA) content in synuclein triple-null and wild-type mice measured by HPLC.*** (A) Triple-null mice exhibited a significantly decreased DA content in the caudate putamen compared with wild-type controls (unpaired t-test, wild-type n=7, triple-null n=7 (*P<0.05). (B) There was no significant difference with genotype in the nucleus accumbens in DA content (unpaired t-test, wild-type n=4, triple null n=4, P=0.8354).

5.2.4. Increased levels of electrically evoked dopamine in the dorsal striatum of synuclein triple-null mice is not due to differences nicotinic receptor function

Since DA content is approximately 30% lower, and release is ~1.5-fold greater, these data indicate deletion of all three synucleins results in a more than 2-fold increase in the releasability of the available DA. Furthermore, this change in DA

synapse function occurs in the CPu, but not in the NAc. To further assess the apparent changes in DA releasability between the two genotypes in the CPu, we assessed candidate mechanisms that might be responsible. nAChR's play a critical role in regulating DA release probability (Rice and Cragg, 2004; Zhang and Sulzer, 2004; Zhou et al., 2001). We assessed the regulation of DA release in wild-type and synuclein triple-null mice across a range of stimulation frequencies. In both genotypes, nAChR antagonist DHβE reduced $[DA]_o$ evoked by low stimulation frequencies (<25 Hz) but enhanced $[DA]_o$ evoked by higher frequencies (>25Hz) in the CPu in a manner to that previously reported (Figure 5.12). Therefore, the observed increase in DA releasability in synuclein triple-null mice in the CPu appears not to be attributable to a difference in nicotinic receptor function.

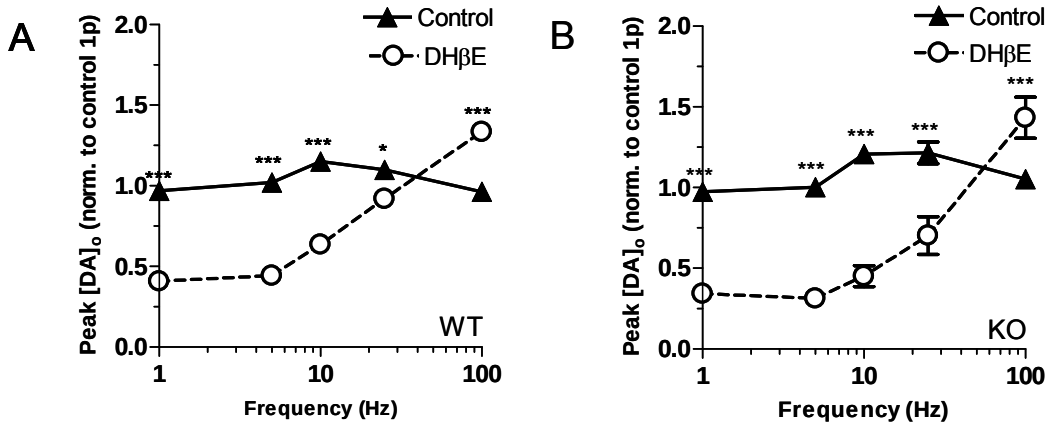


Figure 5.12. ***Cholinergic regulation of DA release in the CPu of in synuclein triple-null and wild-type mice measured by FCV.*** (A) Mean peak $[DA]_o$ SEM± vs. frequency during 5 pulse trains (1-100 Hz) in the dorsal striatum (CPu) of wild-type mice in the presence of DHβE normalised to $[DA]_o$ under control conditions (two-way ANOVA, 3 animals per genotype, *$P<0.05$ at 25 Hz; ***$P<0.001$at all other frequencies); (B) Mean peak $[DA]_o$ SEM± vs. frequency during 5 pulse trains (1-100 Hz) in the dorsal striatum of synuclein triple-null mice in the presence of DHβE normalised to $[DA]_o$ under control conditions (two-way ANOVA, 3 animals per genotype, ***$P<0.001$ at all frequencies).

5.2.5. Increased levels of electrically evoked dopamine in the dorsal striatum of synuclein triple-null mice is not due to differences in uptake of DA

Functional interactions between α-synuclein and the DAT have been reported previously (Fountaine et al., 2008; Fountaine and Wade-Martins, 2007; Lee et al., 2001; Wersinger et al., 2003; Wersinger and Sidhu, 2003). Therefore, we investigated whether the observed increase in evoked $[DA]_o$ in synuclein triple-null mice could be attributed to a reduction in DA uptake. We compared the extracellular DA transients from both genotypes which had been matched for peak $[DA]_o$ (1.0 ± 0.2 μM). The decay phase of the transients is a function of DA uptake by DAT (Giros et al., 1996) which allows ready comparison of any alterations in uptake between the two genotypes. The rate of decay was similar for both genotypes (Figure 5.13, two-way ANOVA, wild-type n=8, triple-null n=7, P>0.05). This suggests the rate of reuptake was not different in synuclein triple-null mice compared to wild-type control mice.

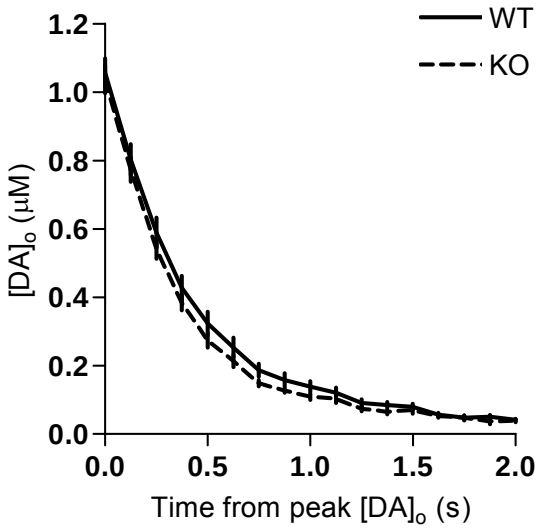


Figure 5.13. ***DA uptake in synuclein triple-null and wild-type mice measured by FCV.*** Comparison of the rates of decay of concentration-matched DA transients suggests that DA uptake rates are not significantly different between the two genotypes (two-way ANOVA, wild-type n=8, triple null n=7 $P>0.05$).

5.2.6. Increased levels of electrically evoked dopamine in the dorsal striatum of synuclein triple-null mice is not due to calcium dependent changes in exocytosis

It has been previously proposed that α-synuclein may act to modify the calcium (Ca^{2+})-dependence of presynaptic neurotransmitter release (Abeliovich et al., 2000). Therefore, we assessed whether the increase in evoked $[DA]_o$ in triple synuclein null mutant mice may be due to changes in the sensitivity of the presynaptic exocytotic machinery to Ca^{2+} caused by the absence of synuclein proteins. We investigated the effect of varying extracellular Ca^{2+} on evoked DA release in both genotypes in the presence of Ca^{2+} concentrations ranging from 0.6 mM to 4.8 mM. Experiments were conducted in the presence of a nAChR antagonist DHβE to ensure any responses seen could be attributed solely to the

activity of DA synapses and not due to any potential Ca^{2+}-mediated effects on cholinergic activity. As predicted, $[DA]_o$ evoked by a single pulse was strongly calcium dependent in both genotypes in a manner that was not significantly different (Figure 5.14, two-way ANOVA, wild-type *n*=4, triple-null *n*=4, P>0.05). We also explored the ratio of release evoked by four pulses at 100 Hz versus a single pulse to identify whether there were differences between genotypes in Ca^{2+} dependent control of release probability during bursts of stimulations. The ratio of $[DA]_o$ evoked by 4p:1p decreased as expected with $[Ca^{2+}]_o$ but in a manner that was not variable with genotype (Figure 5.14, 4p:1p, right hand axis; two-way ANOVA, wild-type *n*=4, triple-null *n*=4, P>0.05).

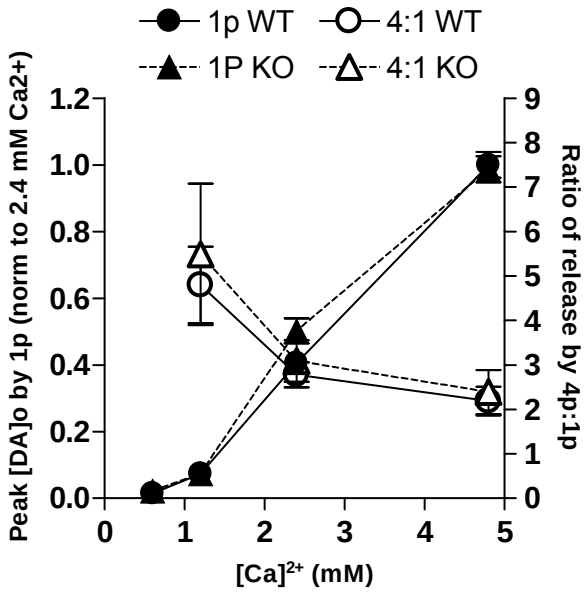


Figure 5.14. ***Calcium dependence in synuclein triple-null and wild-type mice measured by FCV.*** Response of mean peak $[DA]_o$ evoked by a single pulse in the presence of increasing calcium concentrations (0.6mM to 4.8mM) in the dorsal striatum did not significantly differ between genotypes (two-way ANOVA, 4 animals per genotype, *P>0.05*); ratio of release by a burst (4p, 100 Hz) vs. a single pulse (right hand y-axis) as a function of calcium concentration for both genotypes two-way ANOVA, 4 animals per genotype, *P>0.05*).

5.3. Discussion

This Chapter describes the results of the combined behavioural and neurochemical characterisation of midbrain dopaminergic systems in a novel mouse model lacking all three synucleins. Synuclein triple-null mice exhibit a hyperactive phenotype in a novel environment and an overall increased exploratory phenotype compared to their wild-type counterparts. The mice also display an approximate 1.5-fold increase in electrically evoked DA release in the CPu region of the striatum, an increase not seen in the adjacent NAc. This increased release occurs despite a ~30-40% lower DA content in the CPu, a decrease not seen in the NAc. Therefore, observed changes in DA synapse function caused by the deletion of the synuclein proteins is restricted to the dorsal region of the striatum, the region selectively affected in PD.

In a number of parallel collaborative studies, results from the Buchman and Bolam laboratories (University of Cardiff and Anatomical Neuropharmacology Unit (ANU), University of Oxford, respectively) have produced additional data which are integral to the discussion of the results described in this Chapter. These data are summarised below.

(A) Biochemical and anatomical analysis

TH^+-immunostaining combined with stereological counting revealed no differences in the number of midbrain DA neurons in either the SNpc or VTA of synuclein triple-null mice compared to wild-type controls. Further, striatal immunostaining with markers of DA terminals, TH and DAT, and quantitative western blot analysis of striatal tissue proteins did not show any differences in synaptic morphology or

protein levels. In addition to the significantly decreased CPu DA content in synuclein triple-null mice reported here, the levels of the major DA metabolites, DOPAC and HVA were assessed by HPLC in the Buchman laboratory. Results revealed a significantly increased DOPAC/DA ratio and a slightly increased HVA/DA ratio. These occurred in the absence of neuronal loss or obvious biochemical or morphological changes in DA synapses, as confirmed by EM analysis and summarised below. These findings suggest synuclein triple-null mice may exhibit dysfunction in the regulation of DA availability in the nigrostriatal system, since metabolite:DA ratios are often used to indicate that a greater percentage of DA is "turned over" or is released from vesicular stores with access to extracellular and cytosolic metabolic pathways.

(B) Behavioural pharmacology analysis

Data from the Buchman laboratory support the view that presynaptic striatal DA stores are lowered in synuclein triple null mice and argue against a hypothesis of upregulation of postsynaptic transduction mechanisms rather than enhanced presynaptic DA releasability. Administration of 4 mg/kg AMPH, which displaces DA stored in presynaptic vesicles into the extracellular space and whose effects are dependent on the size of presynaptic DA stores, but not exocytosis, had a much less pronounced, and a slower effect on locomotor activity in synuclein triple-null mice than in wild-type controls. However, this activity was significantly greater in synuclein triple null mice, compared to wild-type controls, when the animals were first placed in a novel environment. In contrast, administration of 10 mg/kg cocaine, which acts to block DA uptake but does not induce reverse transport of DA from presynaptic terminal stores, synuclein triple null mice exhibited a similar locomotor

response to wild-type control mice. The effects of dAMPH and cocaine on synuclein triple null mice therefore support the view that presynaptic DA terminals in these mice have lower levels of DA stored in synaptic vesicles. Further, enhancing presynaptic DA storage by administration of 50 mg/kg methyl L-DOPA twenty minutes before placing the mutant mice in a novel non-anxiogenic environment did not change the behaviour of synuclein triple-null mice prior to AMPH administration but restored their level of dAMPH-induced locomotor activity to that of wild-type mice. In addition, direct activation of postsynaptic D1/D2 receptors by apomorphine (APO) showed that a transiently induced 'climbing' behaviour was slightly less marked in synuclein triple null mice compared to wild-type control mice. This suggests that postsynaptic DA signalling may in fact be modestly attenuated in these mice, which is similar to data reported on mice exhibiting a hyperdopaminergic-like behavioural phenotype. These may reporesent mechanisms in which downregulation of postsynaptic D1/D2 heteroreceptors compensates for increased DA levels in the synaptic cleft (Zhuang et al., 2001).

(C) Electron microscopy analysis

Quantitative electron microscopic analysis of dopaminergic profiles in the dorsal striatum of 17 month old synuclein triple-null mice and wild-type controls on TH^+-immunostained sections was performed in the Bolam laboratory. Although synuclein triple-null mice showed trends towards reduction in some parameters, such as the cross sectional area of TH-positive profiles and the number of synaptic vesicles per profile, there were no significant differences in the ultrastructure of dopaminergic synapses in the striatum between the two genotypes. This suggests synucleins do not have an essential role in maintaining the gross ultrastructure of striatal DA synapses.

(D) Analysis of SNARE complexes

A number of recent *in vivo* studies have reported that synucleins may regulate neurotransmitter release through regulation of SNARE complex assembly, and/or the distribution of synaptic SNARE proteins within synapses (Burre et al., 2010; Garcia-Reitbock et al., 2010). However, these data are not entirely consistent and suggest that different synapses may respond differently to changes in the availability of synuclein proteins. Most recently, Burre and colleagues reported that in total brain lysates from aged synuclein triple-null mice, there was an attenuated assembly of SNARE complexes. Experiments performed in the Buchman laboratory therefore assessed whether SNARE complex assembly in the dorsal striatum of synuclein triple null mice was altered. Quantitative analysis of the amount of vSNARE VAMP2/synaptobrevin which co-immunoprecipitated with tSNARE SNAP-25 showed no difference between synuclein triple-null mice and their wild-type counterparts. Further, there were no observed differences between the two genotypes in the distribution of SNAP-25, or of CSP, a co-chaperone protein involved in SNARE complex assembly in the striatum. One possible reason for the contrasting results between the two groups is the sample preparation used – Burre and colleagues report results from whole brain lysates while the observations reported by the Buchman laboratory are from brain samples from the dorsal striatum.

(E) Analysis of MPTP toxicity

Dopaminergic neurons in the SNpc of α-synuclein, γ-synuclein or double α/γ-synuclein null mice, reportedly exhibit a complete or partial resistance to MPTP-induced neurotoxicity (Dauer et al., 2002; Drolet et al., 2004; Fornai et al., 2005;

Robertson et al., 2004; Schluter et al., 2003). Therefore, the Buchman laboratory conducted experiments to assess the sensitivity of midbrain DA neurons to MPTP in mice lacking all three synuclein proteins. Surprisingly, in contrast to α-synuclein, γ-synuclein or double α/γ-synuclein null mice, triple synuclein-null mice were sensitive to sub-chronic administration of MPTP (described previously in (Robertson et al., 2004)), exhibiting a significant loss of TH-positive neurons (56.0±3.81%) compared to wild-type mice (35.4±4.52%). These results indicate that, in contrast to previous reports, the synuclein proteins do not appear to be required for MPTP-induced death of SNpc dopaminergic neurons.

In summary, these collaborative data together reveal that in mice lacking all three synuclein proteins, substantial changes in behaviour and in DA biology in midbrain synapses occur in the absence of overt structural alterations of SNpc DA neurons or their synapses in the CPu. These data suggest that synucleins limit vesicular DA transmission through mechanisms that differ from those reported in other neurons and reveal key differences that vary with dopaminergic neuron subtype.

Synucleins role in regulating DA stores and metabolism

The results suggest synuclein proteins have an important role in DA metabolism in presynaptic terminals. Synuclein triple null mice have significantly lower DA content and a higher DOPAC/DA ratio in the CPu compared to wild-type, single synculein-null, or double synuclein-null mice. Similar reductions in striatal DA content have only been observed in aged (≥ two-years old) α-synuclein knockout mice (Al-Wandi et al., 2010), in α/β-synuclein null mice, where small decreases were observed (Chandra et al., 2004), and also in one line of α-synuclein knockout mice (Abeliovich

et al., 2000). Together with the attenuated responses of synuclein triple null mice to amphetamine, but not cocaine, and reversal of the locomotor effects of amphetamine by L-DOPA pre-treatment, these data suggest that DA stores were decreased in striatal synaptic terminals of these mice. These observations indicate all three synuclein proteins are involved in DA turnover in striatal synaptic terminals; and that while the absence of one or two synucleins can be at least partially or temporarily compensated for, loss of all three leads to the greatest reduction in the capacity of synapses to store DA. In conjunction with the EM data, that vesicle number is not modified, the reduced tissue DA content suggests that in synuclein triple-null mice less DA is stored per vesicle. The cause of the lower DA content is uncertain. There were no significant differences in the number of TH-positive neurons, levels of synaptic proteins, or densities of immunostaining for synaptic markers between synuclein triple-null mice and wild-type controls. Nor were there differences in the frequencies of TH-positive synaptic profiles or numbers of vesicles in the striatum between the two genotypes, as assessed by EM. These results indicate there is no loss of midbrain DA neurons, synapses or synaptic vesicles in synuclein triple null mice. No differences were detected in the level of expression or the *in vivo* activity of TH. Furthermore, DAT function, as measured by FCV, appeared normal. Therefore, while DA synthesis, reuptake and vesicle availability appear normal, it is possible that the lower DA content in the CPu of synuclein triple-null mice may be due to a compromised ability of VMAT2 to sequester DA into synaptic vesicles. This suggestion is consistent with the significantly increased DOPAC/DA ratio, which is an indicator of DA turnover. Compromised VMAT2 function may also explain the observation that reduced DA content is more profound in the CPu than NAc, since the

VMAT2/DAT ratio is lower in SNpc neurons projecting to the CPu, than VTA neurons projecting to the NAc (Miller et al., 1999; Uhl, 1998).

