Synucleins in the Midbrain Dopaminergic System - the Role in Health and Disease

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APPENDIX 1:

Specimen layout for Thesis Summary and Declaration/Statements page to be included in a Thesis

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Abstract

Synucleinopathies are a group of diseases characterised by the presence of insoluble aggregated forms of α-synuclein. The most common of these diseases is Parkinson's disease (PD) which affects approximately 1% of the UK population over the age of 60. Alpha-synuclein has also been linked to the disease through familial mutations and genome wide association studies as well as by its presence in sporadic cases. Although solid evidence exists for a role of α-synuclein in PD, it remains unclear as to how this protein exerts its toxicity on neurons and exactly how this leads to the cell death characteristic of this neurodegenerative disease.

Alpha-synuclein belongs to a family of three proteins which also includes β- and γ-synuclein. These three proteins are highly homologous and evolutionarily conserved, however none of them have a well defined function. Evidence suggests a role for these proteins in synaptic vesicle dynamics but a more specific function remains to be unveiled. However, due to the considerable degree of homology across these three proteins, knockout models have been considered to allow functional compensation of the missing synuclein protein through one of the remaining family members. This has hindered studies from elucidating not only the role of α-synuclein but also β- and γ-synuclein. To overcome this problem triple synuclein knockout mice have been produced and characterised, as described in this thesis. As expected studies of these animals revealed no alterations in the number of dopaminergic neurons in either the substantia nigra pars compacta or ventral tegmental area. Despite this, a significant deficit in striatal dopamine concentrations was detected, regardless of the fact that the levels and function of tyrosine hydroxylase being normal. As well as this triple synuclein null mice were demonstrated to be hyperdopaminergic through various behavioural tests. Work employing physcostimulants and, through a collaboration, using fast scan cyclic voltametry suggested a role for these proteins in normal dopamine release dynamics at the level of the synaptic vesicle.

A previous body of work has indicated that the loss of α- and/or γ-synuclein is able to provide a degree of resistance against the toxic affects of the dopaminergic neurotoxin MPTP. It was therefore hypothesised that the triple synuclein null animals would also display resistance to this toxin. However, these animals were shown to be more sensitive than wild type controls. Importantly it was apparent that animals lacking β-synuclein alone or in combination with other synucleins were the most sensitive to this toxin. Further work revealed a significant deficit in the ability of triple synuclein null mice to store dopamine in their synaptic vesicles.

This may explain the sensitivity to MPP^+, the active metabolite of MPTP, due to the fact it cannot be efficiently stored in synaptic vesicles, which restricts the toxins access to the mitochondria where it normally inhibits complex I, thus leading to cell death. When recombinant β-synuclein was reintroduced the deficit in synaptic vesicle dopamine uptake could be restored. However, β-synuclein can not do this alone and requires incubation with cytosolic factors, suggesting it acts as a chaperone in this role. This may explain why lines of synuclein null mice that specifically have the absence of β-synuclein apparently fair least well when exposed to MPTP.

Finally, in order to assess the extent to which a loss of function role of α-synuclein leads to pathological alteration at the synapse an entirely novel conditional α-synuclein knockout mouse model was produced. Currently no ideal model exists to answer this question as conventional knockout models are based on the knockout of the protein in development. This may allow functional compensatory mechanisms to be established which can be overcome with a conditional knockout approach. As well as this it is important to assess this loss in an aged nervous system, as PD is a disease of aging. It is likely that, as α-synuclein forms insoluble Lewy bodies and undergoes abnormal posttranslational modifications, the amount of normally functioning protein at the synapse is depleted, therefore allowing a loss of function effect to develop. It is hoped this model will allow new insight into the early disease process.

Overall this work further contributes to a body of evidence that suggests the synucleins play an important role in synaptic dopamine handling, particularly at the synaptic vesicle level. It is hoped that the newly established conditional α-synuclein knockout model will produce a new perspective on the loss of function role of α-synuclein in early disease development, an avenue that has yet to be fully explored.

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Abbreviations

AADC - Aromatic L-Amino acid Decarboxylase

ACTB - β-actin

ALS - Amyotrophic Lateral Sclerosis

ATP – Adenosine Triphosphate

CCCP - Carbonyl Cyanidem-Chlorophenylhydrazone

CMA – Chaperone Mediated Autophagy

CMA - Chaperone Mediated Autophagy

CNS - Central Nervous System

CPu – Caudate Putamen

Cre – Cre recombinase

CSP-α - Cysteine String Protein Alpha

DA – Dopamine

DAB - 3, 3'-Diaminobenzidine

dAMP - D-amphetamine

DAT – Dopamine Active Transporter

DOPAC - 3,4-Dihydroxyphenylacetic acid

ER - Oestrogen Receptor

ES - Embryonic Stem cells

FCV - Fast-scan Cyclic Voltammetry

FLP – Flippase

FRT - FLP Recombination Target

GBA – Glucosecerebrosidase

gDNA - Genomic DNA

GPe – External Globus Pallidus

GPi – Internal Globus Pallidus

GWAS - Genome Wide Association Studies

TKO – Triple knockout

HPLC - High-Performance Liquid Chromatography

HSK-tk - Herpes simplex virus thymidine kinase cassette

HVA - Homovanillic acid

INAD - Infantile Neuroaxonal Dystrophy

KO – Knockout

LBs – Lewy Bodies

LIF – Leukaemia Inhibitory Factor

LNs – Lewy Neurites

LoxP - Locus of crossing-over in P1

LRRK2 - Leucine-Rich Repeat Kinase 2

MAPT – Microtubule-Associated Protein Tau

MPP^+ - 1-methyl-4-phenylpyridinium

MPTP - 1-methyl-4-phenyl-1-2-3-6-tetrahyrdopyridine

mtDNA - Mitochondrial DNA

NAC - Non-Amyloid Component

NAc - Nucleus Accumbens

NBIA - Neurodegeneration with Brain Iron Accumulation

NMS – Non Motor Symptoms

NSAID - Non-Steroidal Anti-Inflammatory Drug

PARIS - Parkin Interacting Substrate

PD – Parkinson's disease

PDGFβ - Platelet Derived Growth Factor-β

PGK-1 - Phosphoglycerate Kinase 1

PINK1 - PTEN-Induced Novel Kinase 1

PRA1 - Prenylated Rab Accepter Protein 1

PrP – Prion Protein

Ptn - Putamen

RGCs - Retinal Ganglion Cells

SEM – Standard Error of the Mean

siRNA - Small Interfering RNA

SNARE – Soluble NSF Attachment Protein Receptor

SNCA –synuclein, alpha (non A4 component of amyloid precursor)

SNpc – Substantia Nigra pars compacta

STN – Subthalamic Nucleus

TH – Tyrosine Hydroxolase

UPS - Ubiquitin Proteosome System

VMAT – Vesicular Monoamine Transporter

VTA – Ventral Tegmental Area

WT – Wild Type

Chapter 1

Introduction

Parkinson's Disease

It is now almost 200 years since James Parkinson first described the disease that would later take on his name. He was fortunate to have recognised 6 individuals with similar symptoms who he was able to study closely taking careful histories. From these he was able to clearly state two of the prominent characteristics of the disease as well as describing many other now well recognised features. Firstly, that this was a disease of slow progress that over time caused progressive motor disability and secondly he clearly defined the resting tremor seen in these patients and stated that it was obviously different from other forms of shaking palsy previously described (Parkinson, 1817).

Since this first description, Parkinson's disease (PD) has become widely recognised as the second most common neurodegenerative disease after Alzheimer's disease (Ross *et al.*, 2008b). It is estimated to affect 0.3% of the population in industrialised countries and approximately 1% of the total population over 60 (Rajput, 1992, de Rijk *et al.*, 2000). This gives the lifetime risk of developing the disease at 1.5% (de Rijk *et al.*, 1995). In the UK it is estimated that 127,000 people suffer from the PD, a figure which is likely to increase with our aging society (Parkinson's UK, 2012). The majority of PD sufferers have a mean age of onset of 60 years but in approximately 10% of cases, known as early onset, this age is lowered to 45 years or younger. Following diagnosis of the disease there is an average duration of 15 years until death (Katzenschlager *et al.*, 2008, Samii *et al.*, 2004).

The economic burden of the disease is large, with one study estimating the direct annual cost of the disease on the healthcare system in the UK at £5,993 per year per patient if managed at home. However, should the patient require full time institutionalisation, which is often the case in late disease, this cost rises dramatically to £19,338. The cost of care has also been shown to relate to the stage of disease with patients in the early stages of disease having a care cost of £2971 per year where as at late stage this cost increases to £18,358 (Findley *et al.*, 2003, Findley, 2007). These financial patterns are set to rise with an aging population. Apart from the economic impact of PD, quality of life is significantly reduced for the patient and also has a great impact on the family and carers of the patient. It is therefore key that the disease is better understood allowing more effective treatments to be developed.