Behavioural alterations in synuclein triple null mice

In agreement with numerous preclinical and clinical reports, the ~30% lower striatal DA content was not sufficient to cause behavioural changes in the synuclein triple null mice commonly associated with DA deficiency, such as hypoactivity and hypophagia (Szczypka et al., 1999; Zhou and Palmiter, 1995). Performance of adult synuclein triple-null mice in behavioural tests of motor activity and anxiety were not significantly different from that of their wild-type counterparts. Interestingly, in several behavioural paradigms, synuclein triple-null mice exhibited behaviour reminiscent of an increased, rather than a decreased level of striatal DA. In particular, they exhibited an increased response to novelty; for example, the mice demonstrated increased locomotion in a novel environment in the non-anxiogenic open-field test, when compared with wild-type control mice. These behavioural changes are similar to the observed behavioural phenotype of α-, γ-synuclein double-null mice (Senior et al., 2008) and of that observed in hyperdopaminergic mice expressing reduced levels of DAT (Zhuang et al., 2001), and again imply an important role for synucleins in regulating synaptic DA activity. While the observed behavioural phenotype in synuclein triple-null mice does not readily reconcile with the lower DA content, it is consistent with the increase in evoked DA release.

Synucleins and DA release

As mentioned, the behavioural phenotype of synuclein triple-null mice is consistent with the observed significant increase in evoked DA release in the CPu, measured by

FCV. This increase is remarkably similar to that seen in a study by Senior et al., in which a two-fold increase in electrically evoked DA transients was observed at striatal synapses in α-, γ-synuclein double-null mice (Senior et al., 2008). A number of candidate mechanisms for the causes of the observed increase in extracellular DA concentration in synuclein triple-null mice were assessed in this Chapter. However, the observed increase is not caused by upregulation of nAChR function, or by increased sensitivity of the presynaptic exocytotic machinery to calcium. Further investigations revealed no increases in DA release probability (as determined by measurements of frequency-sensitivity which should be inversely correlated with release probability (Rice and Cragg, 2004)), nor a decrease in the rate of uptake of DA via the DAT in synuclein triple-null mice compared to wild-type control mice. Moreover, when the lower DA content is accounted for, the releasability of the available DA from presynaptic terminals in the CPu is two times greater in the absence of synuclein proteins.

There are a number of possible explanations for the observed release phenotype in synuclein-triple null mice. Most interestingly, a recent study by Greten-Harrison and colleagues reported augmented electrophysiological responses in hippocampal CA1 synapses in young (3 m) synuclein-triple null mice (Greten-Harrison et al., 2010). These mice showed a larger input (stimulus strength)-output (fEPSP slope) relationship (I/O), even when plotted as a function of fibre velocity amplitude (which represents the action potential (AP) firing of Schaffer collaterals), compared with age-matched wild-type control mice. Intriguingly however, in contrast to young mice, aged mice (12 m) showed diminished I/O ratios compared with wild-type controls, and when plotted as a function of fibre velocity amplitude, there were no differences

between the two genotypes in the aged animals. The observations by Greten-Harrison and colleagues support the results in this Chapter, indicating that in young adult synuclein triple-null mice, transmitter release may actually be increased, while in older animals this enhancement is diminished by changes in AP conduction and/or excitability. Greten-Harrison and colleagues also demonstrate that α-synuclein expression can completely restore the synuclein triple null I/O curve to the level of wild-type controls, confirming that synuclein proteins affect basal neurotransmission, and providing further support the argument that deleting synucleins may enhance synaptic neurotransmission in young mice. The enhanced I/O curves reported in young synuclein triple-null mice were not due to changes in release probability, or changes in conduction velocity (related to changes in axonal structure), observed in their study in aged synuclein triple-null mice. However, in contrast with the results from the Buchman laboratory discussed in this Chapter, these mice showed increases in key proteins which regulate vesicle exocytosis, including SNARE proteins complexin I and II and synapsins. Notably also, the proteins which were upregulated in young mice were decreased to wild-type levels, or even diminished, in aged mice. The enhanced releasability may therefore result from the interaction of synuclein proteins with key regulatory elements involved in promoting synaptic transmitter exocytosis. The results from Greten-Harrison et al., which support an enhanced transmitter release in hippocampal neurons, were only observed in young (3 m) mice, but not in older (12 m) mice. The increase in evoked DA release by FCV in the CPu, described in this Chapter, was observed in adult triple synuclein mice aged 9-12 months. A possible reason for the discrepancy may be the different brain regions studied. Since biochemical and morphological distinctions occur between neuron types, it is possible SNpc DA neurons may exhibit delayed changes with age in the

synaptic protein composition and axonal structure in CA1 neurons in synuclein triple-null mice. Therefore, experiments examining effects on evoked DA release in the dorsal striatum in younger (approx 3-6 m) and older (approx 18-24 m) synuclein triple null mice represent an important avenue of future investigation.

There are a number of other potential explanations, which are not mutually exclusive, which may underlie the enhanced releasability from presynaptic terminals with diminished DA content in synuclein triple-null mice. These include an increase in the number of vesicles released, the size of the readily releasable pool versus the reserve pool, and/or the rate of vesicle fusion to the presynaptic membrane. However, ultrastructural analysis does not corroborate this explanation, as no significant differences in the number of synaptic vesicles per dopaminergic profile in the CPu in synuclein triple-null mice and wild-type mice were observed. Instead, these data, together with lower tissue DA content, suggest that vesicles in the CPu of synuclein triple-null mice may have a lower DA vesicular load. That said, even with fewer 'loaded' synaptic vesicles, increased DA releasability could still be achieved if vesicular recycling was dysregulated, leading to an increased readily releasable pool at the expense of the reserve pool. Such a vesicle pool redistribution would be consistent with a recent report by Nemani and colleagues, who observed that modest overexpression of α-synuclein in both cultured neurons and transgenic mice leads to a reduced recycling pool size and inhibition of synaptic vesicle exocytosis (Nemani et al., 2010). Notably, although this inhibitory effect was reportedly seen in cultured midbrain dopaminergic neurons, much of the data from this study was obtained from experiments in hippocampal synapses, which are both morphologically and functionally distinct from striatal dopaminergic synapses. Striatal dopaminergic

synapses lack well-defined, spatially distinct clusters of reserve and recycling vesicles, making ultrastructural analysis much more challenging. Indeed, while the ultrastructural analyses performed by the Bolam laboratory in synuclein triple null mice did not reveal any significant differences in synaptic vesicle distribution in the CPu, a trend towards a closer proximity of vesicles to the active zone, despite a lower vesicle density, was observed.

Another possible explanation for the observed increase in DA releasability from synapses in the CPu in synuclein triple-null mice may be an increased rate of vesicle fusion and/or release of DA, in which SNARE protein complexes play a pivotal role (summarised in (Rizo and Rosenmund, 2008; Sudhof and Rothman, 2009)). This explanation is supported by Greten-Harrison and colleagues, who report increases in key proteins regulating synaptic vesicle exocytosis, discussed above (Greten-Harrison et al., 2010). In contrast however, the results from a recent study by Burre and colleagues which explored the role of synuclein proteins in SNARE complex formation, and included studies with synuclein triple-null mice, do not support this explanation (Burre et al., 2010). This group reported that α-synuclein promotes assembly of SNARE complexes that mediate synaptic vesicle fusion in an activity-dependent manner via an interaction with vesicular SNARE protein VAMP2/synaptobrevin. Further, they observed that aged synuclein triple-null mice formed SNARE complexes much less robustly, and had lower levels of VAMP2/synaptobrevin, relative to young adult synuclein triple-null mice. Moreover, restoring α-synuclein in cultured neurons from these mice helped re-establish SNARE complexes in a dose-dependent manner. The findings by Burre and colleagues contrast with the findings described in this Chapter, which support the view that

synuclein proteins have a role in the inhibition of transmitter release - a view supported by a growing body of evidence highlighted in the Chapter Introduction. In further contrast to the results by Burre et al., immunoprecipitation experiments on synuclein triple-null mice and wild-type controls by the Buchman group revealed no differences between the two genotypes in the levels of vSNARE VAMP2/synaptobrevin.

Synuclein proteins do not appear to be essential for the formation and function of SNARE complexes, since efficient SNARE-dependent fusion of membranes readily occurs in tissues which do not express synucleins, and in invertebrates which lack synucleins. It remains feasible, however, that the presence or absence of synucleins might affect formation of a fusion pore and the fate of a fused vesicle, i.e. kiss-and-run or full-collapse fusion with the presynaptic membrane. An increase in DA release may therefore arise from the release of a greater proportion of contents of each vesicle or the number of DA quanta released per stimulus. Further detailed studies are therefore required to understand if and why any of these mechanisms differ in nigrostriatal, compared to mesolimbic, neurons. Further, the possibility that changes to DA release in the absence of synucleins reflect consequences of the developmental compensation of the nigrostriatal neurons for the absence of synucleins cannot be excluded.

Indeed, an alternative explanation to synucleins having an inhibitory role in neurotransmitter release, which may also reconcile the contrasting results from Burre and colleagues, is that while synaptic vesicle exocytosis becomes compromised in ageing synuclein triple-null mice, robust plasticity in the dopaminergic system applies

an alternative molecular mechanism to achieve compensation for their normal function. Indeed, the Buchman group reported that, in contrast to other brain regions, the abundance of SNARE complexes is not reduced in the CPu in synuclein triple-null mice. Previous reports of SNAP-25 or VAMP2/synaptobrevin mutant mice have demonstrated a remarkable ability of neuronal synapses to adapt and partially restore the function of presynaptic release machinery. It is possible that increased DA releasability in the CPu is the result of such compensation, which may itself be impaired by the absence of synucleins (leading to an 'over'-compensation, and increased releasability). This is plausible, given the documented, but not well defined, role for α-synuclein in synaptic plasticity. With this in mind, elucidating the molecular mechanisms which functionally compensate for the loss of synuclein proteins will drive future experiments with synuclein triple null mice. These studies will help provide an improved understanding of DA neurotransmission in the SNpc and may benefit future research into alternative therapeutic targets for PD. Furthermore, experiments to elucidate the exact mechanisms leading to increased DA releasability are required. For example, the rate of fusion of synaptic vesicles could be explored by labelling vesicles with fluorescent dyes (Aravanis et al., 2003a; Aravanis et al., 2003b; Klingauf et al., 1998; Murthy and Stevens, 1998).

Regionally distinct effects of synuclein deletion: nigrostriatal versus mesolimbic dopamine synapses

A striking aspect of the results described in this Chapter is that the absence of synucleins leads to selective effects on presynaptic DA neurochemistry in the CPu, but not in the NAc. Therefore, the function of synuclein proteins in DA neurons may not only differ substantially between neuron transmitter subtypes (e.g. midbrain DA

neurons vs. hippocampal glutamatergic neurons), but may also be specific to neuron subtypes for a given transmitter. Since SNpc DA neurons degenerate preferentially to those innervating the ventral striatum via the mesolimbic pathway in PD, the regionally distinct effect of synuclein depletion may offer significant new insight into the preferential DA neuron degeneration in PD. While striking, the finding that a mechanism regulating DA neurotransmission might differ between nigrostriatal and mesolimbic neurons is perhaps not surprising. A large body of research suggests that nigrostriatal neurons and mesolimbic neurons differ in many features, such as in embryonic source and stage of ontogenetic development, neuroanatomy of projections and inputs, protein expression levels including DA cell markers (DAT, VMAT2, D2 receptors), ion channels and other proteins, and in their susceptibility to neurodegeneration (reviewed in (Bjorklund and Dunnett, 2007; Korotkova et al., 2004; Liss and Roeper, 2008). Synuclein function may be another feature which variably influences the function of nigrostriatal and mesolimbic neurons.

Another consideration when interpreting the regionally distinct effects of synuclein deletion is that in several behavioural tests, the mice exhibited behaviours reminiscent of increased, rather than decreased levels of striatal DA. Further, the mice also demonstrated behavioural changes traditionally associated with the ventral striatum, where no alterations in DA content or release were observed, whilst exhibiting no differences in motor behaviours traditionally associated with the dorsal striatum. A large contribution of work focusing on the functional division of the striatum comes from classical experiments by Iversen and colleagues (Alpert and Iversen, 1983; Brook and Iversen, 1975; Canales and Iversen, 1998; Iversen, 1980, 1984, 1995; Iversen and Creese, 1975; Iversen et al., 1975; Iversen and Koob, 1977; Kelly et al.,

1975). These include experiments in which 6-OHDA lesions were introduced into the CPu or NAc of the rat, followed by an examination of the effects of AMPH dosing (Kelly et al., 1975). In an unlesioned rat, low doses (1.5 mg/kg) of AMPH primarily impacted the NAc leading to hyperactivity, whereas high doses (5 mg/kg) affected the CPu, producing sterotyped behaviours. In a NAc lesioned rat however, low doses of AMPH failed to produce hyperactivity, whereas hyperactivity was induced in CPu lesioned animals. Conversely, high doses no longer produced sterotyped behaviour in CPu lesioned rats, but did so in NAc lesioned animals. Instead, the CPu lesioned animals demonstrated hyperactivity in response to high doses of AMPH. This work suggests that a competition may occur between the two striatal regions, and the absence of a functioning CPu (i.e. when it is lesioned) allows NAc-mediated behaviours, such as hyperactivity, to come to the fore. It is possible that the 30-40% reduction in DA content in CPu observed in synuclein triple null-mice is similar to a lesion, subsequently unmasking NAc-mediated behaviours. The observation that high doses of AMPH lead to hyperactivity in synuclein triple-null mice is therefore consistent with these experiments. Qualitative examination of AMPH-induced behaviours in synuclein triple-null mice with and without L-DOPA pretreatment remains an important goal for future studies.

Further, a significant degree of well-defined neuronal connectivity between the mesolimbic and nigrostriatal DA pathways has also been described (Ikemoto, 2007). This allows signals to be passed from the mesolimbic pathway in the NAc to influence the CPu via the nigrostriatal pathway (Alexander et al., 1986; DeLong, 1990) through what is proposed to be an elaborate ‘spiral’ organisation of connections between the striatum and ventral midbrain (Haber et al., 2000). This elaborate connectivity means behaviours traditionally associated with one region of the striatum

may be influenced by neuronal interactions with other striatal regions. Examining potential effects of synuclein proteins upon these interactions, which in turn influence behavioural outcomes, will be important when planning future behavioural experiments on synuclein triple null mice. For example, examining motivational behaviours traditionally associated with the ventral striatum, with tests such as rewarded alternation paradigm, may be of interest.

Future experiments with synuclein triple null mice must also focus on investigating potential mechanisms responsible for the observed regional-specificity of increased DA release, seen in the CPu, but not the NAc in the striatum. It is possible that key proteins implicated in regulating DA release and turnover, which interact with synucleins, such as SNARE proteins and VMAT2 differ between the DA neuron populations in the CPu and NAc. In particular, biochemical analysis of the levels of synaptic proteins in both CPu and NAc regions, and further immunoprecipitaton experiments examining the potential interactions of such proteins should be performed.

Despite the abundance of synucleins throughout presynaptic nerve terminals, they appear surprisingly dispensable in neuronal physiology. Synuclein single- and double-null mice present with either a minor phenotype or have alterations which do not compromise survival. Taken together with the results presented in this Chapter, synucleins seem to constitute auxillary, rather than essential, elements of presynaptic machinery. They appear important for the efficiency and plasticity of vertebrate synapses and for the normal function of midbrain dopaminergic neurons, a cell population which remains of paramount interest to research into the pathogenesis of PD.

CHAPTER 6: General Discussion

Synuclein proteins have been known to researchers for over a decade and despite this, the functions of these small, presynaptic proteins remain largely unknown. In particular, the normal and pathological functions of α-synuclein, a protein intimately involved in the pathogenesis of PD which is thought to be involved in regulating DA neurotransmission and synaptic vesicle trafficking, are not fully understood. Studies aimed at exploring the role of α-synuclein in PD have largely focussed on the use of cDNA-based transgenic models which have failed to accurately recapitulate the key pathological hallmarks of PD. In addition, the high degree of functional redundancy existing within the synuclein family limits our understanding of the functions of synuclein proteins, both individually and in combination. Therefore, this thesis addressed the need for the functional characterisation of a novel line of α-synuclein-based transgenic mice created using an alternative approach to express human α-synuclein from the complete SNCA genomic DNA locus. The thesis also addressed the need for a thorough analysis of functional alterations in the nigrostriatal system of synuclein triple knockout mice. Importantly, the phenotypic investigations on synuclein triple-null mice described in Chapter 5 did not necessarily aim to create and characterise a model of PD, but rather, to improve our understanding of the fundamental biology of synuclein proteins.

Interestingly, this thesis describes results in both synuclein-based models which are intriguingly complementary. The studies on the novel α-synuclein transgenic mice to explore the role of synuclein proteins provide evidence that the A30P mutant form of the protein described in this thesis, reveal loss of TH+ neurons specifically in the

SNpc and alterations to DA neurotransmission supporting the view that the A30P form of α-synuclein represents a gain-of-function mutation, and promotes the function of α-synuclein as a negative regulator of DA release at synapses. Moreover, this view is further supported by the studies in mice which lack all three synucleins, described in this thesis. These synuclein null mice exhibit a hyper-dopaminergic like behavioural phenotype and a corresponding two-fold increase in evoked extracellular DA concentration, consistent with a role for synuclein proteins as negative regulators of DA release. Perhaps most interestingly, in both mouse models, there is a region-specific effect on dopamine signalling kinetics specifically in the dorsal striatum, corresponding to the region of the brain selectively vulnerable in PD. This observation is unique amongst all other studies of synuclein transgenic mice and single and double syncuelin knockout mice and will no doubt be of great interest to the neuroscience community.

The results described in this thesis make an important contribution in the field of research into synuclein proteins, not least because they focus on alterations specifically in the nigrostriatal system. Since synucleins are implicated to in PD pathogenesis, and SNpc dopaminergic neurons are selectively vulnerable in PD pathology, understanding the modifications in the midbrain dopaminergic system associated with the loss of all three synuclein proteins is of fundamental importance. The outcomes in this thesis therefore provide major new insight into synuclein functions in the DA system and promise that these complex interactions will be the focus of neuroscience research for many years.

REFERENCES

Parkinson's Study Group, 2000, A randomized controlled trial comparing pramipexole with levodopa in early Parkinson's disease: design and methods of the CALM-PD Study. Parkinson Study Group: Clin Neuropharmacol, v. 23, p. 34-44.

Aarsland, D., E. Tandberg, J. P. Larsen, and J. L. Cummings, 1996, Frequency of dementia in Parkinson disease: Arch Neurol, v. 53, p. 538-42.

Abeliovich, A., Y. Schmitz, I. Farinas, D. Choi-Lundberg, W. H. Ho, P. E. Castillo, N. Shinsky, J. M. Verdugo, M. Armanini, A. Ryan, M. Hynes, H. Phillips, D. Sulzer, and A. Rosenthal, 2000, Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system: Neuron, v. 25, p. 239-52.

Ahmad, M., S. Attoub, M. N. Singh, F. L. Martin, and O. M. El-Agnaf, 2007, Gamma-synuclein and the progression of cancer: Faseb J, v. 21, p. 3419-30.

Ahn, B. H., H. Rhim, S. Y. Kim, Y. M. Sung, M. Y. Lee, J. Y. Choi, B. Wolozin, J. S. Chang, Y. H. Lee, T. K. Kwon, K. C. Chung, S. H. Yoon, S. J. Hahn, M. S. Kim, Y. H. Jo, and D. S. Min, 2002, alpha-Synuclein interacts with phospholipase D isozymes and inhibits pervanadate-induced phospholipase D activation in human embryonic kidney-293 cells: J Biol Chem, v. 277, p. 12334-42.

Al-Wandi, A., N. Ninkina, S. Millership, S. J. Williamson, P. A. Jones, and V. L. Buchman, 2010, Absence of alpha-synuclein affects dopamine metabolism and synaptic markers in the striatum of aging mice: Neurobiol Aging, v. 31, p. 796-804.

Alexander, G. E., M. R. DeLong, and P. L. Strick, 1986, Parallel organization of functionally segregated circuits linking basal ganglia and cortex: Annu Rev Neurosci, v. 9, p. 357-81.

Alim, M. A., Q. L. Ma, K. Takeda, T. Aizawa, M. Matsubara, M. Nakamura, A. Asada, T. Saito, H. Kaji, M. Yoshii, S. Hisanaga, and K. Ueda, 2004, Demonstration of a role for alpha-synuclein as a functional microtubule-associated protein: J Alzheimers Dis, v. 6, p. 435-42; discussion 443-9.

Alimova-Kost, M. V., N. N. Ninkina, S. Imreh, N. V. Gnuchev, J. Adu, A. M. Davies, and V. L. Buchman, 1999, Genomic structure and chromosomal localization of the mouse persyn gene: Genomics, v. 56, p. 224-7.

Alpert, J. E., and S. D. Iversen, 1983, Striatal and limbic dopamine function: evidence from animal studies: Ann Neurol, v. 13, p. 578.

Aravanis, A. M., J. L. Pyle, N. C. Harata, and R. W. Tsien, 2003a, Imaging single synaptic vesicles undergoing repeated fusion events: kissing, running, and kissing again: Neuropharmacology, v. 45, p. 797-813.

Aravanis, A. M., J. L. Pyle, and R. W. Tsien, 2003b, Single synaptic vesicles fusing transiently and successively without loss of identity: Nature, v. 423, p. 643-7.

Auluck, P. K., and N. M. Bonini, 2002, Pharmacological prevention of Parkinson disease in Drosophila: Nat Med, v. 8, p. 1185-6.

Bannon, M. J., 2005, The dopamine transporter: role in neurotoxicity and human disease: Toxicol Appl Pharmacol, v. 204, p. 355-60.

Baptista, M. J., C. O'Farrell, S. Daya, R. Ahmad, D. W. Miller, J. Hardy, M. J. Farrer, and M. R. Cookson, 2003, Co-ordinate transcriptional regulation of dopamine

synthesis genes by alpha-synuclein in human neuroblastoma cell lines: J Neurochem, v. 85, p. 957-68.
Baquet, Z. C., P. C. Bickford, and K. R. Jones, 2005, Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta: J Neurosci, v. 25, p. 6251-9.
Baquet, Z. C., D. Williams, J. Brody, and R. J. Smeyne, 2009, A comparison of model-based (2D) and design-based (3D) stereological methods for estimating cell number in the substantia nigra pars compacta (SNpc) of the C57BL/6J mouse: Neuroscience, v. 161, p. 1082-90.
Barbeau, A., 1962, The pathogenesis of Parkinson's disease: a new hypothesis: Can Med Assoc J, v. 87, p. 802-7.
Bellani, S., V. L. Sousa, G. Ronzitti, F. Valtorta, J. Meldolesi, and E. Chieregatti, 2010, The regulation of synaptic function by alpha-synuclein: Commun Integr Biol, v. 3, p. 106-9.
Bensadoun, J. C., N. Deglon, J. L. Tseng, J. L. Ridet, A. D. Zurn, and P. Aebischer, 2000, Lentiviral vectors as a gene delivery system in the mouse midbrain: cellular and behavioral improvements in a 6-OHDA model of Parkinson's disease using GDNF: Exp Neurol, v. 164, p. 15-24.
Biere, A. L., S. J. Wood, J. Wypych, S. Steavenson, Y. Jiang, D. Anafi, F. W. Jacobsen, M. A. Jarosinski, G. M. Wu, J. C. Louis, F. Martin, L. O. Narhi, and M. Citron, 2000, Parkinson's disease-associated alpha-synuclein is more fibrillogenic than beta- and gamma-synuclein and cannot cross-seed its homologs: J Biol Chem, v. 275, p. 34574-9.
Bjorklund, A., and S. B. Dunnett, 2007, Fifty years of dopamine research: Trends Neurosci, v. 30, p. 185-7.
Björklund, A., and T. Hoekfelt, 1984a, Classical transmitters in the CNS: Handbook of chemical neuroanatomy ; v.002: Amsterdam ; New York, Elsevier, xvii, 463 p. p.
Björklund, A., and T. Hoekfelt, 1984b, Classical transmitters in the CNS, Part I: Handbook of chemical neuroanatomy ; v. 002: Amsterdam ; New York, Elsevier, xvii, 463 p. p.
Bjorklund, L. M., R. Sanchez-Pernaute, S. Chung, T. Andersson, I. Y. Chen, K. S. McNaught, A. L. Brownell, B. G. Jenkins, C. Wahlestedt, K. S. Kim, and O. Isacson, 2002, Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model: Proc Natl Acad Sci U S A, v. 99, p. 2344-9.
Bonifati, V., P. Rizzu, F. Squitieri, E. Krieger, N. Vanacore, J. C. van Swieten, A. Brice, C. M. van Duijn, B. Oostra, G. Meco, and P. Heutink, 2003, DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism: Neurol Sci, v. 24, p. 159-60.
Bower, J. H., D. M. Maraganore, S. K. McDonnell, and W. A. Rocca, 1999, Incidence and distribution of parkinsonism in Olmsted County, Minnesota, 1976-1990: Neurology, v. 52, p. 1214-20.
Braak, H., K. Del Tredici, H. Bratzke, J. Hamm-Clement, D. Sandmann-Keil, and U. Rub, 2002, Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages): J Neurol, v. 249 Suppl 3, p. III/1-5.
Brook, C., and S. D. Iversen, 1975, Changed eating and locomotor behaviour in the rat after 6-hydroxydopamine lesions to the substantia nigra: Neuropharmacology, v. 14, p. 95-105.

Brown, R. G., and C. D. Marsden, 1984, How common is dementia in Parkinson's disease?: Lancet, v. 2, p. 1262-5.
Brown, T. P., P. C. Rumsby, A. C. Capleton, L. Rushton, and L. S. Levy, 2006, Pesticides and Parkinson's disease--is there a link?: Environ Health Perspect, v. 114, p. 156-64.
Buchman, V. L., J. Adu, L. G. Pinon, N. N. Ninkina, and A. M. Davies, 1998a, Persyn, a member of the synuclein family, influences neurofilament network integrity: Nat Neurosci, v. 1, p. 101-3.
Buchman, V. L., H. J. Hunter, L. G. Pinon, J. Thompson, E. M. Privalova, N. N. Ninkina, and A. M. Davies, 1998b, Persyn, a member of the synuclein family, has a distinct pattern of expression in the developing nervous system: J Neurosci, v. 18, p. 9335-41.
Burn, D. J., M. H. Mark, E. D. Playford, D. M. Maraganore, T. R. Zimmerman, Jr., R. C. Duvoisin, A. E. Harding, C. D. Marsden, and D. J. Brooks, 1992, Parkinson's disease in twins studied with 18F-dopa and positron emission tomography: Neurology, v. 42, p. 1894-900.
Burre, J., M. Sharma, T. Tsetsenis, V. Buchman, M. R. Etherton, and T. C. Sudhof, 2010, Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro: Science, v. 329, p. 1663-7.
Cabin, D. E., S. Gispert-Sanchez, D. Murphy, G. Auburger, R. R. Myers, and R. L. Nussbaum, 2005, Exacerbated synucleinopathy in mice expressing A53T SNCA on a Snca null background: Neurobiol Aging, v. 26, p. 25-35.
Cabin, D. E., K. Shimazu, D. Murphy, N. B. Cole, W. Gottschalk, K. L. McIlwain, B. Orrison, A. Chen, C. E. Ellis, R. Paylor, B. Lu, and R. L. Nussbaum, 2002, Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein: J Neurosci, v. 22, p. 8797-807.
Cai, J., A. Donaldson, M. Yang, M. S. German, G. Enikolopov, and L. Iacovitti, 2009, The role of Lmx1a in the differentiation of human embryonic stem cells into midbrain dopamine neurons in culture and after transplantation into a Parkinson's disease model: Stem Cells, v. 27, p. 220-9.
Calabresi, P., D. Centonze, P. Gubellini, A. Pisani, and G. Bernardi, 2000, Acetylcholine-mediated modulation of striatal function: Trends Neurosci, v. 23, p. 120-6.
Campbell, D. G., D. G. Hardie, and P. R. Vulliet, 1986, Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase: J Biol Chem, v. 261, p. 10489-92.
Canales, J. J., and S. D. Iversen, 1998, Behavioural topography in the striatum: differential effects of quinpirole and D-amphetamine microinjections: Eur J Pharmacol, v. 362, p. 111-9.
Cao, X. Q., H. Arai, Y. R. Ren, H. Oizumi, N. Zhang, S. Seike, T. Furuya, T. Yasuda, Y. Mizuno, and H. Mochizuki, 2006, Recombinant human granulocyte colony-stimulating factor protects against MPTP-induced dopaminergic cell death in mice by altering Bcl-2/Bax expression levels: J Neurochem, v. 99, p. 861-7.
Cappai, R., S. L. Leck, D. J. Tew, N. A. Williamson, D. P. Smith, D. Galatis, R. A. Sharples, C. C. Curtain, F. E. Ali, R. A. Cherny, J. G. Culvenor, S. P. Bottomley, C. L. Masters, K. J. Barnham, and A. F. Hill, 2005, Dopamine promotes alpha-synuclein aggregation into SDS-resistant soluble oligomers via a distinct folding pathway: Faseb J, v. 19, p. 1377-9.

Caughey, B., and P. T. Lansbury, 2003, Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders: Annu Rev Neurosci, v. 26, p. 267-98.
Centonze, D., C. Grande, A. Usiello, P. Gubellini, E. Erbs, A. B. Martin, A. Pisani, N. Tognazzi, G. Bernardi, R. Moratalla, E. Borrelli, and P. Calabresi, 2003, Receptor subtypes involved in the presynaptic and postsynaptic actions of dopamine on striatal interneurons: J Neurosci, v. 23, p. 6245-54.
Chan, C. S., J. N. Guzman, E. Ilijic, J. N. Mercer, C. Rick, T. Tkatch, G. E. Meredith, and D. J. Surmeier, 2007, 'Rejuvenation' protects neurons in mouse models of Parkinson's disease: Nature, v. 447, p. 1081-6.
Chandra, S., F. Fornai, H. B. Kwon, U. Yazdani, D. Atasoy, X. Liu, R. E. Hammer, G. Battaglia, D. C. German, P. E. Castillo, and T. C. Sudhof, 2004, Double-knockout mice for alpha- and beta-synucleins: effect on synaptic functions: Proc Natl Acad Sci U S A, v. 101, p. 14966-71.
Chandra, S., G. Gallardo, R. Fernandez-Chacon, O. M. Schluter, and T. C. Sudhof, 2005, Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration: Cell, v. 123, p. 383-96.
Chartier-Harlin, M. C., J. Kachergus, C. Roumier, V. Mouroux, X. Douay, S. Lincoln, C. Levecque, L. Larvor, J. Andrieux, M. Hulihan, N. Waucquier, L. Defebvre, P. Amouyel, M. Farrer, and A. Destee, 2004, Alpha-synuclein locus duplication as a cause of familial Parkinson's disease: Lancet, v. 364, p. 1167-9.
Checkoway, H., K. Powers, T. Smith-Weller, G. M. Franklin, W. T. Longstreth, Jr., and P. D. Swanson, 2002, Parkinson's disease risks associated with cigarette smoking, alcohol consumption, and caffeine intake: Am J Epidemiol, v. 155, p. 732-8.
Chen, X., H. A. de Silva, M. J. Pettenati, P. N. Rao, P. St George-Hyslop, A. D. Roses, Y. Xia, K. Horsburgh, K. Ueda, and T. Saitoh, 1995, The human NACP/alpha-synuclein gene: chromosome assignment to 4q21.3-q22 and TaqI RFLP analysis: Genomics, v. 26, p. 425-7.
Chesselet, M. F., S. Fleming, F. Mortazavi, and B. Meurers, 2008, Strengths and limitations of genetic mouse models of Parkinson's disease: Parkinsonism Relat Disord, v. 14 Suppl 2, p. S84-7.
Chevalier, G., and J. M. Deniau, 1990, Disinhibition as a basic process in the expression of striatal functions: Trends Neurosci, v. 13, p. 277-80.
Choi, P., N. Ostrerova-Golts, D. Sparkman, E. Cochran, J. M. Lee, and B. Wolozin, 2000, Parkin is metabolized by the ubiquitin/proteosome system: Neuroreport, v. 11, p. 2635-8.
Chung, K. K., B. Thomas, X. Li, O. Pletnikova, J. C. Troncoso, L. Marsh, V. L. Dawson, and T. M. Dawson, 2004, S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function: Science, v. 304, p. 1328-31.
Chung, K. K., Y. Zhang, K. L. Lim, Y. Tanaka, H. Huang, J. Gao, C. A. Ross, V. L. Dawson, and T. M. Dawson, 2001, Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease: Nat Med, v. 7, p. 1144-50.
Clayton, D. F., and J. M. George, 1998, The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease: Trends Neurosci, v. 21, p. 249-54.

Coban, A., and N. M. Filipov, 2007, Dopaminergic toxicity associated with oral exposure to the herbicide atrazine in juvenile male C57BL/6 mice: J Neurochem, v. 100, p. 1177-87.
Cole, N. B., D. D. Murphy, J. Lebowitz, L. Di Noto, R. L. Levine, and R. L. Nussbaum, 2005, Metal-catalyzed oxidation of alpha-synuclein: helping to define the relationship between oligomers, protofibrils, and filaments: J Biol Chem, v. 280, p. 9678-90.
Contant, C., D. Umbriaco, S. Garcia, K. C. Watkins, and L. Descarries, 1996, Ultrastructural characterization of the acetylcholine innervation in adult rat neostriatum: Neuroscience, v. 71, p. 937-47.
Contet, C., J. N. Rawlins, and R. M. Deacon, 2001, A comparison of 129S2/SvHsd and C57BL/6JOlaHsd mice on a test battery assessing sensorimotor, affective and cognitive behaviours: implications for the study of genetically modified mice: Behav Brain Res, v. 124, p. 33-46.
Conway, K. A., J. D. Harper, and P. T. Lansbury, 1998, Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease: Nat Med, v. 4, p. 1318-20.
Conway, K. A., S. J. Lee, J. C. Rochet, T. T. Ding, J. D. Harper, R. E. Williamson, and P. T. Lansbury, Jr., 2000a, Accelerated oligomerization by Parkinson's disease linked alpha-synuclein mutants: Ann N Y Acad Sci, v. 920, p. 42-5.
Conway, K. A., S. J. Lee, J. C. Rochet, T. T. Ding, R. E. Williamson, and P. T. Lansbury, Jr., 2000b, Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy: Proc Natl Acad Sci U S A, v. 97, p. 571-6.
Conway, K. A., J. C. Rochet, R. M. Bieganski, and P. T. Lansbury, Jr., 2001, Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct: Science, v. 294, p. 1346-9.
Cooper, A. A., A. D. Gitler, A. Cashikar, C. M. Haynes, K. J. Hill, B. Bhullar, K. Liu, K. Xu, K. E. Strathearn, F. Liu, S. Cao, K. A. Caldwell, G. A. Caldwell, G. Marsischky, R. D. Kolodner, J. Labaer, J. C. Rochet, N. M. Bonini, and S. Lindquist, 2006, Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models: Science, v. 313, p. 324-8.
Cragg, S. J., 2003, Variable dopamine release probability and short-term plasticity between functional domains of the primate striatum: J Neurosci, v. 23, p. 4378-85.
Cragg, S. J., 2005, Singing to the tune of dopamine. Focus on "Properties of dopamine release and uptake in the songbird basal ganglia": J Neurophysiol, v. 93, p. 1827-8.
Cragg, S. J., 2006, Meaningful silences: how dopamine listens to the ACh pause: Trends Neurosci, v. 29, p. 125-31.
Cragg, S. J., and S. A. Greenfield, 1997, Differential autoreceptor control of somatodendritic and axon terminal dopamine release in substantia nigra, ventral tegmental area, and striatum: J Neurosci, v. 17, p. 5738-46.
Cragg, S. J., C. R. Hawkey, and S. A. Greenfield, 1997, Comparison of serotonin and dopamine release in substantia nigra and ventral tegmental area: region and species differences: J Neurochem, v. 69, p. 2378-86.
Cragg, S. J., C. J. Hille, and S. A. Greenfield, 2000, Dopamine release and uptake dynamics within nonhuman primate striatum in vitro: J Neurosci, v. 20, p. 8209-17.

Cragg, S. J., and M. E. Rice, 2004, DAncing past the DAT at a DA synapse: Trends Neurosci, v. 27, p. 270-7.
D'Andrea, M. R., S. Ilyin, and C. R. Plata-Salaman, 2001, Abnormal patterns of microtubule-associated protein-2 (MAP-2) immunolabeling in neuronal nuclei and Lewy bodies in Parkinson's disease substantia nigra brain tissues: Neurosci Lett, v. 306, p. 137-40.
Damier, P., E. C. Hirsch, Y. Agid, and A. M. Graybiel, 1999, The substantia nigra of the human brain. II. Patterns of loss of dopamine-containing neurons in Parkinson's disease: Brain, v. 122 (Pt 8), p. 1437-48.
Dauer, W., N. Kholodilov, M. Vila, A. C. Trillat, R. Goodchild, K. E. Larsen, R. Staal, K. Tieu, Y. Schmitz, C. A. Yuan, M. Rocha, V. Jackson-Lewis, S. Hersch, D. Sulzer, S. Przedborski, R. Burke, and R. Hen, 2002, Resistance of alpha -synuclein null mice to the parkinsonian neurotoxin MPTP: Proc Natl Acad Sci U S A, v. 99, p. 14524-9.
Dauer, W., and S. Przedborski, 2003, Parkinson's disease: mechanisms and models: Neuron, v. 39, p. 889-909.
Davidson, W. S., A. Jonas, D. F. Clayton, and J. M. George, 1998, Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes: J Biol Chem, v. 273, p. 9443-9.
Dawson, T. M., H. S. Ko, and V. L. Dawson, 2010, Genetic animal models of Parkinson's disease: Neuron, v. 66, p. 646-61.
de Rijk, M. C., M. M. Breteler, G. A. Graveland, A. Ott, D. E. Grobbee, F. G. van der Meche, and A. Hofman, 1995, Prevalence of Parkinson's disease in the elderly: the Rotterdam Study: Neurology, v. 45, p. 2143-6.
Deacon, R. M., D. M. Bannerman, B. P. Kirby, A. Croucher, and J. N. Rawlins, 2002a, Effects of cytotoxic hippocampal lesions in mice on a cognitive test battery: Behav Brain Res, v. 133, p. 57-68.
Deacon, R. M., A. Croucher, and J. N. Rawlins, 2002b, Hippocampal cytotoxic lesion effects on species-typical behaviours in mice: Behav Brain Res, v. 132, p. 203-13.
Deacon, R. M., and J. N. Rawlins, 2006, T-maze alternation in the rodent: Nat Protoc, v. 1, p. 7-12.
DeBoer, P., M. J. Heeringa, and E. D. Abercrombie, 1996, Spontaneous release of acetylcholine in striatum is preferentially regulated by inhibitory dopamine D2 receptors: Eur J Pharmacol, v. 317, p. 257-62.
DeLong, M. R., 1990, Primate models of movement disorders of basal ganglia origin: Trends Neurosci, v. 13, p. 281-5.
Dexter, D. T., C. J. Carter, F. R. Wells, F. Javoy-Agid, Y. Agid, A. Lees, P. Jenner, and C. D. Marsden, 1989, Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease: J Neurochem, v. 52, p. 381-9.
Di Ciano, P., R. N. Cardinal, R. A. Cowell, S. J. Little, and B. J. Everitt, 2001, Differential involvement of NMDA, AMPA/kainate, and dopamine receptors in the nucleus accumbens core in the acquisition and performance of pavlovian approach behavior: J Neurosci, v. 21, p. 9471-7.
Di Ciano, P., and B. J. Everitt, 2001, Dissociable effects of antagonism of NMDA and AMPA/KA receptors in the nucleus accumbens core and shell on cocaine-seeking behavior: Neuropsychopharmacology, v. 25, p. 341-60.
Drolet, R. E., B. Behrouz, K. J. Lookingland, and J. L. Goudreau, 2004, Mice lacking alpha-synuclein have an attenuated loss of striatal dopamine following prolonged chronic MPTP administration: Neurotoxicology, v. 25, p. 761-9.