Clinical Features

Classical PD is characterised by the presence of the cardinal features which are bradykinesia, resting tremor, rigidity and postural instability (Factor and Weiner, 2008). For a clinical

diagnosis to be made the patient must present with 2 or more of the cardinal symptoms. However due to the current lack of diagnostic tests for PD the only true confirmation of the disease happens at autopsy in which 80-90% of clinical PD cases are confirmed (Litvan *et al.*, 2003). In most cases the disease course starts with impairment of dexterity with the gradual progression to stiffness and reduced arm swing when walking. However, these symptoms are rarely acknowledged by the patient who often attributes these small changes to the effects of aging. This often causes a lag period between the first symptoms and a diagnosis of PD being made (Lees *et al.*, 2009).

The characteristic resting tremor is seen in 70% of PD patients and normally presents unilaterally but may, in later stages of the disease, become bilateral. It resembles repetitive pill rolling between the thumb and index finger and has a low frequency of 4-7 Hz (Chaudhuri *et al.*, 2003). This differs from essential tremor as it only occurs at rest and is not problematic during activity. The term bradykinesia is used to describe the difficulty in initiating new movement and the general slowness at which activity is performed. In the earlier stages of the disease this tends to affect fine motor movement such as the fastening of buttons and it is also responsible for micrographia, another common feature of PD. As the disease progresses it affects more general movement with patients adopting a shuffling step and also freezing, in which they are unable to initiate any movement. Other symptoms associated with bradykinesia include facial passivity and problems with the laryngeal movement which leads to a monotone voice (Chaudhuri *et al.*, 2003, Samii *et al.*, 2004). PD patients often display rigidity in the trunk and limbs and this refers to the resistance displayed during passive movement, often described as giving a cogwheel type of movement. Through a combination of bradykinesia and rigidity comes the postural instability giving poor balance and bent posture leading to the characteristic gait seen in PD patients. These problems with posture are also an inherent cause of increased falls, a major cause of morbidity in Parkinson's.

PD also displays a vast and diverse array of non-motor symptoms (NMS) which are estimated to be present in up to 90% of PD patients and across all stages of the disease (Chaudhuri *et al.*, 2011). It is generally considered that NMS are under-reported by patients; a recent report suggested up to 62% of NMS were undeclared to healthcare professionals, due to embarrassment or because patients did not realise these symptoms were linked to PD (Mitra *et al.*, 2008). Some of these symptoms are considered to precede the onset of motor symptoms seen in PD such as olfactory dysfunction, rapid eye movement behaviour disorder (RBD), constipation and depression (Chaudhuri *et al.*, 2006). The fact that olfactory

dysfunction may occur as a primary symptom would lend support for the six stage pathological process that occurs in the brain during PD, as proposed by Braak and colleagues, in which the degeneration of the olfactory bulb and the anterior olfactory nucleus occurs in stage one, the earliest preclinical stage proposed (Braak et al., 2003). Witjas and colleagues suggested that the NMS be categorised into three broad subsets of symptoms: dysautonomic, mental (cognitive/phychiatirc) and sensory/pain. Using these criteria they created a questionnaire for PD patients and found that the most frequent NMS were anxiety (66%), drenching sweats (64%), slowness of thinking (58%) and irritability (54%) (Witjas et al., 2002). This is in agreement with the NMSquest study which found similar outcomes and also showed that patients found depression, sleep problems, pain, apathy, memory issues and balance dysfunctions more of a hindrance in everyday life than the motor symptoms of PD. This study also found that patients suffered an average 10-12 NMS, highlighting their importance in the disease. It is unlikely that these NMS are all caused by dopamine loss and the fact that they are generally fairly unresponsive to dopamine treatment is suggestive of the fact that they are likely caused by degeneration of other neurotransmitter pathways in the brain. This makes these symptoms difficult to treat and this often has a detrimental effect on the patients' quality of life.

Of the numerous non-motor symptoms developed throughout the disease process one of the most debilitating can be dementia. It is estimated that up to 80% of late stage PD patients display dementia (Aarsland *et al.*, 2003). This is considered to correlate with staging based on alpha-synuclein pathology proposed by Braak (Braak *et al.*, 2006). As well as dementia being prominent in late stages there are often noted cognitive alterations seen earlier in the disease process, which range from visuospatial problems to those concerned with executive functions (Uc *et al.*, 2005). It is thought that these symptoms do not arise due to the problems in the dopaminergic system, but in other areas of the brain. This is also inferred by the fact that L-DOPA therapy appears to have little effect on cognitive symptoms.

PD Pathogenesis

Classical PD has long been primarily defined by the selective loss of dopaminergic, neuromelanin laden, cells in the substantia nigra pars compacta (SNpc) which was first truly discussed by Greenfield and Bosanquet (Greenfield and Bosanquet, 1953). By the time that the patient presents with symptoms, approximately 70-80% of the SNpc dopaminergic population has already been lost with many of the remaining neurons containing Lewy bodies (LB) and appearing dystrophic (Chaudhuri *et al.*, 2003). This presents a problem for treatment

as these cells cannot be restored and so ideally an earlier diagnosis strategy is required that could be used in-hand with an effective treatment that would halt this loss, thus pausing the dopaminergic symptom progression. However this loss is not solely restricted to the SNpc as was initially believed. It is now apparent that dopaminergic cell loss is seen across other areas of the brain including the locus coeruleus, raphe nuclei, dorsal nuclei of the vagus and basalis of Meynert. Further to this, the loss is not reserved only to dopaminergic neurons, with other catecholaminergic neurons also being affected throughout the disease. Work carried out to measure the levels of both dopamine and noradrenaline in post-mortem brains of idiopathic and postenchephalitic PD patients compared to controls showed a dramatic 70-95% loss in the caudate nucleus and putamen (Ehringer and Hornykiewicz, 1960). The loss of dopaminergic neurons and therefore dopamine in the SNpc accounts for the characteristic movement related problems in PD but fails to explain the more complex non motor symptoms which are more likely explained through the loss of the non-dopaminergic neurons.

It has been proposed that there is a specific pattern to this cell loss, which is now widely accepted. The Braak hypothesis proposes a staging of PD through the use of α-synuclein immunstaining patterns throughout the diseased brain (Braak *et al.*, 2003). This has lead to the belief that PD may begin in the non-dopaminergic structures of the brainstem or possibly even in the peripheral autonomic nervous system, although this remains controversial, with the progressive appearance of abnormal α-synuclein into the midbrain and in late stages of the disease into the cortical regions. The principles of this hypothesis have since been confirmed in a number of other studies (Dickson *et al.*, 2010, Jellinger, 2003). More recently a study of almost 30 PD patients has shown the presence of α-synuclein inclusions in the spinal cord in all but one PD case with neuropathological stages 2-6 which were completely absent in control cases (Del Tredici and Braak, 2012). Further to this it has also been suggested that generally neurons with long unmyelinated axon projections are more susceptible to LB pathology with LB rarely being seen in neurons with myelinated axons (Braak and Tredici, 2004, Orimo *et al.*, 2011). How this spread of α-synuclein occurs throughout the nervous system is still a point of vivid debate, with beliefs that it may be able to spread in a prion-like manner across interconnected neuronal pathways and seed aggregation being most controversial. A study in which normal mice were injected with various forms of α-synuclein demonstrated that a single injection of α-synuclein fibrils was enough to see α-synuclein pathology throughout the brain. This was not the case when monomeric forms of the protein were injected suggesting the form of the protein is important for triggering pathological changes (Luk *et al.*, 2012).

Lewy Bodies

The defining neuropathological hallmark of PD is the Lewy body and it is this structure that is required at post mortem for a definitive diagnosis of the disease. Lewy bodies were first described in 1923 by Fritz Heinrich Lewy in the nucleus basalis of Meynert but are now known to be found throughout the brain of PD patients (Lewy, 1923). It was not until 1997 that the main component of LBs was discovered to be α-synuclein, the importance of which will be discussed in detail below (Spillantini *et al.*, 1997, Lewy, 1923). Ubiquitin and other remnants of the ubiquitin proteosome system are known to be found within these structures along with numerous other proteins but not to the same extent as α-synuclein. Alpha-synuclein is also found in a variety of forms within LBs, including ubiquitinated, phosphorylated and nitrated (Fujiwara *et al.*, 2002, Giasson *et al.*, 2000, Anderson *et al.*, 2006). There are a constant proportion of cells within the SNpc that contain LBs which is an estimated 3-4% of surviving neurons (Greffard *et al.*, 2010).

LBs are found throughout the brain and are categorised based on their location and differences in appearance and structure into either cortical or brainstem LBs. Brainstem LBs are very dense spherical cytoplasmic structures with a hyaline eosinophilic core and a small pale halo. The ultrastructure of these brainstem LBs consists of a dense core of radially arranged 7-20nm filaments whilst the outer edge consists of 10nm filaments. This is contrasting to the cortical LBs which lack a halo and on an ultrastructural level are poorly arranged (Jellinger, 2009). Another variation of the LB is the pale body, which is generally considered to be the precursor of LBs. These are lightly stained eosinophilic structures with a diffuse internal arrangement (Shults, 2006). All of these configurations have α-synuclein staining as a defining feature. Dystrophic neurons that contain LBs often also display Lewy neurites (LNs). These are also immunopositive for α-synuclein and the filaments found in LNs are similar in structure to those in LBs (Spillantini *et al.*, 1998a).

The Nigrostriatal Pathway

The dramatic and selective loss of the dopaminergic neurons of the SNpc ultimately leads to the breakdown of the nigrostriatal pathway which is responsible for the initiation and coordination of voluntary movement thus resulting in the typical movement problems seen in PD. The specific loss of the dopaminergic neurons of the SNpc ultimately leads to the over inhibition of the thalamus through the deregulation between the direct and indirect pathways. The indirect pathway works through the external globus pallidus inhibiting the subthalamic nucleus and thus promoting the inhibition of the thalamocortical pathway leading to

movement initiation. In contrast, the direct pathway promotes movement via the inhibition of the internal globus pallidus, thus reducing its inhibition status and promoting the thalamocortical pathway. The SNpc plays an important role in the regulation of these pathways through a dopaminergic input to the striatum, and the loss of dopamine seen in PD leads to the disinhibition of the indirect pathway and thus failure to initiate the thalamocortical pathway efficiently (Delong, 2000). The breakdown of this circuitry and a further explanation of the outcome can be found in figure 1.1.

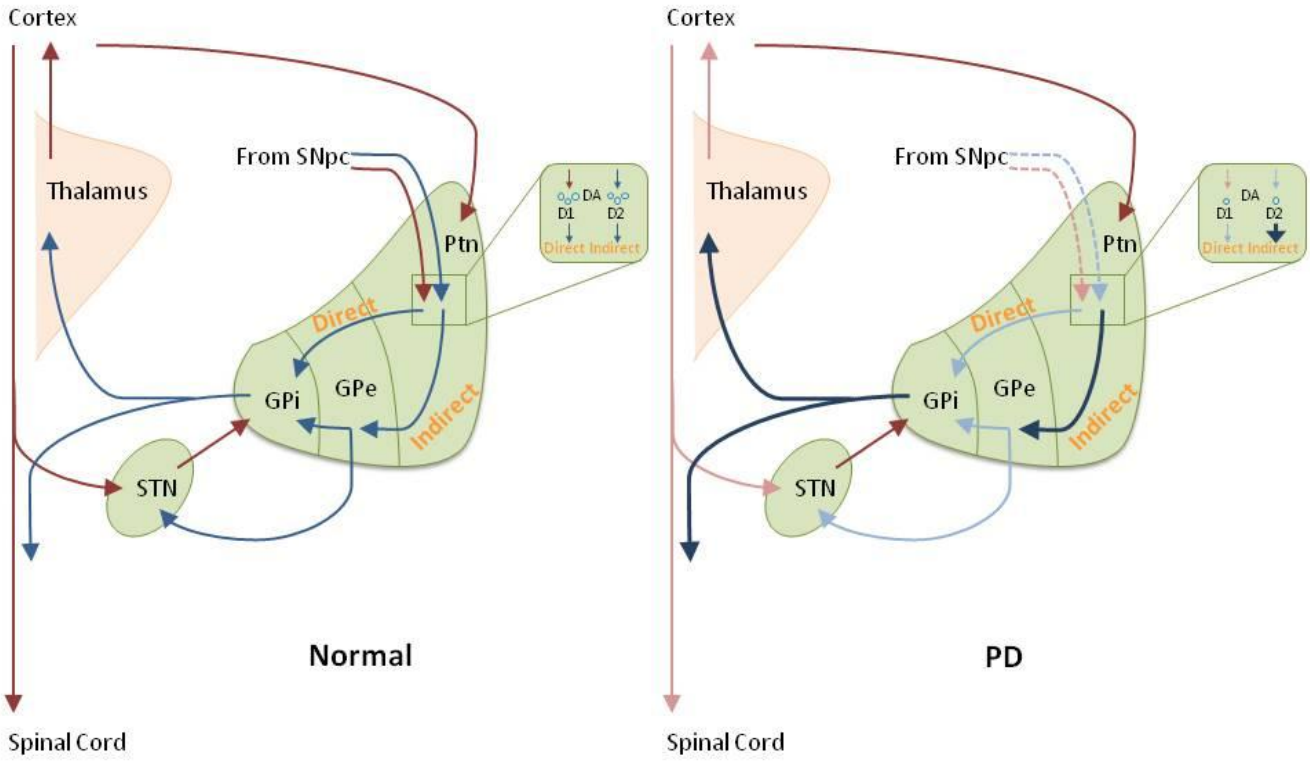


Figure 1.1 **The Nigrostriatal Pathway**

The basal ganglia circuitry under both the normal and PD states. Burgundy arrows represent excitatory pathways and navy represent inhibitory connections. SNpc = subtania nigra pars compacta; DA = dopamine; Ptn = putamen; GPe = external globus pallidus; GPi = internal globus pallidus; STN = subthalamic nucleus. Under normal circumstances the thalamus receives inputs that originate from both the direct and the indirect pathways, which work together in a feedback system to ensure that there is a correct balance between activation and inhibition. The indirect pathway passes through the GPe to the STN in a GABAergic manner from where it then passes to the output nuclei via an excitatory glutaminergic projection. When the direct pathway is activated it ultimately leads to the activation of the thalamus and therefore the cortex whereas activation of the indirect pathway leads to the transient inhibition of both the thalamus and the cortex and therefore movement. The SNpc plays a crucial role in the regulation of these pathways by creating a dopaminergic input to the striatum. The dopamine from the SNpc acts on both the D1 and D2 dopamine receptors. At this point the D1 receptors facilitate the direct pathway and the D2 receptors the indirect pathway. In PD, where the SNpc dopaminergic neurons are selectively lost, so is the nigral input to the striatum. This leads to

the over inhibition of the thalamus due to the lack of inhibition of the GPi and STN and leads to the impairment of movement through the over activation of the indirect pathway. This diagram was adapted from (Delong, 2000).

Sporadic PD and associated risk factors

The vast majority of PD sufferers do not have any family history of the disease and it is therefore considered to be a mainly sporadic disease. Despite the sporadic nature of the disease there are no clear environmental causes and these are still highly controversial within the PD field. However there are a number of risk factors known to be associated with the disease. The main risk factor for the disease, along with many other age related neurodegenerative diseases, is age, with only 10% of cases occurring below the age of 45 and the risk of developing the disease increases with age (de Lau and Breteler, 2006). For example, the incidence of PD is 17.4 per 100,000 person years between the ages of 50 and 59 but this number increases dramatically 93.1 between 70 and 79 years (Bower *et al.*, 1999). Another well established risk factor is that men are more likely to suffer with the disease than women, with some studies finding a 2 fold increase in the likelihood of men developing PD (Baldereschi *et al.*, 2000). However it is as yet unclear why men are more susceptible to the disease.

After the discovery of 1-methyl-4-phenyl-1-2-3-6-tetrahyrdopyridine (MPTP), a selective dopaminergic neurotoxin that gives cell loss in the substantia nigra with a molecular structure recognised as being similar to some pesticides, the hypothesis that environmental insults may have a role in PD became prevalent. Studies have been carried out assessing roles for pesticides, insecticides, well water drinking, heavy metal exposure, rural living, farming, early life infection and head trauma in the onset of PD. However no firm conclusions have been made for any of the mentioned lifestyle risks. There is nevertheless a trend that is suggestive of the fact that long term pesticide exposure may contribute to the onset of PD (de Lau and Breteler, 2006). Paraquat, which is similar structurally to MPTP, is a widely used pesticide associated with increases in oxidative stress leading to the selective loss of SNpc neurons and has been demonstrated to promote the aggregation of α-synuclein (Dinis-Oliveira *et al.*, 2006, McCormack *et al.*, 2002, Manning-Bog *et al.*, 2002). Rotenone, another pesticide, has also been shown to effectively inhibit complex 1 of the mitochondria. Numerous studies have shown that systemic administration of rotenone can cause progressive cell death of the nigrostriatal system in rodents with some describing α-synuclein positive aggregates (Inden *et al.*, 2007, Betarbet *et al.*, 2002) . However it appears that the effects of rotenone are more

unpredictable than those of MPTP and it is for that reason that the latter is the commonly used toxin model (Bove and Perier, 2012). It should be considered that studies used to unveil causative factors in disease are hard to interpret as they are often done retrospectively based on questionnaires that require the patient to remember particular exposures and therefore are prone to recall bias and also suffer from the fact that the individual is rarely only exposed to one particular pesticide. However, a recent study in which large cohorts where carefully constructed to assess the role of paraquat and rotenone exposure was able to demonstrate a clear association of the two pesticides to the development of PD (Tanner *et al.*, 2011).

Three factors that do seem to have consistent results with regards to PD development are the effect of caffeine intake, non-steroidal anti-inflammatory drug (NSAIDs) use and smoking. Studies have consistently shown smokers to have a lower risk of developing PD than non smokers with one 29 year follow-up study showing that the incidence of PD in smokers was less than half of that in non smokers which has been reaffirmed by a large meta-analysis study (Hernan *et al.*, 2002, Morens *et al.*, 1996). It has been suggested that nicotine could act protectively by stimulating dopamine release or possibly acting as an inhibitor of monoamine oxidase B (Quik, 2004). Also, daily intake of caffeine seems to offer some protection from PD which has been clearly demonstrated in numerous studies (Ross *et al.*, 2000, Hernan *et al.*, 2002, de Lau and Breteler, 2006). It has been shown that caffeine is an inhibitor of the adenosine A_2 receptor and in mouse models of PD can improve motor deficits (Ross *et al*., 2000). Numerous studies have also been carried out on the affect of NSAIDs and the development of PD. A recent meta-analysis of seven studies that assessed the use of non aspirin NSAIDs found that people who took these drugs lowered their risk of PD development by 29% with regular use (Gagne and Power, 2010). A study that assessed the combined effect of smoking, caffeine intake and the use of NSAIDs suggested that people who took the largest doses of caffeine and smoking as well as using NSAIDs may reduce their risk of PD by as much as 87% (Powers *et al.*, 2008).