Duda, J. E., B. I. Giasson, Q. Chen, T. L. Gur, H. I. Hurtig, M. B. Stern, S. M. Gollomp, H. Ischiropoulos, V. M. Lee, and J. Q. Trojanowski, 2000, Widespread nitration of pathological inclusions in neurodegenerative synucleinopathies: Am J Pathol, v. 157, p. 1439-45.

Duda, J. E., B. I. Giasson, M. E. Mabon, V. M. Lee, and J. Q. Trojanowski, 2002, Novel antibodies to synuclein show abundant striatal pathology in Lewy body diseases: Ann Neurol, v. 52, p. 205-10.

Eberling, J. L., W. J. Jagust, C. W. Christine, P. Starr, P. Larson, K. S. Bankiewicz, and M. J. Aminoff, 2008, Results from a phase I safety trial of hAADC gene therapy for Parkinson disease: Neurology, v. 70, p. 1980-3.

Edwards, T. L., W. K. Scott, C. Almonte, A. Burt, E. H. Powell, G. W. Beecham, L. Wang, S. Zuchner, I. Konidari, G. Wang, C. Singer, F. Nahab, B. Scott, J. M. Stajich, M. Pericak-Vance, J. Haines, J. M. Vance, and E. R. Martin, 2010, Genome-wide association study confirms SNPs in SNCA and the MAPT region as common risk factors for Parkinson disease: Ann Hum Genet, v. 74, p. 97-109.

Eliezer, D., E. Kutluay, R. Bussell, Jr., and G. Browne, 2001, Conformational properties of alpha-synuclein in its free and lipid-associated states: J Mol Biol, v. 307, p. 1061-73.

Engel, L. S., H. Checkoway, M. C. Keifer, N. S. Seixas, W. T. Longstreth, Jr., K. C. Scott, K. Hudnell, W. K. Anger, and R. Camicioli, 2001, Parkinsonism and occupational exposure to pesticides: Occup Environ Med, v. 58, p. 582-9.

Engelender, S., Z. Kaminsky, X. Guo, A. H. Sharp, R. K. Amaravi, J. J. Kleiderlein, R. L. Margolis, J. C. Troncoso, A. A. Lanahan, P. F. Worley, V. L. Dawson, T. M. Dawson, and C. A. Ross, 1999, Synphilin-1 associates with alpha-synuclein and promotes the formation of cytosolic inclusions: Nat Genet, v. 22, p. 110-4.

Eriksen, J. L., S. Przedborski, and L. Petrucelli, 2005, Gene dosage and pathogenesis of Parkinson's disease: Trends Mol Med, v. 11, p. 91-6.

Exley, R., M. A. Clements, H. Hartung, J. M. McIntosh, and S. J. Cragg, 2008, Alpha6-containing nicotinic acetylcholine receptors dominate the nicotine control of dopamine neurotransmission in nucleus accumbens: Neuropsychopharmacology, v. 33, p. 2158-66.

Exton, J. H., 2002a, Phospholipase D-structure, regulation and function: Rev Physiol Biochem Pharmacol, v. 144, p. 1-94.

Exton, J. H., 2002b, Regulation of phospholipase D: FEBS Lett, v. 531, p. 58-61.

Fallon, J. H., and R. Y. Moore, 1978, Catecholamine innervation of the basal forebrain. IV. Topography of the dopamine projection to the basal forebrain and neostriatum: J Comp Neurol, v. 180, p. 545-80.

Fan, Y., P. Limprasert, I. V. Murray, A. C. Smith, V. M. Lee, J. Q. Trojanowski, B. L. Sopher, and A. R. La Spada, 2006, Beta-synuclein modulates alpha-synuclein neurotoxicity by reducing alpha-synuclein protein expression: Hum Mol Genet, v. 15, p. 3002-11.

Farrer, M., J. Kachergus, L. Forno, S. Lincoln, D. S. Wang, M. Hulihan, D. Maraganore, K. Gwinn-Hardy, Z. Wszolek, D. Dickson, and J. W. Langston, 2004, Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications: Ann Neurol, v. 55, p. 174-9.

Feany, M. B., and W. W. Bender, 2000, A Drosophila model of Parkinson's disease: Nature, v. 404, p. 394-8.

Fernagut, P. O., and M. F. Chesselet, 2004, Alpha-synuclein and transgenic mouse models: Neurobiol Dis, v. 17, p. 123-30.

Filloux, F., and J. J. Townsend, 1993, Pre- and postsynaptic neurotoxic effects of dopamine demonstrated by intrastriatal injection: Exp Neurol, v. 119, p. 79-88.

Fleming, S. M., J. Salcedo, P. O. Fernagut, E. Rockenstein, E. Masliah, M. S. Levine, and M. F. Chesselet, 2004, Early and progressive sensorimotor anomalies in mice overexpressing wild-type human alpha-synuclein: J Neurosci, v. 24, p. 9434-40.

Fleming, S. M., J. Salcedo, C. B. Hutson, E. Rockenstein, E. Masliah, M. S. Levine, and M. F. Chesselet, 2006, Behavioral effects of dopaminergic agonists in transgenic mice overexpressing human wildtype alpha-synuclein: Neuroscience, v. 142, p. 1245-53.

Fornai, F., O. M. Schluter, P. Lenzi, M. Gesi, R. Ruffoli, M. Ferrucci, G. Lazzeri, C. L. Busceti, F. Pontarelli, G. Battaglia, A. Pellegrini, F. Nicoletti, S. Ruggieri, A. Paparelli, and T. C. Sudhof, 2005, Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin-proteasome system and alpha-synuclein: Proc Natl Acad Sci U S A, v. 102, p. 3413-8.

Forno, L. S., 1987, The Lewy body in Parkinson's disease: Adv Neurol, v. 45, p. 35-43.

Forno, L. S., 1996, Neuropathology of Parkinson's disease: J Neuropathol Exp Neurol, v. 55, p. 259-72.

Forno, L. S., L. E. DeLanney, I. Irwin, and J. W. Langston, 1996, Electron microscopy of Lewy bodies in the amygdala-parahippocampal region. Comparison with inclusion bodies in the MPTP-treated squirrel monkey: Adv Neurol, v. 69, p. 217-28.

Fountaine, T. M., L. L. Venda, N. Warrick, H. C. Christian, P. Brundin, K. M. Channon, and R. Wade-Martins, 2008, The effect of alpha-synuclein knockdown on MPP+ toxicity in models of human neurons: Eur J Neurosci, v. 28, p. 2459-73.

Fountaine, T. M., and R. Wade-Martins, 2007, RNA interference-mediated knockdown of alpha-synuclein protects human dopaminergic neuroblastoma cells from MPP(+) toxicity and reduces dopamine transport: J Neurosci Res, v. 85, p. 351-63.

Freed, C. R., R. E. Breeze, N. L. Rosenberg, S. A. Schneck, E. Kriek, J. X. Qi, T. Lone, Y. B. Zhang, J. A. Snyder, T. H. Wells, and et al., 1992, Survival of implanted fetal dopamine cells and neurologic improvement 12 to 46 months after transplantation for Parkinson's disease: N Engl J Med, v. 327, p. 1549-55.

Freed, C. R., P. E. Greene, R. E. Breeze, W. Y. Tsai, W. DuMouchel, R. Kao, S. Dillon, H. Winfield, S. Culver, J. Q. Trojanowski, D. Eidelberg, and S. Fahn, 2001, Transplantation of embryonic dopamine neurons for severe Parkinson's disease: N Engl J Med, v. 344, p. 710-9.

Freeman, T. B., C. W. Olanow, R. A. Hauser, G. M. Nauert, D. A. Smith, C. V. Borlongan, P. R. Sanberg, D. A. Holt, J. H. Kordower, F. J. Vingerhoets, and et al., 1995, Bilateral fetal nigral transplantation into the postcommissural putamen in Parkinson's disease: Ann Neurol, v. 38, p. 379-88.

Funayama, M., K. Hasegawa, H. Kowa, M. Saito, S. Tsuji, and F. Obata, 2002, A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1: Ann Neurol, v. 51, p. 296-301.

Furukawa, K., M. Matsuzaki-Kobayashi, T. Hasegawa, A. Kikuchi, N. Sugeno, Y. Itoyama, Y. Wang, P. J. Yao, I. Bushlin, and A. Takeda, 2006, Plasma membrane ion permeability induced by mutant alpha-synuclein contributes to the degeneration of neural cells: J Neurochem, v. 97, p. 1071-7.

Galvin, J. E., T. M. Schuck, V. M. Lee, and J. Q. Trojanowski, 2001, Differential expression and distribution of alpha-, beta-, and gamma-synuclein in the developing human substantia nigra: Exp Neurol, v. 168, p. 347-55.

Galvin, J. E., K. Uryu, V. M. Lee, and J. Q. Trojanowski, 1999, Axon pathology in Parkinson's disease and Lewy body dementia hippocampus contains alpha-, beta-, and gamma-synuclein: Proc Natl Acad Sci U S A, v. 96, p. 13450-5.

Gao, N., Y. H. Li, X. Li, S. Yu, G. L. Fu, and B. Chen, 2007, Effect of alpha-synuclein on the promoter activity of tyrosine hydroxylase gene: Neurosci Bull, v. 23, p. 53-7.

Garcia-Reitbock, P., O. Anichtchik, A. Bellucci, M. Iovino, C. Ballini, E. Fineberg, B. Ghetti, L. Della Corte, P. Spano, G. K. Tofaris, M. Goedert, and M. G. Spillantini, 2010, SNARE protein redistribution and synaptic failure in a transgenic mouse model of Parkinson's disease: Brain, v. 133, p. 2032-44.

Garris, P. A., E. L. Ciolkowski, and R. M. Wightman, 1994, Heterogeneity of evoked dopamine overflow within the striatal and striatoamygdaloid regions: Neuroscience, v. 59, p. 417-27.

Garris, P. A., and R. M. Wightman, 1994, Different kinetics govern dopaminergic transmission in the amygdala, prefrontal cortex, and striatum: an in vivo voltammetric study: J Neurosci, v. 14, p. 442-50.

Garthwaite, J., S. L. Charles, and R. Chess-Williams, 1988, Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain: Nature, v. 336, p. 385-8.

Gasmi, M., C. D. Herzog, E. P. Brandon, J. J. Cunningham, G. A. Ramirez, E. T. Ketchum, and R. T. Bartus, 2007, Striatal delivery of neurturin by CERE-120, an AAV2 vector for the treatment of dopaminergic neuron degeneration in Parkinson's disease: Mol Ther, v. 15, p. 62-8.

Gasser, T., 2009, Molecular pathogenesis of Parkinson disease: insights from genetic studies: Expert Rev Mol Med, v. 11, p. e22.

George, J. M., H. Jin, W. S. Woods, and D. F. Clayton, 1995, Characterization of a novel protein regulated during the critical period for song learning in the zebra finch: Neuron, v. 15, p. 361-72.

Gerfen, C. R., 1992, The neostriatal mosaic: multiple levels of compartmental organization: Trends Neurosci, v. 15, p. 133-9.

Giasson, B. I., J. E. Duda, M. S. Forman, V. M. Lee, and J. Q. Trojanowski, 2001, Prominent perikaryal expression of alpha- and beta-synuclein in neurons of dorsal root ganglion and in medullary neurons: Exp Neurol, v. 172, p. 354-62.

Giasson, B. I., J. E. Duda, I. V. Murray, Q. Chen, J. M. Souza, H. I. Hurtig, H. Ischiropoulos, J. Q. Trojanowski, and V. M. Lee, 2000, Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions: Science, v. 290, p. 985-9.

Giasson, B. I., J. E. Duda, S. M. Quinn, B. Zhang, J. Q. Trojanowski, and V. M. Lee, 2002, Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein: Neuron, v. 34, p. 521-33.

Giasson, B. I., K. Uryu, J. Q. Trojanowski, and V. M. Lee, 1999, Mutant and wild type human alpha-synucleins assemble into elongated filaments with distinct morphologies in vitro: J Biol Chem, v. 274, p. 7619-22.

Gibb, W. R., and W. H. Poewe, 1986, The centenary of Friederich H. Lewy 1885-1950: Neuropathol Appl Neurobiol, v. 12, p. 217-22.
Giros, B., M. Jaber, S. R. Jones, R. M. Wightman, and M. G. Caron, 1996, Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter: Nature, v. 379, p. 606-12.
Gispert, S., D. Del Turco, L. Garrett, A. Chen, D. J. Bernard, J. Hamm-Clement, H. W. Korf, T. Deller, H. Braak, G. Auburger, and R. L. Nussbaum, 2003, Transgenic mice expressing mutant A53T human alpha-synuclein show neuronal dysfunction in the absence of aggregate formation: Mol Cell Neurosci, v. 24, p. 419-29.
Gitler, A. D., B. J. Bevis, J. Shorter, K. E. Strathearn, S. Hamamichi, L. J. Su, K. A. Caldwell, G. A. Caldwell, J. C. Rochet, J. M. McCaffery, C. Barlowe, and S. Lindquist, 2008, The Parkinson's disease protein alpha-synuclein disrupts cellular Rab homeostasis: Proc Natl Acad Sci U S A, v. 105, p. 145-50.
Goedert, M., 2001a, Alpha-synuclein and neurodegenerative diseases: Nat Rev Neurosci, v. 2, p. 492-501.
Goedert, M., 2001b, Parkinson's disease and other alpha-synucleinopathies: Clin Chem Lab Med, v. 39, p. 308-12.
Gomez-Isla, T., W. B. Growdon, M. McNamara, K. Newell, E. Gomez-Tortosa, E. T. Hedley-Whyte, and B. T. Hyman, 1999, Clinicopathologic correlates in temporal cortex in dementia with Lewy bodies: Neurology, v. 53, p. 2003-9.
Gomez-Isla, T., M. C. Irizarry, A. Mariash, B. Cheung, O. Soto, S. Schrump, J. Sondel, L. Kotilinek, J. Day, M. A. Schwarzschild, J. H. Cha, K. Newell, D. W. Miller, K. Ueda, A. B. Young, B. T. Hyman, and K. H. Ashe, 2003, Motor dysfunction and gliosis with preserved dopaminergic markers in human alpha-synuclein A30P transgenic mice: Neurobiol Aging, v. 24, p. 245-58.
Gomez-Santos, C., M. Barrachina, P. Gimenez-Xavier, E. Dalfo, I. Ferrer, and S. Ambrosio, 2005, Induction of C/EBP beta and GADD153 expression by dopamine in human neuroblastoma cells. Relationship with alpha-synuclein increase and cell damage: Brain Res Bull, v. 65, p. 87-95.
Gorell, J. M., C. C. Johnson, B. A. Rybicki, E. L. Peterson, G. X. Kortsha, G. G. Brown, and R. J. Richardson, 1999a, Occupational exposure to manganese, copper, lead, iron, mercury and zinc and the risk of Parkinson's disease: Neurotoxicology, v. 20, p. 239-47.
Gorell, J. M., B. A. Rybicki, C. Cole Johnson, and E. L. Peterson, 1999b, Occupational metal exposures and the risk of Parkinson's disease: Neuroepidemiology, v. 18, p. 303-8.
Gosavi, N., H. J. Lee, J. S. Lee, S. Patel, and S. J. Lee, 2002, Golgi fragmentation occurs in the cells with prefibrillar alpha-synuclein aggregates and precedes the formation of fibrillar inclusion: J Biol Chem, v. 277, p. 48984-92.
Graham, D. G., 1978, Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones: Mol Pharmacol, v. 14, p. 633-43.
Graham, D. G., S. M. Tiffany, W. R. Bell, Jr., and W. F. Gutknecht, 1978, Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro: Mol Pharmacol, v. 14, p. 644-53.
Greten-Harrison, B., M. Polydoro, M. Morimoto-Tomita, L. Diao, A. M. Williams, E. H. Nie, S. Makani, N. Tian, P. E. Castillo, V. L. Buchman, and S. S. Chandra, 2010, alphabetagamma-Synuclein triple knockout mice reveal age-dependent neuronal dysfunction: Proc Natl Acad Sci U S A, v. 107, p. 19573-8.