The factors affecting the onset of sporadic PD are wide ranging and complex. For these reasons it is hard to pinpoint any exacting cause. One direction that has helped unravel some clues to sporadic PD cases is information gained from familial PD forms.

Genetics of Parkinson's disease

Dominant loci

Although rare, familial PD is estimated to be responsible for 5-10% of PD cases with more than 13 loci and 9 genes being identified as potentially causative (Lesage and Brice, 2009). Table 1.1 summarises the genetic factors of PD. Loci that have been associated with PD have been given the title "PARK" loci and it is these that will be discussed below.

PARK1 is designated to the autosomal dominant missense mutations that have been identified in α-synuclein gene. The first and most frequently occurring α-synuclein mutation to be recognised was the A53T point mutation that was discovered in a large Greek/Italian kindred (Polymeropoulos *et al.*, 1997). Following this, two further missense mutations in α-synuclein were also discovered the A30P and E46K which were found in German and Spanish kindred, respectively (Krüger R *et al.*, 1998, Zarranz *et al.*, 2004). The mean onset of the disease with these missense mutations is 46 years and although generally this familial form is phenotypically very similar to idiopathic forms of PD there are some deviations from this. Commonly α-synuclein point mutations are associated with the development of dementia and the characteristic resting tremor may be absent (Kay *et al.*, 2008). Triplication and duplication mutations of the entire α-synuclein gene (SNCA) have also been found to display autosomal dominant inheritance in families from diverse origins (Singleton *et al.*, 2003, Chartier-Harlin *et al.*, 2004). These were originally designated as PARK4 as they were considered to be a novel locus on the short arm of chromosome 4, however this was later disproved. Triplications and duplications in the SNCA locus have shown to vary in size and contain variable numbers of other genes, suggesting that these mutations are *de novo* rearrangements across the various families. The phenotype in these cases does appear to be dosage responsive with onset seen in patients carrying a triplication in their thirties and in duplication cases in their fifties (Hardy *et al.*, 2009). Patients that have a triplication of SNCA tend to have a faster disease progression and prominent dementia, but generally their symptoms match idiopathic PD whereas patients harbouring the duplication of SNCA tend to have pathology restricted more to the brainstem, thus resulting in a more movement disorder based disease pathway (Fuchs *et al.*, 2007, Ross *et al.*, 2008a). This implies that increased wild type α-synuclein expression alone is enough to cause disease.

Another possible link between PD and α-synuclein may be found in nucleotide polymorphisms seen in the promoter region of SNCA. A number of studies have shown these polymorphisms

to have a role in the risk of developing PD. A cell based study of Rep1, a dinucleotide repeat sequence 10kb prior to the translational start of α-synuclein, found that alternations in its sequence could alter the expression level of α-synuclein up to 3 fold (Chiba-Falek and Nussbaum, 2001). This was later confirmed as a risk factor for PD in a study involving over 6000 individuals (Maraganore *et al.*, 2006).

A second gene that has been clearly linked to PD encodes the leucine-rich repeat kinase 2 protein (LRRK2). Autosomal dominant mutations in this gene have been located on the PARK8 locus and are estimated to account for up to 10% of familial PD and a small number of apparently sporadic cases (Lesage and Brice, 2009). This is a large 2527 amino acid protein that has multiple domains including RAS, WD-40 and domains with GTPase and kinase activities. The presence of these numerous domains makes it likely that the LRRK2 protein has a role in cellular signalling (Taymans and Cookson, 2010). The Park 8 locus was designated to chromosome 12p11.2-q13 in 2002 when genome-wide linkage studies found the association in a large Japanese kindred (Funayama et al., 2002). Following this it wasn't until 2004 that the first mutations were recognised – these being the Y1699C and R1441C missense mutations (Paisán-Ruíz *et al.*, 2004, Zimprich *et al.*, 2004, Lesage and Brice, 2009). Since then more than 40 mutations have been identified in the LRRK2 gene most of which are missense. However as yet it is not entirely clear if all of these are pathogenic, but at least 7 of these identified mutations have been demonstrated to be pathogenic and as may be expected these are clustered in the conserved functional domains of the protein (Kay et al., 2008).

The most frequently occurring LRRK2 mutation is the G2019S but this differs depending on ethnic background being very rare in Asian and South African populations but accounting for 30-40% of familial and sporadic PD cases in North Africa and 10-30% in Ashkenazi Jews (Lesage and Brice, 2009). The mutation affects the kinase domain and it is considered to have a simple gain of function mechanism by increasing the kinase activity of the protein within the cell (Greggio et al., 2006).However it is not clear whether this is the case for all mutations, particularly those that fall outside of the kinase domain. The G2019S mutation results in late onset PD indistinguishable from the disease course seen in sporadic PD cases and at post-mortem there is a typical dispersion of LBs and LNs. Incomplete penetrance is seen with the G2019S mutation, estimated to be below 50%. This penetrance is age dependent meaning the older the carrier the higher the likelihood of developing PD (Bonifati, 2007). This incomplete penetrance is likely to account for a number of apparently sporadic cases.

Another possible autosomal dominant PD associated gene is UCHL-1. However there has only been one clearly evidenced mutation as yet found. This is the I93M missense mutation discovered in a German family (Leroy *et al.*, 1998). This mutation results in a partial loss of function in the catalytic activity of the thiol protease. Since then further studies, including a large meta-analysis, have shown a polymorphism in the UCHL-1 gene to be associated with sporadic PD (DeStefano *et al.*, 2003). At current it is considered that UCHL-1 could be a rather rare high penetrance PD associated gene.

Dominant Loci				
Gene/Loci	Examples of Known Mutations	Chromosome (human)	Clinical Manifestation	Reference
SNCA PARK 1/4	A53T A30P E46K Duplication Triplication	4q21	Typical PD but can present in some cases with related dementia	(Polymeropoulos *et al.*, 1997) (Krüger R *et al.*, 1998) (Zarranz *et al.*, 2004) (Chartier-Harlin *et al.*, 2004) (Singleton *et al.*, 2003)
LRRK2 PARK 8	G2019S Y1699C R1441C	12q12	Typical PD	(Kachergus *et al.*, 2005) (Zimprich *et al.*, 2004)
UCHL-1 PARK 5	I93M	4p13	Typical PD	(Leroy *et al.*, 1998)
Recessive Loci				
Parkin PARK 2	T240R R42P	6q26	Early Onset PD with slow progression	(Kitada *et al.*, 1998) (Terreni *et al.*, 2001)
PINK1 PARK 6	C92F G386A G409V	1p35-36	Early Onset PD with slow progression and in some cases severe depression	(Valente *et al.*, 2004) (Ibáñez *et al.*, 2006)
DJ-1 PARK 7	L10P L166P	1p36	Early Onset PD with slow progression	(Guo *et al.*, 2008) (Bonifati *et al.*, 2003)
ATP13A2 PARK9	F182L G504R	1p36	A more aggressive form of PD with numerous complications including dementia	(Ning *et al.*, 2008) (Di Fonzo *et al.*, 2007)
PLA2G6 PARK14	R632W R747W R741Q	22q13	A more aggressive form of PD with numerous complications	(Sina *et al.*, 2009) (Paisan-Ruiz *et al.*, 2009)
FBXO7 PARK15	T22M R378G	22q12	PD with additional pyramidal symptoms	(Di Fonzo *et al.*, 2009) (Shojaee *et al.*, 2008)

Table1.1 **Summary of mutations known to be associated with PD**

Recessive Loci

As well as the dominant loci in PD a number of small families have allowed the recognition of recessive loci that are also responsible for the disease. The first of these to be discovered was parkin (PARK2), mapped to chromosome 6, which is responsible for the autosomal recessive juvenile form of PD and was identified in a Japanese family (Kitada *et al.*, 1998). The most common cause of autosomal recessive PD is mutations in the Parkin gene, with over 100 pathogenic mutations now identified and together are responsible for almost half of all autosomal recessive PD cases, meaning that it is the only recessive PD mutation with a reasonable amount of information from post mortem analysis (Nuytemans *et al.*, 2010). This has revealed that a typical pattern of neuronal loss is seen with some cases reporting apparent α-synuclein and ubiquitin positive inclusions but there are no known examples of Lewy bodies in these patients (Sasaki *et al.*, 2004, Gouider-Khouja *et al.*, 2003). Patients with parkin mutations typically have onset of PD between 20-50 years of age, but the disease progress is relatively slow and responds well to L-DOPA therapy (Cookson *et al.*, 2008).

Two further autosomal recessive PD associated genes have also been identified. PTEN-induced novel kinase 1 (PINK1) was denoted as PARK6 and was first described in three families from Italy and Spain in 2004 (Valente *et al.*, 2004). Numerous mutations have been identified in the PINK1 gene that suggests a loss of function role is likely to be the contributing factor in the disease. The phenotype seen in PINK1 cases is very similar to those caused by parkin mutations but currently only a single PINK1 patient has been autopsied, which showed LB pathology but more cases will be required to confirm this result (Samaranch *et al.*, 2010).

Another autosomal recessive gene known to be associated with PD is DJ-1 (PARK7) which was identified in 2003 in both Italian and Dutch isolates (Bonifati *et al.*, 2003). The original study showed the Dutch case had a deletion within the DJ-1 gene, meaning that they had no expression of the protein product and the Italian case had a missense mutation causing the substitution of a highly conserved leucine for a proline, resulting in a non-functional protein product. DJ-1 mutations are very rare, accounting for approximately 0.8% of familial PD cases which give rise to a similar disease course as parkin and PINK1 mutations (Kilarski *et al.*, 2012). However although the number of individuals affected by this mutation is low it may still allow for an insight into PD.

It has recently been estimated that early onset PD resulting from mutations in parkin, PINK1 and DJ-1 account for a 5.1% rate of pathogenic mutation in UK patients (Kilarski *et al.*, 2012).

These three recessive PD linked genes are all conserved and possibly come together having complimentary functions involving mitochondria quality assurance. Parkin is an E3 ubiquitin ligase which labels other proteins for proteasomal degradation (Cookson *et al.*, 2008). However it has also been linked to the mitochondrial pathway with a suggested interaction with PINK1. Early Drosophila studies that knocked out the homolog of parkin resulted in loss of flight muscles and male sterility, which were attributed to the fact that the earliest changes to be consistently seen in the muscles and developing spermatids were swollen mitochondria with disintegrated cristae (Greene *et al.*, 2003). A further study also showed that parkin knockout Drosophila have an increased sensitivity to oxidative stress induced using paraquat supporting a potential role for parkin in mitochondria and oxidative stress (Pesah *et al.*, 2004). Following on from these studies parkin knockout mice were demonstrated to have mitochondrial dysfunction (Palacino *et al.*, 2004). It was later realised that parkin and PINK1 may well interact in some way when PINK1 knockout Drosophila were revealed to have the same phenotypes as their parkin equivalents and importantly that over-expression of parkin could rescue the PINK1 phenotype but the reverse was not true suggesting for the first time that PINK1 acted upstream of parkin in an interplaying pathway (Park *et al.*, 2006, Clark *et al.*, 2006). The first work to help reveal a potential role for parkin was shown in HEK293 and HeLa cells treated with the mitochondrial uncoupling agent carbonyl cyanidem-chlorophenylhydrazone (CCCP). After just one hour of treatment parkin redistributed so that it localised with the depolarised mitochondria and began the process of mitophagy which has since been shown with other mitochondrial depolarising agents (Narendra *et al.*, 2008). Since this it has been shown that PINK1 is required for parkin to be recruited to the mitochondria by various studies (Geisler *et al.*, 2010). It is now considered that PINK1 is found on the outer mitochondrial membrane due to its amino-terminal mitochondrial targeting sequence where it is imported into the inner membrane of the normal functioning mitochondria which results in it becoming degraded. Under conditions in which the mitochondrial membrane potential is lowered, such as dysfunction of the electron transfer chain and mutations in the mitochondrial DNA, PINK1 is prevented from reaching the inner membrane and so builds up on the outer membrane (Narendra and Youle, 2011). Following this a recent study has found that once depolarisation of the mitochondrial membrane has occurred PINK1 undergoes autophosphorylation at two serine residues which are imperative to the recruitment of parkin to the mitochondria, following which it ubiquitinates the mitochondria marking it for mitophagy (Okatsu *et al.*, 2012).

It is apparent that PINK1 disease mutations block the ability of the protein to recruit parkin. Similarly, mutations in parkin inhibit its recruitment to the mitochondria entirely or stop it from inducing mitophagy (Cookson, 2012). Other recent evidence from a conditional parkin knockout mouse model proposes that parkin regulates the turnover of a zinc finger protein known as parkin interacting substrate (PARIS), which is able to accumulate when parkin is mutated or absent. PARIS is able to bind to DNA and control the transcription of PGC1α, a transcriptional coactivator for a number of nuclear genes involved in the control of mitochondria and oxidative metabolism suggesting another possible role for parkin. It was found that over expression of PARIS was enough to incur loss of dopaminergic neurons in the SNpc which could be reversed by expression of parkin (Shin *et al.*, 2011).

It is less clear where DJ-1 may fit into this picture however; there has been long standing evidence linking it with oxidative stress, with studies showing DJ-1 deficient mice to be more sensitive to mitochondrial toxins such as MPTP (Kim *et al.*, 2005). Other more recent work has demonstrated that in the absence of DJ-1 the appearance of mitochondria become aberrant and these apparent problems arising from DJ-1 seem to be dependent of the amount of reactive oxygen species. This study found that this phenotype could be rescued with the expression of either parkin or PINK1 therefore possibly linking DJ-1 to faulty mitochondria clearance (Irrcher *et al.*, 2010). Further to this, loss of DJ-1 results in an increase of stress induced parkin recruitment to the mitochondria, which in turn leads to increased mitophagy. This can be returned to normal levels with the expression of wild type DJ-1. It is still unclear how this interaction with parkin may occur and there is as yet no evidence to suggest that DJ-1 acts directly with PINK1 in any way (Joselin *et al.*, 2012).

Although the role of mitochondria appears important from what can be learnt from the parkin, PINK1 and DJ-1 it is unlikely to be the sole cause of PD. These cases contribute to only a very small percentage of the overall number of patients suffering PD and other hallmarks are more robust across the majority of cases.

Other genes to be associated with autosomal recessive PD have recently been identified. These account for the ATP13A2; PARK9, PLA2G6; PARK14 and FBXO7; PARK15 loci. These again count for a very small proportion of PD cases overall and as above cause juvenile onset of the disease often with extrapyramidal symptoms. It is probably correct to consider these mutations as causing a disease that includes Parkinsonism as a symptom rather than causing classical PD.

The ATP13A2 mutations were first described in a Jordanian family and causes a loss of function in the encoded protein that results in a complex phenotype (Ramirez *et al.*, 2006). The gene encodes a transmembrane lysosomal type 5 P-type ATPase protein and, due to the phenotype given by the mutation resembling that of a lysosomal storage disease, it is considered that it may help show the link between PD and lysosomal storage disorders. Recent work using fibroblasts from patients with the L3292 and L6025 mutations has shown some revealing results. Both mutated fibroblasts and neuronal cells with ATP13A2 knockdown showed impaired lysosomal function which leads to an abundance of improperly degraded autolysosomes and autophagic vacuoles (Dehay *et al.*, 2012). This further legitimises the possible role of the lysosomal pathway in PD.

Mutations in the FBX07 gene were first recorded in a Persian family and since have also been noted in Italian kindred (Shojaee *et al.*, 2008, Di Fonzo *et al.*, 2009). The gene encodes an E3 ubiquitin ligase similar to parkin and the diseases progress is very similar to that seen in patients possessing a parkin mutation.

PLA2G6 mutations had already been identified for causing infantile neuroaxonal dystrophy (INAD) and neurodegeneration with brain iron accumulation (NBIA) but in 2009 a new mutation, R632W, was shown to be the casual mutation for PD presenting with dystonia in an Iranian family (Sina *et al.*, 2009). Since then a handful of other mutations in this gene associated with familial PD have also been recognised.

Gaucher's and Parkinson's Disease

Gaucher's disease is a lysosomal storage disease caused by a recessive mutation in glucosecerebrosidase (GBA). This results in a lysosomal build up of GBA, which causes most notably liver damage among other symptoms and can result in an array of neurological symptoms. At autopsy it has been noted that LBs are sometimes present in the brains of these patients (Wong *et al.*, 2004). More interestingly a study found that the parents and second degree relatives of these patients frequently had PD and in such cases they were always GBA mutation carriers (Goker-Alpan *et al.*, 2004). This initial study lead to further investigation and it emerged that a heterozygous loss of function of GBA, meaning a reduction in GBA function of 40-50%, resulted in approximately a 5 fold increased risk to developing PD. In Jewish populations where these mutations are more common they can account for up to 25% of all PD cases (Neumann *et al.*, 2009). This is of interest in PD research as a biochemical pathway that leads to the problems in Gaucher's is already clearly defined.