Groenewegen, H. J., H. W. Berendse, and S. N. Haber, 1993, Organization of the output of the ventral striatopallidal system in the rat: ventral pallidal efferents: Neuroscience, v. 57, p. 113-42.
Guenther, K., R. M. Deacon, V. H. Perry, and J. N. Rawlins, 2001, Early behavioural changes in scrapie-affected mice and the influence of dapsone: Eur J Neurosci, v. 14, p. 401-9.
Guo, J., C. Shou, L. Meng, B. Jiang, B. Dong, L. Yao, Y. Xie, J. Zhang, Y. Chen, D. R. Budman, and Y. E. Shi, 2007, Neuronal protein synuclein gamma predicts poor clinical outcome in breast cancer: Int J Cancer, v. 121, p. 1296-305.
Haber, S. N., J. L. Fudge, and N. R. McFarland, 2000, Striatonigrostriatal pathways in primates form an ascending spiral from the shell to the dorsolateral striatum: J Neurosci, v. 20, p. 2369-82.
Hancock, D. B., E. R. Martin, J. M. Stajich, R. Jewett, M. A. Stacy, B. L. Scott, J. M. Vance, and W. K. Scott, 2007, Smoking, caffeine, and nonsteroidal anti-inflammatory drugs in families with Parkinson disease: Arch Neurol, v. 64, p. 576-80.
Hashimoto, M., L. J. Hsu, Y. Xia, A. Takeda, A. Sisk, M. Sundsmo, and E. Masliah, 1999a, Oxidative stress induces amyloid-like aggregate formation of NACP/alpha-synuclein in vitro: Neuroreport, v. 10, p. 717-21.
Hashimoto, M., E. Rockenstein, M. Mante, M. Mallory, and E. Masliah, 2001, beta-Synuclein inhibits alpha-synuclein aggregation: a possible role as an anti-parkinsonian factor: Neuron, v. 32, p. 213-23.
Hashimoto, M., A. Takeda, L. J. Hsu, T. Takenouchi, and E. Masliah, 1999b, Role of cytochrome c as a stimulator of alpha-synuclein aggregation in Lewy body disease: J Biol Chem, v. 274, p. 28849-52.
Hastings, T. G., D. A. Lewis, and M. J. Zigmond, 1996, Reactive dopamine metabolites and neurotoxicity: implications for Parkinson's disease: Adv Exp Med Biol, v. 387, p. 97-106.
Haycock, J. W., 1990, Phosphorylation of tyrosine hydroxylase in situ at serine 8, 19, 31, and 40: J Biol Chem, v. 265, p. 11682-91.
Hayflick, L., 2000, The future of ageing: Nature, v. 408, p. 267-9.
Hebb, C. O., 1957, Biochemical evidence for the neural function of acetylcholine: Physiol Rev, v. 37, p. 196-220.
Heimer, L., 2000, Basal forebrain in the context of schizophrenia: Brain Res Brain Res Rev, v. 31, p. 205-35.
Heimer, L., D. S. Zahm, L. Churchill, P. W. Kalivas, and C. Wohltmann, 1991, Specificity in the projection patterns of accumbal core and shell in the rat: Neuroscience, v. 41, p. 89-125.
Hely, M. A., J. G. Morris, W. G. Reid, D. J. O'Sullivan, P. M. Williamson, D. Rail, G. A. Broe, and S. Margrie, 1994, The Sydney Multicentre Study of Parkinson's disease: a randomised, prospective five year study comparing low dose bromocriptine with low dose levodopa-carbidopa: J Neurol Neurosurg Psychiatry, v. 57, p. 903-10.
Heuser, J. E., and T. S. Reese, 1973, Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction: J Cell Biol, v. 57, p. 315-44.
Hietanen, M., and H. Teravainen, 1988, The effect of age of disease onset on neuropsychological performance in Parkinson's disease: J Neurol Neurosurg Psychiatry, v. 51, p. 244-9.

Hindle, J. V., 2010, Ageing, neurodegeneration and Parkinson's disease: Age Ageing, v. 39, p. 156-61.
Hodara, R., E. H. Norris, B. I. Giasson, A. J. Mishizen-Eberz, D. R. Lynch, V. M. Lee, and H. Ischiropoulos, 2004, Functional consequences of alpha-synuclein tyrosine nitration: diminished binding to lipid vesicles and increased fibril formation: J Biol Chem, v. 279, p. 47746-53.
Holt, D. J., A. M. Graybiel, and C. B. Saper, 1997, Neurochemical architecture of the human striatum: J Comp Neurol, v. 384, p. 1-25.
Hoozemans, J. J., E. S. van Haastert, P. Eikelenboom, R. A. de Vos, J. M. Rozemuller, and W. Scheper, 2007, Activation of the unfolded protein response in Parkinson's disease: Biochem Biophys Res Commun, v. 354, p. 707-11.
Hornykiewicz, O., 1963, [The tropical localization and content of noradrenalin and dopamine (3-hydroxytyramine) in the substantia nigra of normal persons and patients with Parkinson's disease]: Wien Klin Wochenschr, v. 75, p. 309-12.
Hsu, L. J., M. Mallory, Y. Xia, I. Veinbergs, M. Hashimoto, M. Yoshimoto, L. J. Thal, T. Saitoh, and E. Masliah, 1998, Expression pattern of synucleins (non-Abeta component of Alzheimer's disease amyloid precursor protein/alpha-synuclein) during murine brain development: J Neurochem, v. 71, p. 338-44.
Ichimura, T., T. Isobe, T. Okuyama, N. Takahashi, K. Araki, R. Kuwano, and Y. Takahashi, 1988, Molecular cloning of cDNA coding for brain-specific 14-3-3 protein, a protein kinase-dependent activator of tyrosine and tryptophan hydroxylases: Proc Natl Acad Sci U S A, v. 85, p. 7084-8.
Ichimura, T., T. Isobe, T. Okuyama, T. Yamauchi, and H. Fujisawa, 1987, Brain 14-3-3 protein is an activator protein that activates tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in the presence of Ca2+,calmodulin-dependent protein kinase II: FEBS Lett, v. 219, p. 79-82.
Ikarashi, Y., A. Takahashi, H. Ishimaru, T. Arai, and Y. Maruyama, 1997, Regulation of dopamine D1 and D2 receptors on striatal acetylcholine release in rats: Brain Res Bull, v. 43, p. 107-15.
Ikemoto, S., 2007, Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex: Brain Res Rev, v. 56, p. 27-78.
Irizarry, M. C., T. W. Kim, M. McNamara, R. E. Tanzi, J. M. George, D. F. Clayton, and B. T. Hyman, 1996, Characterization of the precursor protein of the non-A beta component of senile plaques (NACP) in the human central nervous system: J Neuropathol Exp Neurol, v. 55, p. 889-95.
Iversen, S. D., 1980, Brain chemistry and behaviour: Psychol Med, v. 10, p. 527-39.
Iversen, S. D., 1984, Behavioural effects of manipulation of basal ganglia neurotransmitters: Ciba Found Symp, v. 107, p. 183-200.
Iversen, S. D., 1995, Interactions between excitatory amino acids and dopamine systems in the forebrain: implications for schizophrenia and Parkinson's disease: Behav Pharmacol, v. 6, p. 478-491.
Iversen, S. D., and I. Creese, 1975, Behavioral correlates of dopaminergic supersensitivity: Adv Neurol, v. 9, p. 81-92.
Iversen, S. D., P. H. Kelly, R. J. Miller, and P. Seviour, 1975, Proceedings: Amphetamine and apomorphine responses in the rat after lesion of mesolimbic or striatal dopamine neurones: Br J Pharmacol, v. 54, p. 244P.

Iversen, S. D., and G. F. Koob, 1977, Behavioral implications of dopaminergic neurons in the mesolimbic system: Adv Biochem Psychopharmacol, v. 16, p. 209-14.
Iwai, A., E. Masliah, M. Yoshimoto, N. Ge, L. Flanagan, H. A. de Silva, A. Kittel, and T. Saitoh, 1995, The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system: Neuron, v. 14, p. 467-75.
Iwata, A., M. Maruyama, I. Kanazawa, and N. Nukina, 2001, alpha-Synuclein affects the MAPK pathway and accelerates cell death: J Biol Chem, v. 276, p. 45320-9.
Jakes, R., M. G. Spillantini, and M. Goedert, 1994, Identification of two distinct synucleins from human brain: FEBS Lett, v. 345, p. 27-32.
Jakowec, M. W., K. Nixon, E. Hogg, T. McNeill, and G. M. Petzinger, 2004, Tyrosine hydroxylase and dopamine transporter expression following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration of the mouse nigrostriatal pathway: J Neurosci Res, v. 76, p. 539-50.
Jenco, J. M., A. Rawlingson, B. Daniels, and A. J. Morris, 1998, Regulation of phospholipase D2: selective inhibition of mammalian phospholipase D isoenzymes by alpha- and beta-synucleins: Biochemistry, v. 37, p. 4901-9.
Jenner, P., 2007, Oxidative stress and Parkinson's disease: Handb Clin Neurol, v. 83, p. 507-20.
Jenner, P., D. T. Dexter, J. Sian, A. H. Schapira, and C. D. Marsden, 1992, Oxidative stress as a cause of nigral cell death in Parkinson's disease and incidental Lewy body disease. The Royal Kings and Queens Parkinson's Disease Research Group: Ann Neurol, v. 32 Suppl, p. S82-7.
Jensen, P. H., H. Hager, M. S. Nielsen, P. Hojrup, J. Gliemann, and R. Jakes, 1999, alpha-synuclein binds to Tau and stimulates the protein kinase A-catalyzed tau phosphorylation of serine residues 262 and 356: J Biol Chem, v. 274, p. 25481-9.
Jensen, P. H., K. Islam, J. Kenney, M. S. Nielsen, J. Power, and W. P. Gai, 2000, Microtubule-associated protein 1B is a component of cortical Lewy bodies and binds alpha-synuclein filaments: J Biol Chem, v. 275, p. 21500-7.
Jensen, P. H., M. S. Nielsen, R. Jakes, C. G. Dotti, and M. Goedert, 1998, Binding of alpha-synuclein to brain vesicles is abolished by familial Parkinson's disease mutation: J Biol Chem, v. 273, p. 26292-4.
Ji, H., Y. E. Liu, T. Jia, M. Wang, J. Liu, G. Xiao, B. K. Joseph, C. Rosen, and Y. E. Shi, 1997, Identification of a breast cancer-specific gene, BCSG1, by direct differential cDNA sequencing: Cancer Res, v. 57, p. 759-64.
Jia, T., Y. E. Liu, J. Liu, and Y. E. Shi, 1999, Stimulation of breast cancer invasion and metastasis by synuclein gamma: Cancer Res, v. 59, p. 742-7.
Jo, E., N. Fuller, R. P. Rand, P. St George-Hyslop, and P. E. Fraser, 2002, Defective membrane interactions of familial Parkinson's disease mutant A30P alpha-synuclein: J Mol Biol, v. 315, p. 799-807.
Jones, D. H., B. Bax, A. Fensome, and S. Cockcroft, 1999, ADP ribosylation factor 1 mutants identify a phospholipase D effector region and reveal that phospholipase D participates in lysosomal secretion but is not sufficient for recruitment of coatomer I: Biochem J, v. 341 (Pt 1), p. 185-92.
Kahle, P. J., M. Neumann, L. Ozmen, and C. Haass, 2000a, Physiology and pathophysiology of alpha-synuclein. Cell culture and transgenic animal

models based on a Parkinson's disease-associated protein: Ann N Y Acad Sci, v. 920, p. 33-41.
Kahle, P. J., M. Neumann, L. Ozmen, V. Muller, H. Jacobsen, A. Schindzielorz, M. Okochi, U. Leimer, H. van Der Putten, A. Probst, E. Kremmer, H. A. Kretzschmar, and C. Haass, 2000b, Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha -synuclein in human and transgenic mouse brain: J Neurosci, v. 20, p. 6365-73.
Kahle, P. J., M. Neumann, L. Ozmen, V. Muller, H. Jacobsen, W. Spooren, B. Fuss, B. Mallon, W. B. Macklin, H. Fujiwara, M. Hasegawa, T. Iwatsubo, H. A. Kretzschmar, and C. Haass, 2002, Hyperphosphorylation and insolubility of alpha-synuclein in transgenic mouse oligodendrocytes: EMBO Rep, v. 3, p. 583-8.
Kalivendi, S. V., S. Cunningham, S. Kotamraju, J. Joseph, C. J. Hillard, and B. Kalyanaraman, 2004, Alpha-synuclein up-regulation and aggregation during MPP+-induced apoptosis in neuroblastoma cells: intermediacy of transferrin receptor iron and hydrogen peroxide: J Biol Chem, v. 279, p. 15240-7.
Kalivendi, S. V., D. Yedlapudi, C. J. Hillard, and B. Kalyanaraman, 2010, Oxidants induce alternative splicing of alpha-synuclein: Implications for Parkinson's disease: Free Radic Biol Med, v. 48, p. 377-83.
Kanda, S., J. F. Bishop, M. A. Eglitis, Y. Yang, and M. M. Mouradian, 2000, Enhanced vulnerability to oxidative stress by alpha-synuclein mutations and C-terminal truncation: Neuroscience, v. 97, p. 279-84.
Kawamata, H., P. J. McLean, N. Sharma, and B. T. Hyman, 2001, Interaction of alpha-synuclein and synphilin-1: effect of Parkinson's disease-associated mutations: J Neurochem, v. 77, p. 929-34.
Kawamoto, Y., I. Akiguchi, S. Nakamura, Y. Honjyo, H. Shibasaki, and H. Budka, 2002, 14-3-3 proteins in Lewy bodies in Parkinson disease and diffuse Lewy body disease brains: J Neuropathol Exp Neurol, v. 61, p. 245-53.
Kelly, P. H., P. W. Seviour, and S. D. Iversen, 1975, Amphetamine and apomorphine responses in the rat following 6-OHDA lesions of the nucleus accumbens septi and corpus striatum: Brain Res, v. 94, p. 507-22.
Kim, K. S., S. Y. Choi, H. Y. Kwon, M. H. Won, T. C. Kang, and J. H. Kang, 2002, Aggregation of alpha-synuclein induced by the Cu,Zn-superoxide dismutase and hydrogen peroxide system: Free Radic Biol Med, v. 32, p. 544-50.
Kiskinis, E., and K. Eggan, 2010, Progress toward the clinical application of patient-specific pluripotent stem cells: J Clin Invest, v. 120, p. 51-9.
Kitada, T., S. Asakawa, N. Hattori, H. Matsumine, Y. Yamamura, S. Minoshima, M. Yokochi, Y. Mizuno, and N. Shimizu, 1998, Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism: Nature, v. 392, p. 605-8.
Klingauf, J., E. T. Kavalali, and R. W. Tsien, 1998, Kinetics and regulation of fast endocytosis at hippocampal synapses: Nature, v. 394, p. 581-5.
Koller, W. C., J. T. Hutton, E. Tolosa, and R. Capilldeo, 1999, Immediate-release and controlled-release carbidopa/levodopa in PD: a 5-year randomized multicenter study. Carbidopa/Levodopa Study Group: Neurology, v. 53, p. 1012-9.
Kordower, J. H., N. M. Kanaan, Y. Chu, R. Suresh Babu, J. Stansell, 3rd, B. T. Terpstra, C. E. Sortwell, K. Steece-Collier, and T. J. Collier, 2006, Failure of proteasome inhibitor administration to provide a model of Parkinson's disease in rats and monkeys: Ann Neurol, v. 60, p. 264-8.

Korotkova, T. M., A. A. Ponomarenko, R. E. Brown, and H. L. Haas, 2004, Functional diversity of ventral midbrain dopamine and GABAergic neurons: Mol Neurobiol, v. 29, p. 243-59.
Kruger, R., W. Kuhn, T. Muller, D. Woitalla, M. Graeber, S. Kosel, H. Przuntek, J. T. Epplen, L. Schols, and O. Riess, 1998, Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease: Nat Genet, v. 18, p. 106-8.
Ktistakis, N. T., H. A. Brown, M. G. Waters, P. C. Sternweis, and M. G. Roth, 1996, Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles: J Cell Biol, v. 134, p. 295-306.
Kuhn, D. M., R. E. Arthur, Jr., D. M. Thomas, and L. A. Elferink, 1999, Tyrosine hydroxylase is inactivated by catechol-quinones and converted to a redox-cycling quinoprotein: possible relevance to Parkinson's disease: J Neurochem, v. 73, p. 1309-17.
Kulnane, L. S., E. J. Lehman, B. J. Hock, K. D. Tsuchiya, and B. T. Lamb, 2002, Rapid and efficient detection of transgene homozygosity by FISH of mouse fibroblasts: Mamm Genome, v. 13, p. 223-6.
Kuo, Y. M., Z. Li, Y. Jiao, N. Gaborit, A. K. Pani, B. M. Orrison, B. G. Bruneau, B. I. Giasson, R. J. Smeyne, M. D. Gershon, and R. L. Nussbaum, 2010, Extensive enteric nervous system abnormalities in mice transgenic for artificial chromosomes containing Parkinson disease-associated alpha-synuclein gene mutations precede central nervous system changes: Hum Mol Genet, v. 19, p. 1633-50.
Kuopio, A. M., R. J. Marttila, H. Helenius, and U. K. Rinne, 1999, Environmental risk factors in Parkinson's disease: Mov Disord, v. 14, p. 928-39.
Laitinen, L. V., A. T. Bergenheim, and M. I. Hariz, 1992, Ventroposterolateral pallidotomy can abolish all parkinsonian symptoms: Stereotact Funct Neurosurg, v. 58, p. 14-21.
Lang, A. E., 2000, Surgery for levodopa-induced dyskinesias: Ann Neurol, v. 47, p. S193-9; discussion S199-202.
Lange, K. W., W. D. Rausch, W. Gsell, M. Naumann, E. Oestreicher, and P. Riederer, 1994, Neuroprotection by dopamine agonists: J Neural Transm Suppl, v. 43, p. 183-201.
Langston, J. W., P. Ballard, J. W. Tetrud, and I. Irwin, 1983, Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis: Science, v. 219, p. 979-80.
Larsen, K. E., Y. Schmitz, M. D. Troyer, E. Mosharov, P. Dietrich, A. Z. Quazi, M. Savalle, V. Nemani, F. A. Chaudhry, R. H. Edwards, L. Stefanis, and D. Sulzer, 2006, Alpha-synuclein overexpression in PC12 and chromaffin cells impairs catecholamine release by interfering with a late step in exocytosis: J Neurosci, v. 26, p. 11915-22.
Lashuel, H. A., and H. Hirling, 2006, Rescuing defective vesicular trafficking protects against alpha-synuclein toxicity in cellular and animal models of Parkinson's disease: ACS Chem Biol, v. 1, p. 420-4.
Lashuel, H. A., B. M. Petre, J. Wall, M. Simon, R. J. Nowak, T. Walz, and P. T. Lansbury, Jr., 2002, Alpha-synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils: J Mol Biol, v. 322, p. 1089-102.
Lavedan, C., 1998, The synuclein family: Genome Res, v. 8, p. 871-80.
Lavedan, C., S. Buchholtz, G. Auburger, R. L. Albin, A. Athanassiadou, J. Blancato, J. A. Burguera, R. E. Ferrell, V. Kostic, E. Leroy, B. Leube, L. Mota-Vieira,

T. Papapetropoulos, M. A. Pericak-Vance, J. Pinkus, W. K. Scott, G. Ulm, J. Vasconcelos, J. J. Vilchez, R. L. Nussbaum, and M. H. Polymeropoulos, 1998a, Absence of mutation in the beta- and gamma-synuclein genes in familial autosomal dominant Parkinson's disease: DNA Res, v. 5, p. 401-2.

Lavedan, C., E. Leroy, A. Dehejia, S. Buchholtz, A. Dutra, R. L. Nussbaum, and M. H. Polymeropoulos, 1998b, Identification, localization and characterization of the human gamma-synuclein gene: Hum Genet, v. 103, p. 106-12.

Le, W. D., P. Xu, J. Jankovic, H. Jiang, S. H. Appel, R. G. Smith, and D. K. Vassilatis, 2003, Mutations in NR4A2 associated with familial Parkinson disease: Nat Genet, v. 33, p. 85-9.

Lee, C. S., H. Sauer, and A. Bjorklund, 1996, Dopaminergic neuronal degeneration and motor impairments following axon terminal lesion by instrastriatal 6-hydroxydopamine in the rat: Neuroscience, v. 72, p. 641-53.

Lee, F. J., F. Liu, Z. B. Pristupa, and H. B. Niznik, 2001, Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis: Faseb J, v. 15, p. 916-26.

Lee, M. K., W. Stirling, Y. Xu, X. Xu, D. Qui, A. S. Mandir, T. M. Dawson, N. G. Copeland, N. A. Jenkins, and D. L. Price, 2002, Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53 --> Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice: Proc Natl Acad Sci U S A, v. 99, p. 8968-73.