Low-risk loci and Genome Wide Association Studies

Genome wide association studies (GWAS) have allowed us to undertake large scale studies of the genetic variability in the normal population. Generally the loci that have been found in association with PD by this strategy were found in approximately 5% of the population and at most increase the risk of disease by no more than 2 fold (Hardy, 2010). Over the next decades it can be expected that genetic variants of smaller effect size will be detected using larger population studies. A number of GWAS studies have been performed with regards to PD and have all been on a relatively small scale. From these studies two loci have been consistently shown to be relevant in the susceptibility of PD, these being SNCA and MAPT, which encodes the microtubule associated protein tau (Simon-Sanchez et al., 2009, Satake et al., 2009, Saad et al., 2011, UK Parkinson's Disease Consortium et al., 2011, Edwards et al., 2010, Pankratz et al., 2009). MAPT association was seen in studies using samples from people of European descent but not with Asian descent, which is likely to do with the expression variation of the two tau haplotypes (Simon-Sanchez et al., 2009, Satake et al., 2009). It would appear from these studies that an increased expression of α-synuclein by 10% would be enough to increase the likelihood of developing PD by 40%. The fact that the MAPT locus has been consistently shown to be associated with PD is more surprising however it is not that unusual to find tau pathology in the brains of PD patients. Mutations in the MAPT gene have also been shown to cause frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP -17), possibly supporting the fact that the gene has been consistently shown in association with PD (Hutton et al., 1998). However some caution should be held regarding this GWAS result as it is a large haplotype that has been assumed to have MAPT as its causative agent despite the fact that there are a number of other genes within this region.

Further to these initial findings a number of other genes have now been shown to associate with PD through GWAS. This includes LRRK2, thus furthering the credibility to this gene having a role in sporadic disease. Recently the first meta-analysis of the PD GWAS studies has been undertaken which found 11 loci to reach genome wide significance. This is a useful method in allowing studies that were underpowered individually to be combined and analysed to give new insights to PD associated genes. Of these genes 6 were already known (SNCA, MAPT, HLA-DRB5, BSTX1, GAK and LRRK2) and another 5 of the identified were novel genes (ACMSD, STK39, MCCC1/LAMP3,SYT11, and CCDC62/HIP1R)(International Parkinson's Disease Genomics Consortium *et al.*, 2011). Studies such as this help to enlighten our knowledge on other possible pathways that as yet were unidentified in the pathogenesis of PD and may ultimately lead to novel therapeutic strategies. However these results still only account for a small

amount of risk and the main gene to get pulled out consistently in all the GWAS studies was SNCA.

Pathways to Parkinson's Disease

There are three main pathways that have gained appreciation when considering the molecular basis to PD. These involve the mitochondria, the protein degradation system and the role of improper protein folding. The following section aims to briefly discuss the current views behind each possible pathway in PD and their probable overlap.

Mitochondia

In cells such as neurons that have a high energy load that are required to respond quickly and efficiently to various inputs it is extremely important that mitochondria are tightly quality controlled. Various different routes have implicated the role of mitochondria in the pathogenesis of PD being investigated. Interest was first sparked with the discovery of severe Parkinsonism in a group of drug users in the 1980s. A batch of heroin had become contaminated with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) which was inadvertently injected by users who went on to develop the initial symptoms of PD within 4-14 days. Following their admission to hospital they were examined and all showed slowness of movement, abnormal posture, cogwheel rigidity and in one case a classic Parkinsonian tremor. All patients responded to L-DOPA therapy and when this treatment was withdrawn reverted to their original Parkinsonian state. Gas chromatography and mass spectroscopy analysis of drug samples revealed various quantities of MPTP (Langston *et al.*, 1983). Post mortem examination of patients who had self administered MPTP showed loss of dopaminergic neurons in the SNpc but there was no evidence of LB pathology in the 3 patients described (Langston *et al.*, 1999). However a mouse model using continuous MPTP infusion has shown LB pathology but these results have proved hard to replicate and the LB structures do not take the appearance of a traditional LB (Fornai *et al.*, 2005). It has also been reported that in non-human primates administered MPTP there was the presence of α-synuclein positive immunoreactivity but this has also been refuted in another study making it hard to draw firm conclusions (Halliday *et al.*, 2009, Purisai *et al.*, 2005). It is likely that the regime of administration of MPTP is responsible for these variations. With MPTP recapitulating one of the major pathogenic hallmarks of PD it was of great excitement when the pathway of its action was revealed. This mechanism of action is illustrated in figure 1.2 and has been shown in many models to be selectively toxic to the dopaminergic neurons of the SNpc making it a widely used toxin for PD modelling.

Rotenone, a pesticide, is another complex I inhibitor that is also commonly used as a modelling agent for PD. As it became apparent that MPTP worked through inhibiting complex I (NADH ubiquinoneoxidoreductase) of the mitochondrial electron transport chain, interest fast developed around the functioning of mitochondria in PD patients. A study by Schapira and colleagues showed a significant reduction in the activity of complex I in the substantia nigra of PD brains at post mortem, a result that was also later demonstrated in the frontal cortex (Schapira *et al.*, 1989, Parker *et al.*, 2008). However some caution should be taken as this deficit has not been seen in all PD patients.

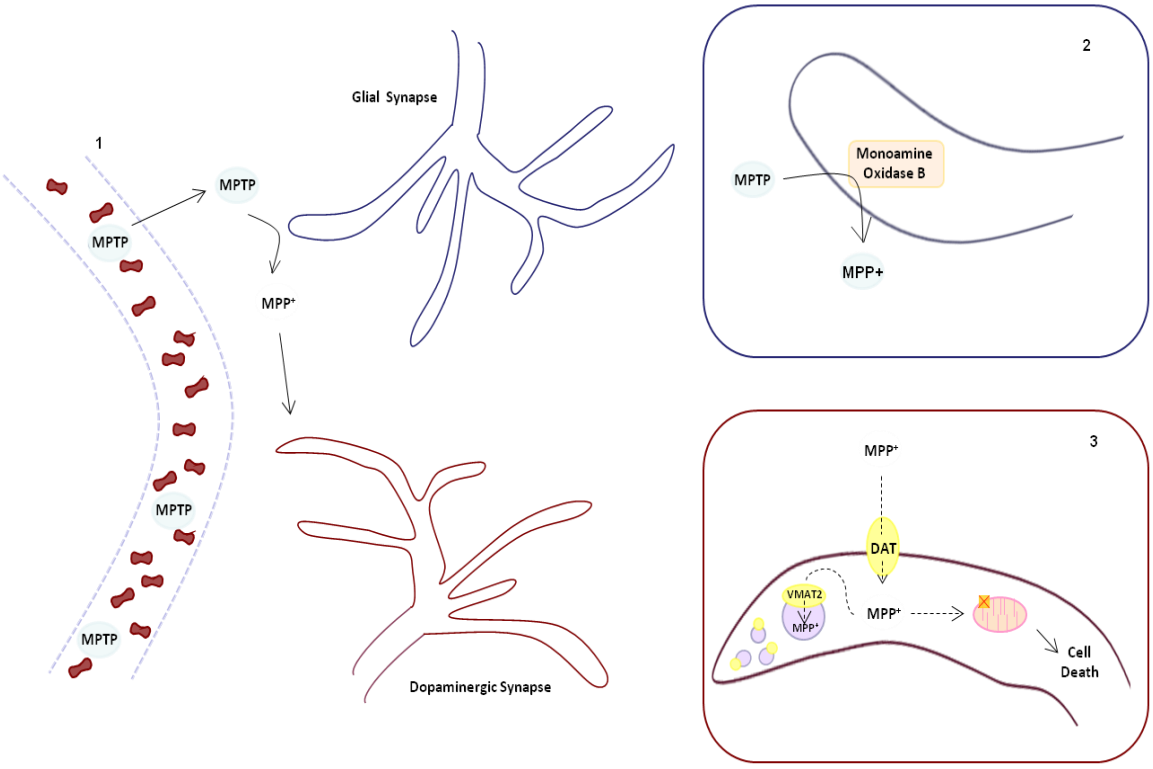


***Figure 1.2* MPTP Mechanism of Action**

A diagrammatic representation of the action of MPTP on dopaminergic neurons. As can be seen at (1) MPTP that has entered the bloodstream can cross the blood brain barrier and enter the brain. Once in the brain it is converted, mainly via glial cells, to its active metabolite 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase B (2). MPP^+ is then released and is selectively taken up by dopaminergic neurons in a DAT dependent manner (3). Once MPP^+ has

entered the neuron there are two possible routes it can follow. It may either inhibit complex 1 of the mitochondria, which leads to cell death or it may be sequestered into synaptic vesicles, represented as purple circles, in a VMAT2 dependent approach.

Since mitochondria appear to have some importance in PD pathogenesis mitochondrial DNA (mtDNA), which encodes a number of protein components involved in the electron transport chain, has been investigated. Although some studies have proved inconclusive, a study that used laser dissection of specifically SNpc dopaminergic neurons showed that there was an increased proportion of mtDNA deletions than in age-matched controls which may have led to mitochondrial dysfunction (Bender *et al.*, 2006). This could have resulted from increased oxidative stress in these neurons, which is also a result of mitochondrial dysfunction but could also be the result of other stresses on the cell. Dopaminergic neurons are considered to be at a higher risk of oxidative stress through the oxidisation of cytoplasmic DA and it is known that PD patients show free radical mediated damage to various cellular components. Thus any factor that may increase cytoplasmic DA concentrations may also generate a vicious circle of oxidative stress leading to mitochondrial dysfunction and ultimately cell apoptosis through a lack of ATP. However this scenario doesn't necessarily apply to other non dopaminergic neurons that are affected during PD and so is unlikely to be the full story.

As has already been discussed, there is evidence from genetic forms of PD supporting the role of mitochondrial dysfunction. Recent work on parkin, PINK1 and DJ-1 highlight the importance of well controlled mitophagy with dysfunction possibly leading to neuronal death. However there is still debate as to how relevant this is in an *in vivo* situation away from the cell culture environment that has thus far shed light on this pathway.

Roles that lead to alterations in the normal function of mitochondria have also been suggested for LRRK2 and α-synuclein. Those for α-synuclein will be discussed in detail in a later section. Recently a study by Wang et al has implicated over-expression of WT LRRK2 in the fragmentation of mitochondria through its part in the regulation of the fission/fusion pathway. This effect was further enhanced by LRRK2 mutants associated with PD. This increased fragmentation was dependent on the increased recruitment of Drp1 to the mitochondria but it is as yet unclear if these effects are a direct or indirect action of LRRK2 (Wang *et al.*, 2012). Prior to this fibroblasts from LRRK2 patients with the G2019S mutation had shown alterations in mitochondrial membrane potential and lowered ATP production, suggesting it may somehow be involved in altered mitochondrial dynamics (Mortiboys *et al.*, 2010). However this study was small involving only 5 patients and more *in vivo* work will need to be done to convincingly link LRRK2 to mitochondrial dysfunction.

Impaired protein degradation

There are two main cellular pathways that are normally used for the clearance of unwanted proteins and organelles. These are the ubiquitin proteosome system (UPS) and the autophagy-lysosome pathway which when blocked or dysregulated in some manner can cause extensive problems for the cell. In PD and various other neurodegenerative disorders there is protein aggregation, resulting in well defined pathological hallmarks. So it seems plausible that dysfunction of the degradation systems could either cause this or escalate the problem.

There is evidence that in sporadic PD there are reductions in levels of 20/26S proteosomes and proteosome activity in the striatum and substania nigra in comparison to control subjects (McNaught *et al.*, 2003). However it is unclear as to whether these changes are specific to late stage disease only. The fact that LBs contain ubiquitinated α-synuclein may also support the reasoning for alterations to the UPS system. However it could also be reliant on clearance via the lysosome due to the fact that the LB structure is too dense for the UPS to gain efficient access and remove it from the cell. It is known that wild type α-synuclein is cleared through chaperone mediated autophagy (CMA) but dysfunction in this system can lead to its aggregation. Further to this A53T and A30P mutant α-synuclein are able to inhibit degradation through the CMA pathway (Cuervo *et al.*, 2004). This in turn is likely to put strain on the UPS putting further stress on the cell.

Other evidence that has linked the UPS to PD is the disease causing mutations in UCHL-I and parkin. UCHL-I is a ubiquitin hydrolase but may also act as a ubiquitin ligase (Liu et al., 2002). It is as yet unclear to how this protein contributes to familial PD but it is likely to act within the UPS system. As parkin has been identified as an E3 ubiquitin ligase its dysfunction in familial PD is considered to interrupt the normal UPS. Apart from this recent evidence, discussed above, highlights parkin's role in mitophagy, a process that is reliant on the proteasome. Recent work by Bedford *et al* has demonstrated that the conditional knockout of the PSMC1, a protein which is an essential subunit to the 26S proteasome, in the subtantia nigra allows the development of Lewy-like inclusions and significant neurodegeneration pointing to the importance of a properly functioning ubiquitin proteosome system in normal neuronal homeostasis (Bedford *et al.*, 2008). The importance in normal protein degradation by the lysosome has also been linked to PD through mutations in the ATP13A2 and GBA.

Protein aggregation

Protein aggregation is apparent in the majority of neurodegenerative disorders, which is suggestive for the fact that it is an important component of the pathogenesis that leads to neuronal dysfunction and death. In PD the majority, but not all, cases have aggregated α-

synuclein in the form of LBs and LNs but it is still unclear how these aggregates lead to disease. It has been suggested that the aggregates themselves act protectively by accumulating toxic α-synuclein thus inhibiting them from causing neuronal death.

Although it is as yet unclear how protein aggregation alone induces cell death it is apparent that these aggregates are stressful to the cellular environment. It may be that a cascade of events within a cell may occur which cause a vicious cycle of events to ensue. For example dopamine is able to spontaneously self oxidise, which if not dealt with properly would lead to increased oxidative stress and thus cause problems for mitochondrial function which can lead to increased α-synuclein aggregation (Betarbet *et al.*, 2000, Dawson and Dawson, 2003). This aggregation in turn leads to an array of problems with regards to normal protein degradation and so this overall dysfunction eventually leads to cell death.

Recently there has been alot of debate on whether or not certain α-synuclein species could be transmissible from cell to cell. This would possibly explain the spread of LB pathology throughout the brain as proposed in Braak hypothesis and would allow the proposition that the disease could begin in one dysfunctioning cell and lead to a disease of the nervous system. Some of the strongest evidence for this hypothesis comes from PD patients who received foetal mesencephalic dopaminergic neuron grafts and at post mortem have been found to have LB pathology within the grafts (Li *et al.*, 2008). This would suggest that somehow the unhealthly LB laden tissue of the patient was able to "infect" the healthy graft possibly in a prion like manner. Very recently (Luk *et al.*, 2012)have demonstrated the ability of fibril forms of α-synuclein to seed the spread of the protein through interconnected neuronal pathways and show that there is associated loss of dopaminergic neurons, decreased dopamine concentrations and motor deficit in these animals. However they were not able to demonstrate any adverse affects when they used monomeric forms of α-synuclein for injections showing that the form of the protein appears important in relation to pathology (Luk *et al.*, 2012). This may have large implications on the importance of the forms involved in protein aggregation in PD and how we think about the disease process.

The Synucleins

The synuclein family consists of three highly evolutionary conserved, highly homologous proteins: α-synuclein, β-synuclein and γ-synuclein. Currently the function of these proteins remains to be fully elucidated. All of the synucleins are small natively unfolded proteins and consist of a highly conserved amino-terminal with a more varied carboxy-terminal. The amino terminal contains a variable number of 11-residue imperfect repeats which are highlighted in figure 1.3. This section aims to introduce the synuclein family in detail to provide context to this thesis.

Alpha-Synuclein

Alpha-synuclein structure and aggregation properties

Within the synuclein family α-synuclein was the first to be discovered and is the best studied and described. In 1988 α-synuclein was first identified and cloned from *Torpedo californica* and was also identified in a rat brain cDNA library (Maroteaux *et al.*, 1988). Following this a 35 amino acid peptide was identified in plaques from Alzheimer's disease patients and once isolated and sequenced was named the non-amyloid-beta component precursor protein which is now known to create the hydrophobic region of α-synuclein (Uéda *et al.*, 1993). Only in 1994 was the 140 amino acid human ortholog finally described as α-synuclein (Jakes *et al.*, 1994). Since then it has been found in a vast array of species including zebra finches, where it was named Synelfin and is upregulated during periods of song learning. However, there is no homolog of the protein, or any other synuclein, expressed in invertebrates. The protein is expressed widely throughout presynaptic nerve terminals of the central nervous system including the hippocampus, cerebral cortex, amygdala, midbrain, olfactory bulb and has been suggested to account for up to 1% of total protein (George *et al.*, 1995, George, 2001, Lia *et al.*, 2002).

The SNCA gene has been mapped in humans to chromosome 4q21.3-q22 and in mice to chromosome 6 (Chen *et al.*, 1995, Touchman *et al.*, 2001). SNCA consists of 7 exons, 5 of which are coding and there is evidence of alternate splicing between exons 4 and 6 (Campion *et al.*, 1995, Uéda *et al.*, 1994). The sequence of α-synuclein is 95.3% identical between human and rodents meaning only 6 amino acids differ, demonstrating the high degree of conservation across species. One interesting alteration between the sequences is that in rodents the amino acid in position 53 is a threonine whereas in the human protein it is an alanine and substitution of the alanine to a threonine is known to cause familial PD (Lavedan,

1998). Alpha-synuclein has 6 KTKEGV imperfect repeats and, as with the other synucleins, appears to have a natively unfolded structure. However, more recently there has been some controversy over the presence of a tetrameric form of α-synuclein which has been described in two separate studies in which the form was purified from *E. coli* or red blood cells and human cell lines (Wang *et al.*, 2011, Bartels *et al.*, 2011). These studies both claim that this conformation of α-synuclein is the predominant form. However, another carefully constructed investigation using both *E. coli* expressed forms and protein purified under various conditions from human and rodent brain tissue agreed with the previous view that the majority of α-synuclein in the central nervous system is natively unfolded (Fauvet *et al.*, 2012).

The N-terminus, amino acids 1-67, consists of two alpha-helical domains which are thought to be responsible for the apolipoprotein-like class-A2 helix which allows the protein to take on an alpha-helical conformation which is important when binding to lipid membranes (George, 2001). It has been shown that the substitution of certain residues with charged ones can disrupt this ability to associate with lipids and in another study the A30P mutant form of α-synuclein was demonstrated to lose the ability to bind synaptic vesicles (Perrin *et al.*, 2000, Jensen *et al.*, 1998). Residues 61-95 are responsible for the hydrophobic middle region also termed the non-amyoid β component of the protein which allows the transition from a random coil structure to β-sheet structure and Aβ-like fibrils (Serpell *et al.*, 2000, Conway *et al.*, 2000, Giasson *et al.*, 2001). The remaining C-terminal, residues 96-140, is rich in proline, aspartic and glutamic acids and appears to be important for possible chaperone activity of the protein (Dev *et al.*, 2003).