Lehmensiek, V., E. M. Tan, S. Liebau, T. Lenk, H. Zettlmeisl, J. Schwarz, and A. Storch, 2006, Dopamine transporter-mediated cytotoxicity of 6-hydroxydopamine in vitro depends on expression of mutant alpha-synucleins related to Parkinson's disease: Neurochem Int, v. 48, p. 329-40.

Lehmensiek, V., E. M. Tan, J. Schwarz, and A. Storch, 2002, Expression of mutant alpha-synucleins enhances dopamine transporter-mediated MPP+ toxicity in vitro: Neuroreport, v. 13, p. 1279-83.

Lennox, G., J. Lowe, M. Landon, E. J. Byrne, R. J. Mayer, and R. B. Godwin-Austen, 1989a, Diffuse Lewy body disease: correlative neuropathology using anti-ubiquitin immunocytochemistry: J Neurol Neurosurg Psychiatry, v. 52, p. 1236-47.

Lennox, G., J. Lowe, K. Morrell, M. Landon, and R. J. Mayer, 1989b, Anti-ubiquitin immunocytochemistry is more sensitive than conventional techniques in the detection of diffuse Lewy body disease: J Neurol Neurosurg Psychiatry, v. 52, p. 67-71.

Leroy, E., D. Anastasopoulos, S. Konitsiotis, C. Lavedan, and M. H. Polymeropoulos, 1998a, Deletions in the Parkin gene and genetic heterogeneity in a Greek family with early onset Parkinson's disease: Hum Genet, v. 103, p. 424-7.

Leroy, E., R. Boyer, G. Auburger, B. Leube, G. Ulm, E. Mezey, G. Harta, M. J. Brownstein, S. Jonnalagada, T. Chernova, A. Dehejia, C. Lavedan, T. Gasser, P. J. Steinbach, K. D. Wilkinson, and M. H. Polymeropoulos, 1998b, The ubiquitin pathway in Parkinson's disease: Nature, v. 395, p. 451-2.

Leroy, E., R. Boyer, and M. H. Polymeropoulos, 1998c, Intron-exon structure of ubiquitin c-terminal hydrolase-L1: DNA Res, v. 5, p. 397-400.

Lesage, S., and A. Brice, 2009, Parkinson's disease: from monogenic forms to genetic susceptibility factors: Hum Mol Genet, v. 18, p. R48-59.

LeWitt, P. A., and D. C. Taylor, 2008, Protection against Parkinson's disease progression: clinical experience: Neurotherapeutics, v. 5, p. 210-25.

Li, J., V. N. Uversky, and A. L. Fink, 2001, Effect of familial Parkinson's disease point mutations A30P and A53T on the structural properties, aggregation, and fibrillation of human alpha-synuclein: Biochemistry, v. 40, p. 11604-13.
Li, J., M. Zhu, A. B. Manning-Bog, D. A. Di Monte, and A. L. Fink, 2004, Dopamine and L-dopa disaggregate amyloid fibrils: implications for Parkinson's and Alzheimer's disease: Faseb J, v. 18, p. 962-4.
Li, W., and M. K. Lee, 2005, Antiapoptotic property of human alpha-synuclein in neuronal cell lines is associated with the inhibition of caspase-3 but not caspase-9 activity: J Neurochem, v. 93, p. 1542-50.
Liberatore, G. T., V. Jackson-Lewis, S. Vukosavic, A. S. Mandir, M. Vila, W. G. McAuliffe, V. L. Dawson, T. M. Dawson, and S. Przedborski, 1999, Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease: Nat Med, v. 5, p. 1403-9.
Lindvall, O., P. Brundin, H. Widner, S. Rehncrona, B. Gustavii, R. Frackowiak, K. L. Leenders, G. Sawle, J. C. Rothwell, C. D. Marsden, and et al., 1990, Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease: Science, v. 247, p. 574-7.
Lindvall, O., H. Widner, S. Rehncrona, P. Brundin, P. Odin, B. Gustavii, R. Frackowiak, K. L. Leenders, G. Sawle, J. C. Rothwell, and et al., 1992, Transplantation of fetal dopamine neurons in Parkinson's disease: one-year clinical and neurophysiological observations in two patients with putaminal implants: Ann Neurol, v. 31, p. 155-65.
Liou, H. H., M. C. Tsai, C. J. Chen, J. S. Jeng, Y. C. Chang, S. Y. Chen, and R. C. Chen, 1997, Environmental risk factors and Parkinson's disease: a case-control study in Taiwan: Neurology, v. 48, p. 1583-8.
Liscovitch, M., M. Czarny, G. Fiucci, and X. Tang, 2000, Phospholipase D: molecular and cell biology of a novel gene family: Biochem J, v. 345 Pt 3, p. 401-15.
Liss, B., and J. Roeper, 2008, Individual dopamine midbrain neurons: functional diversity and flexibility in health and disease: Brain Res Rev, v. 58, p. 314-21.
Lo Bianco, C., J. L. Ridet, B. L. Schneider, N. Deglon, and P. Aebischer, 2002, alpha -Synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease: Proc Natl Acad Sci U S A, v. 99, p. 10813-8.
Lotharius, J., S. Barg, P. Wiekop, C. Lundberg, H. K. Raymon, and P. Brundin, 2002, Effect of mutant alpha-synuclein on dopamine homeostasis in a new human mesencephalic cell line: J Biol Chem, v. 277, p. 38884-94.
Lotharius, J., and P. Brundin, 2002a, Impaired dopamine storage resulting from alpha-synuclein mutations may contribute to the pathogenesis of Parkinson's disease: Hum Mol Genet, v. 11, p. 2395-407.
Lotharius, J., and P. Brundin, 2002b, Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein: Nat Rev Neurosci, v. 3, p. 932-42.
Lowe, J., H. McDermott, M. Landon, R. J. Mayer, and K. D. Wilkinson, 1990, Ubiquitin carboxyl-terminal hydrolase (PGP 9.5) is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative diseases: J Pathol, v. 161, p. 153-60.
Macintosh, F. C., 1941, The distribution of acetylcholine in the peripheral and the central nervous system: J Physiol, v. 99, p. 436-42.
Manning-Bog, A. B., A. L. McCormack, J. Li, V. N. Uversky, A. L. Fink, and D. A. Di Monte, 2002, The herbicide paraquat causes up-regulation and aggregation

of alpha-synuclein in mice: paraquat and alpha-synuclein: J Biol Chem, v. 277, p. 1641-4.
Maraganore, D. M., M. de Andrade, A. Elbaz, M. J. Farrer, J. P. Ioannidis, R. Kruger, W. A. Rocca, N. K. Schneider, T. G. Lesnick, S. J. Lincoln, M. M. Hulihan, J. O. Aasly, T. Ashizawa, M. C. Chartier-Harlin, H. Checkoway, C. Ferrarese, G. Hadjigeorgiou, N. Hattori, H. Kawakami, J. C. Lambert, T. Lynch, G. D. Mellick, S. Papapetropoulos, A. Parsian, A. Quattrone, O. Riess, E. K. Tan, and C. Van Broeckhoven, 2006, Collaborative analysis of alpha-synuclein gene promoter variability and Parkinson disease: Jama, v. 296, p. 661-70.
Maraganore, D. M., M. de Andrade, T. G. Lesnick, K. J. Strain, M. J. Farrer, W. A. Rocca, P. V. Pant, K. A. Frazer, D. R. Cox, and D. G. Ballinger, 2005, High-resolution whole-genome association study of Parkinson disease: Am J Hum Genet, v. 77, p. 685-93.
Marek, K., D. Jennings, and J. Seibyl, 2002, Do dopamine agonists or levodopa modify Parkinson's disease progression?: Eur J Neurol, v. 9 Suppl 3, p. 15-22.
Marks, W. J., Jr., J. L. Ostrem, L. Verhagen, P. A. Starr, P. S. Larson, R. A. Bakay, R. Taylor, D. A. Cahn-Weiner, A. J. Stoessl, C. W. Olanow, and R. T. Bartus, 2008, Safety and tolerability of intraputaminal delivery of CERE-120 (adeno-associated virus serotype 2-neurturin) to patients with idiopathic Parkinson's disease: an open-label, phase I trial: Lancet Neurol, v. 7, p. 400-8.
Maroteaux, L., J. T. Campanelli, and R. H. Scheller, 1988, Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal: J Neurosci, v. 8, p. 2804-15.
Maroteaux, L., and R. H. Scheller, 1991, The rat brain synucleins; family of proteins transiently associated with neuronal membrane: Brain Res Mol Brain Res, v. 11, p. 335-43.
Marttila, R. J., J. Kaprio, M. Koskenvuo, and U. K. Rinne, 1988a, Parkinson's disease in a nationwide twin cohort: Neurology, v. 38, p. 1217-9.
Marttila, R. J., H. Lorentz, and U. K. Rinne, 1988b, Oxygen toxicity protecting enzymes in Parkinson's disease. Increase of superoxide dismutase-like activity in the substantia nigra and basal nucleus: J Neurol Sci, v. 86, p. 321-31.
Marx, F. P., C. Holzmann, K. M. Strauss, L. Li, O. Eberhardt, E. Gerhardt, M. R. Cookson, D. Hernandez, M. J. Farrer, J. Kachergus, S. Engelender, C. A. Ross, K. Berger, L. Schols, J. B. Schulz, O. Riess, and R. Kruger, 2003, Identification and functional characterization of a novel R621C mutation in the synphilin-1 gene in Parkinson's disease: Hum Mol Genet, v. 12, p. 1223-31.
Maskri, L., X. Zhu, S. Fritzen, K. Kuhn, C. Ullmer, P. Engels, M. Andriske, C. C. Stichel, and H. Lubbert, 2004, Influence of different promoters on the expression pattern of mutated human alpha-synuclein in transgenic mice: Neurodegener Dis, v. 1, p. 255-65.
Masliah, E., A. Iwai, M. Mallory, K. Ueda, and T. Saitoh, 1996, Altered presynaptic protein NACP is associated with plaque formation and neurodegeneration in Alzheimer's disease: Am J Pathol, v. 148, p. 201-10.
Masliah, E., E. Rockenstein, I. Veinbergs, M. Mallory, M. Hashimoto, A. Takeda, Y. Sagara, A. Sisk, and L. Mucke, 2000, Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders: Science, v. 287, p. 1265-9.
Matsuda, W., T. Furuta, K. C. Nakamura, H. Hioki, F. Fujiyama, R. Arai, and T. Kaneko, 2009, Single nigrostriatal dopaminergic neurons form widely spread

and highly dense axonal arborizations in the neostriatum: J Neurosci, v. 29, p. 444-53.
Matsuoka, Y., M. Vila, S. Lincoln, A. McCormack, M. Picciano, J. LaFrancois, X. Yu, D. Dickson, W. J. Langston, E. McGowan, M. Farrer, J. Hardy, K. Duff, S. Przedborski, and D. A. Di Monte, 2001, Lack of nigral pathology in transgenic mice expressing human alpha-synuclein driven by the tyrosine hydroxylase promoter: Neurobiol Dis, v. 8, p. 535-9.
Mayeux, R., J. Chen, E. Mirabello, K. Marder, K. Bell, G. Dooneief, L. Cote, and Y. Stern, 1990, An estimate of the incidence of dementia in idiopathic Parkinson's disease: Neurology, v. 40, p. 1513-7.
Mayeux, R., J. B. Williams, Y. Stern, and L. Cote, 1984, Depression and Parkinson's disease: Adv Neurol, v. 40, p. 241-50.
Mazzulli, J. R., A. J. Mishizen, B. I. Giasson, D. R. Lynch, S. A. Thomas, A. Nakashima, T. Nagatsu, A. Ota, and H. Ischiropoulos, 2006, Cytosolic catechols inhibit alpha-synuclein aggregation and facilitate the formation of intracellular soluble oligomeric intermediates: J Neurosci, v. 26, p. 10068-78.
McCormack, A. L., J. G. Atienza, L. C. Johnston, J. K. Andersen, S. Vu, and D. A. Di Monte, 2005, Role of oxidative stress in paraquat-induced dopaminergic cell degeneration: J Neurochem, v. 93, p. 1030-7.
McCormack, A. L., J. G. Atienza, J. W. Langston, and D. A. Di Monte, 2006, Decreased susceptibility to oxidative stress underlies the resistance of specific dopaminergic cell populations to paraquat-induced degeneration: Neuroscience, v. 141, p. 929-37.
McDonald, W. M., I. H. Richard, and M. R. DeLong, 2003, Prevalence, etiology, and treatment of depression in Parkinson's disease: Biol Psychiatry, v. 54, p. 363-75.
McDonnell, L., C. Maginnis, S. Lewis, N. Pickering, M. Antoniak, R. Hubbard, I. Lawson, and J. Britton, 2003, Occupational exposure to solvents and metals and Parkinson's disease: Neurology, v. 61, p. 716-7.
McNaught, K. S., and P. Jenner, 2001, Proteasomal function is impaired in substantia nigra in Parkinson's disease: Neurosci Lett, v. 297, p. 191-4.
Melrose, H. L., S. J. Lincoln, G. M. Tyndall, and M. J. Farrer, 2006, Parkinson's disease: a rethink of rodent models: Exp Brain Res, v. 173, p. 196-204.
Michel, P. P., and F. Hefti, 1990, Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture: J Neurosci Res, v. 26, p. 428-35.
Millar, J., J. A. Stamford, Z. L. Kruk, and R. M. Wightman, 1985, Electrochemical, pharmacological and electrophysiological evidence of rapid dopamine release and removal in the rat caudate nucleus following electrical stimulation of the median forebrain bundle: Eur J Pharmacol, v. 109, p. 341-8.
Miller, G. W., J. D. Erickson, J. T. Perez, S. N. Penland, D. C. Mash, D. B. Rye, and A. I. Levey, 1999, Immunochemical analysis of vesicular monoamine transporter (VMAT2) protein in Parkinson's disease: Exp Neurol, v. 156, p. 138-48.
Montastruc, J. L., M. E. Llau, O. Rascol, and J. M. Senard, 1994, Drug-induced parkinsonism: a review: Fundam Clin Pharmacol, v. 8, p. 293-306.
Mori, F., K. Tanji, M. Yoshimoto, H. Takahashi, and K. Wakabayashi, 2002, Demonstration of alpha-synuclein immunoreactivity in neuronal and glial cytoplasm in normal human brain tissue using proteinase K and formic acid pretreatment: Exp Neurol, v. 176, p. 98-104.

Mosharov, E. V., K. E. Larsen, E. Kanter, K. A. Phillips, K. Wilson, Y. Schmitz, D. E. Krantz, K. Kobayashi, R. H. Edwards, and D. Sulzer, 2009, Interplay between cytosolic dopamine, calcium, and alpha-synuclein causes selective death of substantia nigra neurons: Neuron, v. 62, p. 218-29.

Mosharov, E. V., R. G. Staal, J. Bove, D. Prou, A. Hananiya, D. Markov, N. Poulsen, K. E. Larsen, C. M. Moore, M. D. Troyer, R. H. Edwards, S. Przedborski, and D. Sulzer, 2006, Alpha-synuclein overexpression increases cytosolic catecholamine concentration: J Neurosci, v. 26, p. 9304-11.

Moussa, C. E., F. Mahmoodian, Y. Tomita, and A. Sidhu, 2008, Dopamine differentially induces aggregation of A53T mutant and wild type alpha-synuclein: insights into the protein chemistry of Parkinson's disease: Biochem Biophys Res Commun, v. 365, p. 833-9.

Murphy, D. D., S. M. Rueter, J. Q. Trojanowski, and V. M. Lee, 2000, Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons: J Neurosci, v. 20, p. 3214-20.

Murthy, V. N., and C. F. Stevens, 1998, Synaptic vesicles retain their identity through the endocytic cycle: Nature, v. 392, p. 497-501.

Nakajo, S., K. Omata, T. Aiuchi, T. Shibayama, I. Okahashi, H. Ochiai, Y. Nakai, K. Nakaya, and Y. Nakamura, 1990, Purification and characterization of a novel brain-specific 14-kDa protein: J Neurochem, v. 55, p. 2031-8.

Nakajo, S., S. Shioda, Y. Nakai, and K. Nakaya, 1994, Localization of phosphoneuroprotein 14 (PNP 14) and its mRNA expression in rat brain determined by immunocytochemistry and in situ hybridization: Brain Res Mol Brain Res, v. 27, p. 81-6.

Nauta, W. J., G. P. Smith, R. L. Faull, and V. B. Domesick, 1978, Efferent connections and nigral afferents of the nucleus accumbens septi in the rat: Neuroscience, v. 3, p. 385-401.

Nelson, E. L., C. L. Liang, C. M. Sinton, and D. C. German, 1996, Midbrain dopaminergic neurons in the mouse: computer-assisted mapping: J Comp Neurol, v. 369, p. 361-71.

Nemani, V. M., W. Lu, V. Berge, K. Nakamura, B. Onoa, M. K. Lee, F. A. Chaudhry, R. A. Nicoll, and R. H. Edwards, 2010, Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis: Neuron, v. 65, p. 66-79.

Neumann, M., P. J. Kahle, B. I. Giasson, L. Ozmen, E. Borroni, W. Spooren, V. Muller, S. Odoy, H. Fujiwara, M. Hasegawa, T. Iwatsubo, J. Q. Trojanowski, H. A. Kretzschmar, and C. Haass, 2002, Misfolded proteinase K-resistant hyperphosphorylated alpha-synuclein in aged transgenic mice with locomotor deterioration and in human alpha-synucleinopathies: J Clin Invest, v. 110, p. 1429-39.

Ninkina, N., O. Peters, S. Millership, H. Salem, H. van der Putten, and V. L. Buchman, 2009, Gamma-synucleinopathy: neurodegeneration associated with overexpression of the mouse protein: Hum Mol Genet, v. 18, p. 1779-94.

Nishioka, K., C. Wider, C. Vilarino-Guell, A. I. Soto-Ortolaza, S. J. Lincoln, J. M. Kachergus, B. Jasinska-Myga, O. A. Ross, A. Rajput, C. A. Robinson, T. J. Ferman, Z. K. Wszolek, D. W. Dickson, and M. J. Farrer, 2010, Association of alpha-, beta-, and gamma-Synuclein with diffuse lewy body disease: Arch Neurol, v. 67, p. 970-5.

Norris, E. H., B. I. Giasson, R. Hodara, S. Xu, J. Q. Trojanowski, H. Ischiropoulos, and V. M. Lee, 2005, Reversible inhibition of alpha-synuclein fibrillization by dopaminochrome-mediated conformational alterations: J Biol Chem, v. 280, p. 21212-9.
Nutt, J. G., 2001, Fluctuations in response to treatment for Parkinson's disease: Adv Neurol, v. 86, p. 361-6.
Nutt, J. G., and T. G. Nygaard, 2001, Response to levodopa treatment in dopa-responsive dystonia: Arch Neurol, v. 58, p. 905-10.
Obeso, J. A., M. C. Rodriguez-Oroz, C. G. Goetz, C. Marin, J. H. Kordower, M. Rodriguez, E. C. Hirsch, M. Farrer, A. H. Schapira, and G. Halliday, 2010, Missing pieces in the Parkinson's disease puzzle: Nat Med, v. 16, p. 653-61.
Ohtake, H., P. Limprasert, Y. Fan, O. Onodera, A. Kakita, H. Takahashi, L. T. Bonner, D. W. Tsuang, I. V. Murray, V. M. Lee, J. Q. Trojanowski, A. Ishikawa, J. Idezuka, M. Murata, T. Toda, T. D. Bird, J. B. Leverenz, S. Tsuji, and A. R. La Spada, 2004, Beta-synuclein gene alterations in dementia with Lewy bodies: Neurology, v. 63, p. 805-11.
Olanow, C. W., C. G. Goetz, J. H. Kordower, A. J. Stoessl, V. Sossi, M. F. Brin, K. M. Shannon, G. M. Nauert, D. P. Perl, J. Godbold, and T. B. Freeman, 2003, A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease: Ann Neurol, v. 54, p. 403-14.
Olanow, C. W., and J. Jankovic, 2005, Neuroprotective therapy in Parkinson's disease and motor complications: a search for a pathogenesis-targeted, disease-modifying strategy: Mov Disord, v. 20 Suppl 11, p. S3-10.
Olanow, C. W., J. H. Kordower, A. E. Lang, and J. A. Obeso, 2009, Dopaminergic transplantation for Parkinson's disease: current status and future prospects: Ann Neurol, v. 66, p. 591-6.
Oorschot, D. E., 1996, Total number of neurons in the neostriatal, pallidal, subthalamic, and substantia nigral nuclei of the rat basal ganglia: a stereological study using the cavalieri and optical disector methods: J Comp Neurol, v. 366, p. 580-99.
Ostrerova, N., L. Petrucelli, M. Farrer, N. Mehta, P. Choi, J. Hardy, and B. Wolozin, 1999, alpha-Synuclein shares physical and functional homology with 14-3-3 proteins: J Neurosci, v. 19, p. 5782-91.
Paganini-Hill, A., 2001, Risk factors for parkinson's disease: the leisure world cohort study: Neuroepidemiology, v. 20, p. 118-24.
Pankratz, N., J. B. Wilk, J. C. Latourelle, A. L. DeStefano, C. Halter, E. W. Pugh, K. F. Doheny, J. F. Gusella, W. C. Nichols, T. Foroud, and R. H. Myers, 2009, Genomewide association study for susceptibility genes contributing to familial Parkinson disease: Hum Genet, v. 124, p. 593-605.
Parent, A., and L. N. Hazrati, 1995, Functional anatomy of the basal ganglia. I. The cortico-basal ganglia-thalamo-cortical loop: Brain Res Brain Res Rev, v. 20, p. 91-127.
Park, J., S. Y. Kim, G. H. Cha, S. B. Lee, S. Kim, and J. Chung, 2005, Drosophila DJ-1 mutants show oxidative stress-sensitive locomotive dysfunction: Gene, v. 361, p. 133-9.
Park, J. Y., and P. T. Lansbury, Jr., 2003, Beta-synuclein inhibits formation of alpha-synuclein protofibrils: a possible therapeutic strategy against Parkinson's disease: Biochemistry, v. 42, p. 3696-700.
Park, S. M., H. Y. Jung, H. O. Kim, H. Rhim, S. R. Paik, K. C. Chung, J. H. Park, and J. Kim, 2002, Evidence that alpha-synuclein functions as a negative regulator

of Ca(++)-dependent alpha-granule release from human platelets: Blood, v. 100, p. 2506-14.
Parkinson, J., 1817, An essay on the shaking palsy: London, Sherwood Neely and Jones, 66 p.
Parkinson, J. A., M. C. Olmstead, L. H. Burns, T. W. Robbins, and B. J. Everitt, 1999, Dissociation in effects of lesions of the nucleus accumbens core and shell on appetitive pavlovian approach behavior and the potentiation of conditioned reinforcement and locomotor activity by D-amphetamine: J Neurosci, v. 19, p. 2401-11.
Parkkinen, L., T. Kauppinen, T. Pirttila, J. M. Autere, and I. Alafuzoff, 2005a, Alpha-synuclein pathology does not predict extrapyramidal symptoms or dementia: Ann Neurol, v. 57, p. 82-91.
Parkkinen, L., T. Pirttila, M. Tervahauta, and I. Alafuzoff, 2005b, Widespread and abundant alpha-synuclein pathology in a neurologically unimpaired subject: Neuropathology, v. 25, p. 304-14.
Parsons, T. D., S. A. Rogers, A. J. Braaten, S. P. Woods, and A. I. Troster, 2006, Cognitive sequelae of subthalamic nucleus deep brain stimulation in Parkinson's disease: a meta-analysis: Lancet Neurol, v. 5, p. 578-88.
Paxinos, G., and K. B. J. Franklin, 2001, The mouse brain in stereotaxic coordinates: San Diego, Calif. ; London, Academic, xxv, [264] p.
Paxinou, E., Q. Chen, M. Weisse, B. I. Giasson, E. H. Norris, S. M. Rueter, J. Q. Trojanowski, V. M. Lee, and H. Ischiropoulos, 2001, Induction of alpha-synuclein aggregation by intracellular nitrative insult: J Neurosci, v. 21, p. 8053-61.
Payton, J. E., R. J. Perrin, D. F. Clayton, and J. M. George, 2001, Protein-protein interactions of alpha-synuclein in brain homogenates and transfected cells: Brain Res Mol Brain Res, v. 95, p. 138-45.
Pendleton, R. G., F. Parvez, M. Sayed, and R. Hillman, 2002, Effects of pharmacological agents upon a transgenic model of Parkinson's disease in Drosophila melanogaster: J Pharmacol Exp Ther, v. 300, p. 91-6.
Peng, X., R. Tehranian, P. Dietrich, L. Stefanis, and R. G. Perez, 2005, Alpha-synuclein activation of protein phosphatase 2A reduces tyrosine hydroxylase phosphorylation in dopaminergic cells: J Cell Sci, v. 118, p. 3523-30.
Perez, R. G., J. C. Waymire, E. Lin, J. J. Liu, F. Guo, and M. J. Zigmond, 2002, A role for alpha-synuclein in the regulation of dopamine biosynthesis: J Neurosci, v. 22, p. 3090-9.
Perrin, R. J., W. S. Woods, D. F. Clayton, and J. M. George, 2000, Interaction of human alpha-Synuclein and Parkinson's disease variants with phospholipids. Structural analysis using site-directed mutagenesis: J Biol Chem, v. 275, p. 34393-8.
Piccini, P., and D. J. Brooks, 1999, Etiology of Parkinson's disease: contributions from 18F-DOPA positron emission tomography: Adv Neurol, v. 80, p. 227-31.
Pisani, A., P. Bonsi, D. Centonze, P. Gubellini, G. Bernardi, and P. Calabresi, 2003, Targeting striatal cholinergic interneurons in Parkinson's disease: focus on metabotropic glutamate receptors: Neuropharmacology, v. 45, p. 45-56.
Polymeropoulos, M. H., C. Lavedan, E. Leroy, S. E. Ide, A. Dehejia, A. Dutra, B. Pike, H. Root, J. Rubenstein, R. Boyer, E. S. Stenroos, S. Chandrasekharappa, A. Athanassiadou, T. Papapetropoulos, W. G. Johnson, A. M. Lazzarini, R. C. Duvoisin, G. Di Iorio, L. I. Golbe, and R. L. Nussbaum, 1997, Mutation in the

alpha-synuclein gene identified in families with Parkinson's disease: Science, v. 276, p. 2045-7.
Powers, K. M., D. M. Kay, S. A. Factor, C. P. Zabetian, D. S. Higgins, A. Samii, J. G. Nutt, A. Griffith, B. Leis, J. W. Roberts, E. D. Martinez, J. S. Montimurro, H. Checkoway, and H. Payami, 2008, Combined effects of smoking, coffee, and NSAIDs on Parkinson's disease risk: Mov Disord, v. 23, p. 88-95.
Ramirez, A., A. Heimbach, J. Grundemann, B. Stiller, D. Hampshire, L. P. Cid, I. Goebel, A. F. Mubaidin, A. L. Wriekat, J. Roeper, A. Al-Din, A. M. Hillmer, M. Karsak, B. Liss, C. G. Woods, M. I. Behrens, and C. Kubisch, 2006, Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase: Nat Genet, v. 38, p. 1184-91.
Rappley, I., A. D. Gitler, P. E. Selvy, M. J. LaVoie, B. D. Levy, H. A. Brown, S. Lindquist, and D. J. Selkoe, 2009, Evidence that alpha-synuclein does not inhibit phospholipase D: Biochemistry, v. 48, p. 1077-83.
Rascol, O., P. Payoux, J. Ferreira, and C. Brefel-Courbon, 2002, The management of patients with early Parkinson's disease: Parkinsonism Relat Disord, v. 9, p. 61-7.
Redmond, D. E., Jr., K. B. Bjugstad, Y. D. Teng, V. Ourednik, J. Ourednik, D. R. Wakeman, X. H. Parsons, R. Gonzalez, B. C. Blanchard, S. U. Kim, Z. Gu, S. A. Lipton, E. A. Markakis, R. H. Roth, J. D. Elsworth, J. R. Sladek, Jr., R. L. Sidman, and E. Y. Snyder, 2007, Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells: Proc Natl Acad Sci U S A, v. 104, p. 12175-80.
Reynolds, J. N., B. I. Hyland, and J. R. Wickens, 2001, A cellular mechanism of reward-related learning: Nature, v. 413, p. 67-70.
Rice, M. E., and S. J. Cragg, 2004, Nicotine amplifies reward-related dopamine signals in striatum: Nat Neurosci, v. 7, p. 583-4.
Richfield, E. K., M. J. Thiruchelvam, D. A. Cory-Slechta, C. Wuertzer, R. R. Gainetdinov, M. G. Caron, D. A. Di Monte, and H. J. Federoff, 2002, Behavioral and neurochemical effects of wild-type and mutated human alpha-synuclein in transgenic mice: Exp Neurol, v. 175, p. 35-48.
Riggs, J. E., 1993, Smoking and Alzheimer's disease: protective effect or differential survival bias?: Lancet, v. 342, p. 793-4.
Rizo, J., and C. Rosenmund, 2008, Synaptic vesicle fusion: Nat Struct Mol Biol, v. 15, p. 665-74.
Robertson, D. C., O. Schmidt, N. Ninkina, P. A. Jones, J. Sharkey, and V. L. Buchman, 2004, Developmental loss and resistance to MPTP toxicity of dopaminergic neurones in substantia nigra pars compacta of gamma-synuclein, alpha-synuclein and double alpha/gamma-synuclein null mutant mice: J Neurochem, v. 89, p. 1126-36.
Rockenstein, E., M. Mallory, M. Hashimoto, D. Song, C. W. Shults, I. Lang, and E. Masliah, 2002, Differential neuropathological alterations in transgenic mice expressing alpha-synuclein from the platelet-derived growth factor and Thy-1 promoters: J Neurosci Res, v. 68, p. 568-78.
Rosenberg, P. A., 1988, Catecholamine toxicity in cerebral cortex in dissociated cell culture: J Neurosci, v. 8, p. 2887-94.
Saggu, H., J. Cooksey, D. Dexter, F. R. Wells, A. Lees, P. Jenner, and C. D. Marsden, 1989, A selective increase in particulate superoxide dismutase activity in parkinsonian substantia nigra: J Neurochem, v. 53, p. 692-7.

Saint-Cyr, J. A., L. L. Trepanier, R. Kumar, A. M. Lozano, and A. E. Lang, 2000, Neuropsychological consequences of chronic bilateral stimulation of the subthalamic nucleus in Parkinson's disease: Brain, v. 123 (Pt 10), p. 2091-108.

Samii, A., J. G. Nutt, and B. R. Ransom, 2004, Parkinson's disease: Lancet, v. 363, p. 1783-93.

Satake, W., Y. Nakabayashi, I. Mizuta, Y. Hirota, C. Ito, M. Kubo, T. Kawaguchi, T. Tsunoda, M. Watanabe, A. Takeda, H. Tomiyama, K. Nakashima, K. Hasegawa, F. Obata, T. Yoshikawa, H. Kawakami, S. Sakoda, M. Yamamoto, N. Hattori, M. Murata, Y. Nakamura, and T. Toda, 2009, Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease: Nat Genet, v. 41, p. 1303-7.

Savitt, J. M., V. L. Dawson, and T. M. Dawson, 2006, Diagnosis and treatment of Parkinson disease: molecules to medicine: J Clin Invest, v. 116, p. 1744-54.

Schapira, A. H., J. M. Cooper, D. Dexter, J. B. Clark, P. Jenner, and C. D. Marsden, 1990, Mitochondrial complex I deficiency in Parkinson's disease: J Neurochem, v. 54, p. 823-7.

Schapira, A. H., J. M. Cooper, D. Dexter, P. Jenner, J. B. Clark, and C. D. Marsden, 1989, Mitochondrial complex I deficiency in Parkinson's disease: Lancet, v. 1, p. 1269.

Schapira, A. H., M. Gu, J. W. Taanman, S. J. Tabrizi, T. Seaton, M. Cleeter, and J. M. Cooper, 1998, Mitochondria in the etiology and pathogenesis of Parkinson's disease: Ann Neurol, v. 44, p. S89-98.

Schapira, A. H., V. M. Mann, J. M. Cooper, D. Krige, P. J. Jenner, and C. D. Marsden, 1992, Mitochondrial function in Parkinson's disease. The Royal Kings and Queens Parkinson's Disease Research Group: Ann Neurol, v. 32 Suppl, p. S116-24.

Schlossmacher, M. G., M. P. Frosch, W. P. Gai, M. Medina, N. Sharma, L. Forno, T. Ochiishi, H. Shimura, R. Sharon, N. Hattori, J. W. Langston, Y. Mizuno, B. T. Hyman, D. J. Selkoe, and K. S. Kosik, 2002, Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies: Am J Pathol, v. 160, p. 1655-67.

Schluter, O. M., F. Fornai, M. G. Alessandri, S. Takamori, M. Geppert, R. Jahn, and T. C. Sudhof, 2003, Role of alpha-synuclein in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism in mice: Neuroscience, v. 118, p. 985-1002.

Schmidt, A., M. Wolde, C. Thiele, W. Fest, H. Kratzin, A. V. Podtelejnikov, W. Witke, W. B. Huttner, and H. D. Soling, 1999, Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid: Nature, v. 401, p. 133-41.

Senior, S. L., N. Ninkina, R. Deacon, D. Bannerman, V. L. Buchman, S. J. Cragg, and R. Wade-Martins, 2008, Increased striatal dopamine release and hyperdopaminergic-like behaviour in mice lacking both alpha-synuclein and gamma-synuclein: Eur J Neurosci, v. 27, p. 947-57.

Sharon, R., I. Bar-Joseph, M. P. Frosch, D. M. Walsh, J. A. Hamilton, and D. J. Selkoe, 2003, The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson's disease: Neuron, v. 37, p. 583-95.

Sharon, R., M. S. Goldberg, I. Bar-Josef, R. A. Betensky, J. Shen, and D. J. Selkoe, 2001, alpha-Synuclein occurs in lipid-rich high molecular weight complexes,

binds fatty acids, and shows homology to the fatty acid-binding proteins: Proc Natl Acad Sci U S A, v. 98, p. 9110-5.
Shibasaki, Y., D. A. Baillie, D. St Clair, and A. J. Brookes, 1995, High-resolution mapping of SNCA encoding alpha-synuclein, the non-A beta component of Alzheimer's disease amyloid precursor, to human chromosome 4q21.3-->q22 by fluorescence in situ hybridization: Cytogenet Cell Genet, v. 71, p. 54-5.
Shibayama-Imazu, T., I. Okahashi, K. Omata, S. Nakajo, H. Ochiai, Y. Nakai, T. Hama, Y. Nakamura, and K. Nakaya, 1993, Cell and tissue distribution and developmental change of neuron specific 14 kDa protein (phosphoneuroprotein 14): Brain Res, v. 622, p. 17-25.
Shimura, H., N. Hattori, S. Kubo, Y. Mizuno, S. Asakawa, S. Minoshima, N. Shimizu, K. Iwai, T. Chiba, K. Tanaka, and T. Suzuki, 2000, Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase: Nat Genet, v. 25, p. 302-5.
Silver, D. E., and S. Ruggieri, 1998, Initiating therapy for Parkinson's disease: Neurology, v. 50, p. S18-22; discussion S44-8.
Silvestri, L., V. Caputo, E. Bellacchio, L. Atorino, B. Dallapiccola, E. M. Valente, and G. Casari, 2005, Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism: Hum Mol Genet, v. 14, p. 3477-92.
Simon-Sanchez, J., C. Schulte, J. M. Bras, M. Sharma, J. R. Gibbs, D. Berg, C. Paisan-Ruiz, P. Lichtner, S. W. Scholz, D. G. Hernandez, R. Kruger, M. Federoff, C. Klein, A. Goate, J. Perlmutter, M. Bonin, M. A. Nalls, T. Illig, C. Gieger, H. Houlden, M. Steffens, M. S. Okun, B. A. Racette, M. R. Cookson, K. D. Foote, H. H. Fernandez, B. J. Traynor, S. Schreiber, S. Arepalli, R. Zonozi, K. Gwinn, M. van der Brug, G. Lopez, S. J. Chanock, A. Schatzkin, Y. Park, A. Hollenbeck, J. Gao, X. Huang, N. W. Wood, D. Lorenz, G. Deuschl, H. Chen, O. Riess, J. A. Hardy, A. B. Singleton, and T. Gasser, 2009, Genome-wide association study reveals genetic risk underlying Parkinson's disease: Nat Genet, v. 41, p. 1308-12.
Singh, N., V. Pillay, and Y. E. Choonara, 2007, Advances in the treatment of Parkinson's disease: Prog Neurobiol, v. 81, p. 29-44.
Singleton, A. B., M. Farrer, J. Johnson, A. Singleton, S. Hague, J. Kachergus, M. Hulihan, T. Peuralinna, A. Dutra, R. Nussbaum, S. Lincoln, A. Crawley, M. Hanson, D. Maraganore, C. Adler, M. R. Cookson, M. Muenter, M. Baptista, D. Miller, J. Blancato, J. Hardy, and K. Gwinn-Hardy, 2003, alpha-Synuclein locus triplication causes Parkinson's disease: Science, v. 302, p. 841.
Smeyne, M., Y. Jiao, K. R. Shepherd, and R. J. Smeyne, 2005, Glia cell number modulates sensitivity to MPTP in mice: Glia, v. 52, p. 144-52.
Smith, W. W., H. Jiang, Z. Pei, Y. Tanaka, H. Morita, A. Sawa, V. L. Dawson, T. M. Dawson, and C. A. Ross, 2005, Endoplasmic reticulum stress and mitochondrial cell death pathways mediate A53T mutant alpha-synuclein-induced toxicity: Hum Mol Genet, v. 14, p. 3801-11.
Soldner, F., D. Hockemeyer, C. Beard, Q. Gao, G. W. Bell, E. G. Cook, G. Hargus, A. Blak, O. Cooper, M. Mitalipova, O. Isacson, and R. Jaenisch, 2009, Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors: Cell, v. 136, p. 964-77.
Sopher, B. L., K. L. Koszdin, M. E. McClain, S. B. Myrick, R. A. Martinez, A. C. Smith, and A. R. La Spada, 2001, Genomic organization, chromosome

location, and expression analysis of mouse beta-synuclein, a candidate for involvement in neurodegeneration: Cytogenet Cell Genet, v. 93, p. 117-23.
Souza, J. M., B. I. Giasson, Q. Chen, V. M. Lee, and H. Ischiropoulos, 2000, Dityrosine cross-linking promotes formation of stable alpha -synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies: J Biol Chem, v. 275, p. 18344-9.
Specht, C. G., and R. Schoepfer, 2001, Deletion of the alpha-synuclein locus in a subpopulation of C57BL/6J inbred mice: BMC Neurosci, v. 2, p. 11.
Spillantini, M. G., R. A. Crowther, R. Jakes, M. Hasegawa, and M. Goedert, 1998, alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies: Proc Natl Acad Sci U S A, v. 95, p. 6469-73.
Spillantini, M. G., A. Divane, and M. Goedert, 1995, Assignment of human alpha-synuclein (SNCA) and beta-synuclein (SNCB) genes to chromosomes 4q21 and 5q35: Genomics, v. 27, p. 379-81.
Spillantini, M. G., M. L. Schmidt, V. M. Lee, J. Q. Trojanowski, R. Jakes, and M. Goedert, 1997, Alpha-synuclein in Lewy bodies: Nature, v. 388, p. 839-40.
Stamford, J. A., 1985, In vivo voltammetry: promise and perspective: Brain Res, v. 357, p. 119-35.
Stamford, J. A., Z. Kruk, and J. Millar, 1986a, An in vivo voltammetric comparison of the effects of three psychomotor stimulants on electrically evoked neostriatal dopamine release: Brain Res, v. 366, p. 350-3.
Stamford, J. A., Z. L. Kruk, and J. Millar, 1986b, Measurement of stimulated dopamine release in the rat by in vivo voltammetry: the influence of stimulus duration on drug responses: Neurosci Lett, v. 69, p. 70-3.
Stamford, J. A., Z. L. Kruk, and J. Millar, 1986c, Sub-second striatal dopamine release measured by in vivo voltammetry: Brain Res, v. 381, p. 351-5.
Stamford, J. A., Z. L. Kruk, J. Millar, and R. M. Wightman, 1984, Striatal dopamine uptake in the rat: in vivo analysis by fast cyclic voltammetry: Neurosci Lett, v. 51, p. 133-8.
Stefanis, L., K. E. Larsen, H. J. Rideout, D. Sulzer, and L. A. Greene, 2001, Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death: J Neurosci, v. 21, p. 9549-60.
Steidl, J. V., T. Gomez-Isla, A. Mariash, K. H. Ashe, and L. M. Boland, 2003, Altered short-term hippocampal synaptic plasticity in mutant alpha-synuclein transgenic mice: Neuroreport, v. 14, p. 219-23.
Strauss, K. M., L. M. Martins, H. Plun-Favreau, F. P. Marx, S. Kautzmann, D. Berg, T. Gasser, Z. Wszolek, T. Muller, A. Bornemann, H. Wolburg, J. Downward, O. Riess, J. B. Schulz, and R. Kruger, 2005, Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease: Hum Mol Genet, v. 14, p. 2099-111.
Sudhof, T. C., and J. E. Rothman, 2009, Membrane fusion: grappling with SNARE and SM proteins: Science, v. 323, p. 474-7.
Sugama, S., L. Yang, B. P. Cho, L. A. DeGiorgio, S. Lorenzl, D. S. Albers, M. F. Beal, B. T. Volpe, and T. H. Joh, 2003, Age-related microglial activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurodegeneration in C57BL/6 mice: Brain Res, v. 964, p. 288-94.
Sung, Y. H., and D. Eliezer, 2007, Residual structure, backbone dynamics, and interactions within the synuclein family: J Mol Biol, v. 372, p. 689-707.

Surmeier, D. J., 2007, Calcium, ageing, and neuronal vulnerability in Parkinson's disease: Lancet Neurol, v. 6, p. 933-8.
Surmeier, D. J., 2010, alpha-Synuclein at the synaptic gate: Neuron, v. 65, p. 3-4.
Swerdlow, R. H., J. K. Parks, S. W. Miller, J. B. Tuttle, P. A. Trimmer, J. P. Sheehan, J. P. Bennett, Jr., R. E. Davis, and W. D. Parker, Jr., 1996, Origin and functional consequences of the complex I defect in Parkinson's disease: Ann Neurol, v. 40, p. 663-71.
Szczypka, M. S., M. A. Rainey, D. S. Kim, W. A. Alaynick, B. T. Marck, A. M. Matsumoto, and R. D. Palmiter, 1999, Feeding behavior in dopamine-deficient mice: Proc Natl Acad Sci U S A, v. 96, p. 12138-43.
Taira, T., Y. Saito, T. Niki, S. M. Iguchi-Ariga, K. Takahashi, and H. Ariga, 2004, DJ-1 has a role in antioxidative stress to prevent cell death: EMBO Rep, v. 5, p. 213-8.
Takahashi, H., and K. Wakabayashi, 2001, The cellular pathology of Parkinson's disease: Neuropathology, v. 21, p. 315-22.
Takahashi, M., and T. Yamada, 1999, Viral etiology for Parkinson's disease--a possible role of influenza A virus infection: Jpn J Infect Dis, v. 52, p. 89-98.
Takahashi, R., Y. Imai, N. Hattori, and Y. Mizuno, 2003, Parkin and endoplasmic reticulum stress: Ann N Y Acad Sci, v. 991, p. 101-6.
Tanner, C. M., R. Ottman, S. M. Goldman, J. Ellenberg, P. Chan, R. Mayeux, and J. W. Langston, 1999, Parkinson disease in twins: an etiologic study: Jama, v. 281, p. 341-6.
Tehranian, R., S. E. Montoya, A. D. Van Laar, T. G. Hastings, and R. G. Perez, 2006, Alpha-synuclein inhibits aromatic amino acid decarboxylase activity in dopaminergic cells: J Neurochem, v. 99, p. 1188-96.
Thiruchelvam, M., A. McCormack, E. K. Richfield, R. B. Baggs, A. W. Tank, D. A. Di Monte, and D. A. Cory-Slechta, 2003, Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype: Eur J Neurosci, v. 18, p. 589-600.
Thomas, B., and M. F. Beal, 2007, Parkinson's disease: Hum Mol Genet, v. 16 Spec No. 2, p. R183-94.
Tompkins, M. M., and W. D. Hill, 1997, Contribution of somal Lewy bodies to neuronal death: Brain Res, v. 775, p. 24-9.
Touchman, J. W., A. Dehejia, O. Chiba-Falek, D. E. Cabin, J. R. Schwartz, B. M. Orrison, M. H. Polymeropoulos, and R. L. Nussbaum, 2001, Human and mouse alpha-synuclein genes: comparative genomic sequence analysis and identification of a novel gene regulatory element: Genome Res, v. 11, p. 78-86.
Tsigelny, I. F., P. Bar-On, Y. Sharikov, L. Crews, M. Hashimoto, M. A. Miller, S. H. Keller, O. Platoshyn, J. X. Yuan, and E. Masliah, 2007, Dynamics of alpha-synuclein aggregation and inhibition of pore-like oligomer development by beta-synuclein: Febs J, v. 274, p. 1862-77.
Ueda, K., H. Fukushima, E. Masliah, Y. Xia, A. Iwai, M. Yoshimoto, D. A. Otero, J. Kondo, Y. Ihara, and T. Saitoh, 1993, Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease: Proc Natl Acad Sci U S A, v. 90, p. 11282-6.
Uhl, G. R., 1998, Hypothesis: the role of dopaminergic transporters in selective vulnerability of cells in Parkinson's disease: Ann Neurol, v. 43, p. 555-60.

Usuda, I., K. Tanaka, and T. Chiba, 1998, Efferent projections of the nucleus accumbens in the rat with special reference to subdivision of the nucleus: biotinylated dextran amine study: Brain Res, v. 797, p. 73-93.
Uversky, V. N., 2003, A protein-chameleon: conformational plasticity of alpha-synuclein, a disordered protein involved in neurodegenerative disorders: J Biomol Struct Dyn, v. 21, p. 211-34.
Uversky, V. N., 2007, Neuropathology, biochemistry, and biophysics of alpha-synuclein aggregation: J Neurochem, v. 103, p. 17-37.
Uversky, V. N., J. Li, and A. L. Fink, 2001, Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular NK between Parkinson's disease and heavy metal exposure: J Biol Chem, v. 276, p. 44284-96.
Uversky, V. N., J. Li, P. Souillac, I. S. Millett, S. Doniach, R. Jakes, M. Goedert, and A. L. Fink, 2002, Biophysical properties of the synucleins and their propensities to fibrillate: inhibition of alpha-synuclein assembly by beta- and gamma-synucleins: J Biol Chem, v. 277, p. 11970-8.
Valente, E. M., P. M. Abou-Sleiman, V. Caputo, M. M. Muqit, K. Harvey, S. Gispert, Z. Ali, D. Del Turco, A. R. Bentivoglio, D. G. Healy, A. Albanese, R. Nussbaum, R. Gonzalez-Maldonado, T. Deller, S. Salvi, P. Cortelli, W. P. Gilks, D. S. Latchman, R. J. Harvey, B. Dallapiccola, G. Auburger, and N. W. Wood, 2004, Hereditary early-onset Parkinson's disease caused by mutations in PINK1: Science, v. 304, p. 1158-60.
van der Putten, H., K. H. Wiederhold, A. Probst, S. Barbieri, C. Mistl, S. Danner, S. Kauffmann, K. Hofele, W. P. Spooren, M. A. Ruegg, S. Lin, P. Caroni, B. Sommer, M. Tolnay, and G. Bilbe, 2000, Neuropathology in mice expressing human alpha-synuclein: J Neurosci, v. 20, p. 6021-9.
Vijitruth, R., M. Liu, D. Y. Choi, X. V. Nguyen, R. L. Hunter, and G. Bing, 2006, Cyclooxygenase-2 mediates microglial activation and secondary dopaminergic cell death in the mouse MPTP model of Parkinson's disease: J Neuroinflammation, v. 3, p. 6.
Volles, M. J., and P. T. Lansbury, Jr., 2002, Vesicle permeabilization by protofibrillar alpha-synuclein is sensitive to Parkinson's disease-linked mutations and occurs by a pore-like mechanism: Biochemistry, v. 41, p. 4595-602.
Volles, M. J., S. J. Lee, J. C. Rochet, M. D. Shtilerman, T. T. Ding, J. C. Kessler, and P. T. Lansbury, Jr., 2001, Vesicle permeabilization by protofibrillar alpha-synuclein: implications for the pathogenesis and treatment of Parkinson's disease: Biochemistry, v. 40, p. 7812-9.
Voorn, P., L. J. Vanderschuren, H. J. Groenewegen, T. W. Robbins, and C. M. Pennartz, 2004, Putting a spin on the dorsal-ventral divide of the striatum: Trends Neurosci, v. 27, p. 468-74.
Wakabayashi, K., Y. S. Piao, S. Hayashi, A. Kakita, M. Yamada, and H. Takahashi, 2001, Ubiquitinated neuronal inclusions in the neostriatum in patients with amyotrophic lateral sclerosis with and without dementia--a study of 60 patients 31 to 87 years of age: Clin Neuropathol, v. 20, p. 47-52.
Walters, T. L., I. Irwin, K. Delfani, J. W. Langston, and A. M. Janson, 1999, Diethyldithiocarbamate causes nigral cell loss and dopamine depletion with nontoxic doses of MPTP: Exp Neurol, v. 156, p. 62-70.
Wang, H. Q., and R. Takahashi, 2007, Expanding insights on the involvement of endoplasmic reticulum stress in Parkinson's disease: Antioxid Redox Signal, v. 9, p. 553-61.

Ward, C. D., R. C. Duvoisin, S. E. Ince, J. D. Nutt, R. Eldridge, and D. B. Calne, 1983, Parkinson's disease in 65 pairs of twins and in a set of quadruplets: Neurology, v. 33, p. 815-24.
Weinreb, P. H., W. Zhen, A. W. Poon, K. A. Conway, and P. T. Lansbury, Jr., 1996, NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded: Biochemistry, v. 35, p. 13709-15.
Wersinger, C., A. Jeannotte, and A. Sidhu, 2006a, Attenuation of the norepinephrine transporter activity and trafficking via interactions with alpha-synuclein: Eur J Neurosci, v. 24, p. 3141-52.
Wersinger, C., D. Prou, P. Vernier, and A. Sidhu, 2003, Modulation of dopamine transporter function by alpha-synuclein is altered by impairment of cell adhesion and by induction of oxidative stress: Faseb J, v. 17, p. 2151-3.
Wersinger, C., M. Rusnak, and A. Sidhu, 2006b, Modulation of the trafficking of the human serotonin transporter by human alpha-synuclein: Eur J Neurosci, v. 24, p. 55-64.
Wersinger, C., and A. Sidhu, 2003, Attenuation of dopamine transporter activity by alpha-synuclein: Neurosci Lett, v. 340, p. 189-92.
Woolf, N. J., 1991, Cholinergic systems in mammalian brain and spinal cord: Prog Neurobiol, v. 37, p. 475-524.
Xia, Y., T. Saitoh, K. Ueda, S. Tanaka, X. Chen, M. Hashimoto, L. Hsu, C. Conrad, M. Sundsmo, M. Yoshimoto, L. Thal, R. Katzman, and E. Masliah, 2001, Characterization of the human alpha-synuclein gene: Genomic structure, transcription start site, promoter region and polymorphisms: J Alzheimers Dis, v. 3, p. 485-494.
Xu, J., S. Y. Kao, F. J. Lee, W. Song, L. W. Jin, and B. A. Yankner, 2002a, Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease: Nat Med, v. 8, p. 600-6.
Xu, P. Y., R. Liang, J. Jankovic, C. Hunter, Y. X. Zeng, T. Ashizawa, D. Lai, and W. D. Le, 2002b, Association of homozygous 7048G7049 variant in the intron six of Nurr1 gene with Parkinson's disease: Neurology, v. 58, p. 881-4.
Yaffe, M. B., 2002, How do 14-3-3 proteins work?-- Gatekeeper phosphorylation and the molecular anvil hypothesis: FEBS Lett, v. 513, p. 53-7.
Yamaguchi, H., and J. Shen, 2007, Absence of dopaminergic neuronal degeneration and oxidative damage in aged DJ-1-deficient mice: Mol Neurodegener, v. 2, p. 10.
Yamamoto, M., 1998, Do dopamine agonists provide neuroprotection?: Neurology, v. 51, p. S10-2.
Yang, Y., S. Gehrke, M. E. Haque, Y. Imai, J. Kosek, L. Yang, M. F. Beal, I. Nishimura, K. Wakamatsu, S. Ito, R. Takahashi, and B. Lu, 2005, Inactivation of Drosophila DJ-1 leads to impairments of oxidative stress response and phosphatidylinositol 3-kinase/Akt signaling: Proc Natl Acad Sci U S A, v. 102, p. 13670-5.
Yavich, L., M. Oksman, H. Tanila, P. Kerokoski, M. Hiltunen, T. van Groen, J. Puolivali, P. T. Mannisto, A. Garcia-Horsman, E. MacDonald, K. Beyreuther, T. Hartmann, and P. Jakala, 2005, Locomotor activity and evoked dopamine release are reduced in mice overexpressing A30P-mutated human alpha-synuclein: Neurobiol Dis, v. 20, p. 303-13.
Yavich, L., H. Tanila, S. Vepsalainen, and P. Jakala, 2004, Role of alpha-synuclein in presynaptic dopamine recruitment: J Neurosci, v. 24, p. 11165-70.

Yu, S., X. Zuo, Y. Li, C. Zhang, M. Zhou, Y. A. Zhang, K. Ueda, and P. Chan, 2004, Inhibition of tyrosine hydroxylase expression in alpha-synuclein-transfected dopaminergic neuronal cells: Neurosci Lett, v. 367, p. 34-9.

Zarranz, J. J., J. Alegre, J. C. Gomez-Esteban, E. Lezcano, R. Ros, I. Ampuero, L. Vidal, J. Hoenicka, O. Rodriguez, B. Atares, V. Llorens, E. Gomez Tortosa, T. del Ser, D. G. Munoz, and J. G. de Yebenes, 2004, The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia: Ann Neurol, v. 55, p. 164-73.

Zeng, B. Y., A. D. Medhurst, M. Jackson, S. Rose, and P. Jenner, 2005, Proteasomal activity in brain differs between species and brain regions and changes with age: Mech Ageing Dev, v. 126, p. 760-6.

Zhang, H., and D. Sulzer, 2004, Frequency-dependent modulation of dopamine release by nicotine: Nat Neurosci, v. 7, p. 581-2.

Zhang, L., V. L. Dawson, and T. M. Dawson, 2006, Role of nitric oxide in Parkinson's disease: Pharmacol Ther, v. 109, p. 33-41.

Zheng, K., B. Heydari, and D. K. Simon, 2003, A common NURR1 polymorphism associated with Parkinson disease and diffuse Lewy body disease: Arch Neurol, v. 60, p. 722-5.

Zhou, F. M., Y. Liang, and J. A. Dani, 2001, Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum: Nat Neurosci, v. 4, p. 1224-9.

Zhou, Q. Y., and R. D. Palmiter, 1995, Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic: Cell, v. 83, p. 1197-209.

Zhou, Y., G. Gu, D. R. Goodlett, T. Zhang, C. Pan, T. J. Montine, K. S. Montine, R. H. Aebersold, and J. Zhang, 2004, Analysis of alpha-synuclein-associated proteins by quantitative proteomics: J Biol Chem, v. 279, p. 39155-64.

Zhu, M., J. Li, and A. L. Fink, 2003, The association of alpha-synuclein with membranes affects bilayer structure, stability, and fibril formation: J Biol Chem, v. 278, p. 40186-97.

Zhuang, X., R. S. Oosting, S. R. Jones, R. R. Gainetdinov, G. W. Miller, M. G. Caron, and R. Hen, 2001, Hyperactivity and impaired response habituation in hyperdopaminergic mice: Proc Natl Acad Sci U S A, v. 98, p. 1982-7.

Zimprich, A., B. Muller-Myhsok, M. Farrer, P. Leitner, M. Sharma, M. Hulihan, P. Lockhart, A. Strongosky, J. Kachergus, D. B. Calne, J. Stoessl, R. J. Uitti, R. F. Pfeiffer, C. Trenkwalder, N. Homann, E. Ott, K. Wenzel, F. Asmus, J. Hardy, Z. Wszolek, and T. Gasser, 2004, The PARK8 locus in autosomal dominant parkinsonism: confirmation of linkage and further delineation of the disease-containing interval: Am J Hum Genet, v. 74, p. 11-9.

Zou, L., J. Xu, J. Jankovic, Y. He, S. H. Appel, and W. Le, 2000, Pramipexole inhibits lipid peroxidation and reduces injury in the substantia nigra induced by the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in C57BL/6 mice: Neurosci Lett, v. 281, p. 167-70.