It is apparent that α-synuclein has the propensity to aggregate, doing so 20 times faster than γ-synuclein (Uversky *et al.*, 2002). The wild type protein has been shown *in vitro* to form insoluble aggregates, within 280 hours of incubation, as have mutant forms with A53T and A30P mutants showing the faster dynamics of 100 hours and 180 hours, respectively (Conway *et al.*, 2000, Narhi *et al.*, 1999). These dynamics are dependent on the incubation temperature, agitation, concentration of the protein and what form of the protein is used with some seeding aggregation more readily than others. It has recently been shown that fibril forms of α-synuclein, but not monomeric forms, can seed the spread of the protein with associated loss of dopaminergic neurons and decreased dopamine concentrations also occurring. This highlights the potential importance of the proteins form in contributing to the disease process (Luk *et al.*, 2012).

Various forms of the protein including oligomers, protofibrils and pore-like structures have also been described as causing toxicity which has lead to debate over just how toxic LBs are, if at all. It has been suggested that the production of LBs may actually be a protective mechanism of the neuron employed to limit the impact of more toxic pre-fibrilar stages (Tompkins and Hill, 1997). For instance, studies of oligomeric forms both *in vivo* and *in vitro* situations have shown that they are more toxic than species forming fibrils more readily (Winner *et al.*, 2011). This is also true of protofibrils which have been shown to form more willingly in A53T and A30P variants of α-synuclein, supporting their capacity to cause disease, than in the wild type form and are able to form annular pore-like and tubular protofibril structures (Conway *et al.*, 2000, Lashuel *et al.*, 2002). These protofibril structures are known to be able to bind and permeabilise lipid membranes which could have drastic knock on effects for the neuron by affecting its normal ion homeostasis. More specifically in dopaminergic neurons this could allow synaptic vesicles to be permeabilised, which would ultimately lead to the leakage of DA and oxidative stress within the cell (Volles *et al.*, 2001). Interestingly, in PD patients significantly increased levels of plasma α-synuclein oligomers have been recorded, hinting at a link with disease (El-Agnaf *et al.*, 2006). Other evidence that supports non-fibrilar forms of α-synuclein being the toxic species is the fact that not all cases of familial PD cases harbour LBs, which is supported by animal models in which α-synuclein aggregates are present but with no associated neuronal loss (Lee *et al.*, 2002).

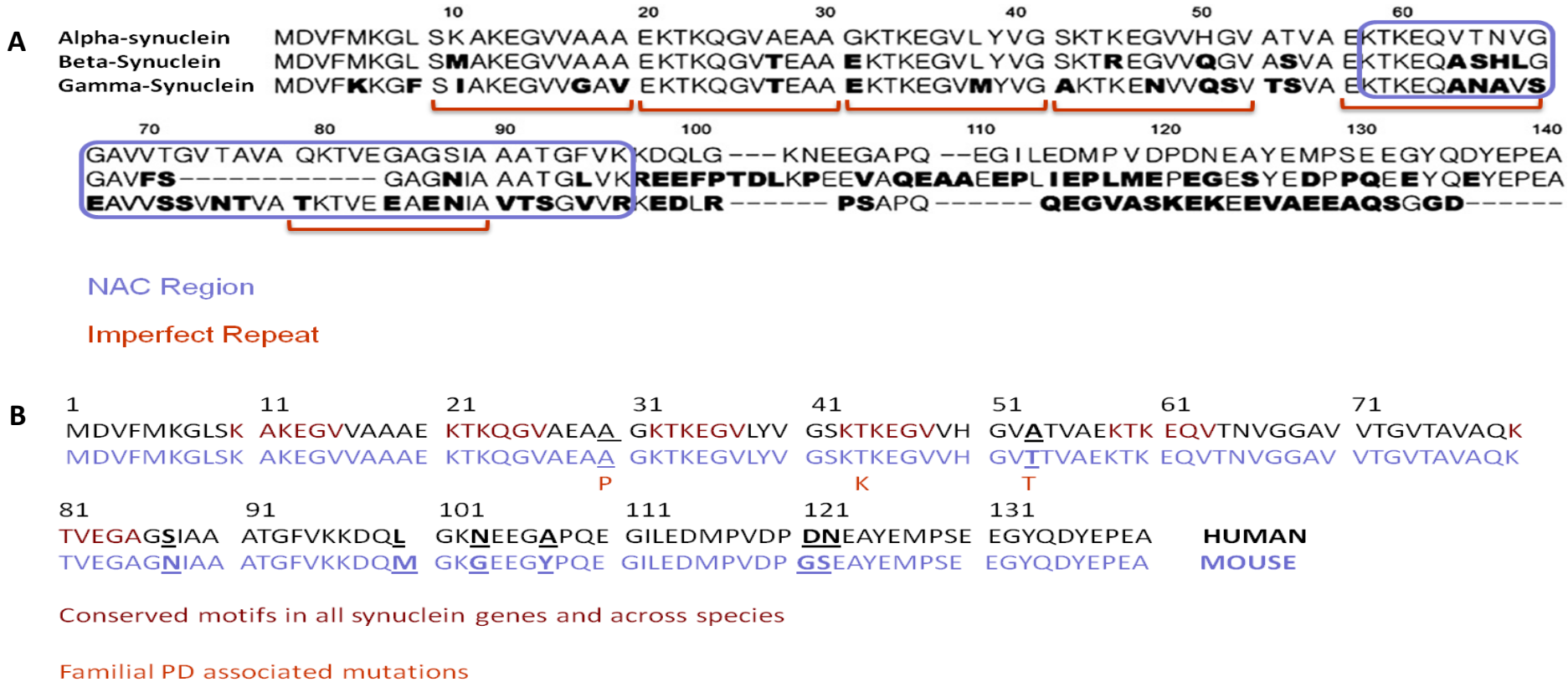


Figure1.3 Synuclein Amino Acid Sequence Comparisons

A) Comparison of the amino acid structures of alpha-, beta- and gamma-synuclein. Highlighted are the imperfect repeats and the NAC region which is not complete in beta-synuclein. This figure was adapted from (Sung and Eliezer, 2007). (B) Protein sequence of both mouse and human alpha-synuclein. Differences between the two sequences are underlined. Highlighted are the conserved repeats and the mutations that are associated with familial PD, A30P, E46K and A53T. Diagram adapted from (Dev *et al.*, 2003).

Alpha-synuclein and disease

As has already been discussed it is well established that α-synuclein has a prominent role in the disease process leading to PD. As well as this, it is considered to be a causative factor in a group of neurodegenerative diseases now collectively referred to as synucleinopathies (Spillantini *et al.*, 1998b). This group of diseases is defined by the presence of aggregated α-synuclein forms and includes Lewy body dementia, multiple system atrophy (MSA), Lewy body variant of Alzheimer's disease and Hallervorden-Spatz syndrome. In all cases specific subpopulations of neurons or glia have characteristic α-synuclein inclusions. This group of diseases is reviewed by Galvin *et al* should further detail be required (Galvin *et al.*, 2001).

Alpha-synuclein function

The function of α-synuclein is still very much speculated however a vast number of possible roles have been discussed. One of the earliest established ideas was the role of α-synuclein in synaptic plasticity. The first evidence were obtained in studies of a zebra finch where α-synuclein becomes significantly unregulated during a critical period of juvenile development in which bird song is learnt from a tutor. During this time the RNA levels increased vastly in circuitry of the brain involved in song learning (George *et al.*, 1995).

The majority of focus has been on the role of α-synuclein in relation to dopamine and in particular its synthesis, release and reuptake. From this it has been deemed that α-synuclein likely has a role in vesicle pool dynamics. Early research demonstrated α-synuclein could bind to synaptic vesicles purified from brain homogenates and that the mutant A30P, but not A53T, had an impaired ability to do so (Jensen *et al.*, 1998). Since then further evidence has been gathered that highlights the possible importance of α-synuclein interactions with vesicles. In yeast models α-synuclein over-expression was shown to increase the number of cytoplasmic lipid droplet and cause vesicle accumulation thus inhibiting ER-Golgi transport, which was later verified in mammalian cell lines with A53T α-synuclein causing stronger inhibition (Gitler *et al.*, 2008, Outeiro and Lindquist, 2003). This work found that co-expression of Ykt6p, which is a vesicle associated SNARE protein involved in the fusion of vesicles, ameliorated the inhibitory effect of α-synuclein. Further to this α-synuclein has been shown to co-localise with prenylated Rab accepter protein 1 (PRA1) which, when this co-localisation occurs, localises vesicles to the periphery of the cytosol (Lee *et al.*, 2011).

Alpha-synuclein has also been implicated in the normal release of neurotransmitters into the synaptic cleft, a subject that will be discussed in detail below. Animals produced that lack α-

synuclein alone show alterations in synaptic dopamine handling as do animals lacking multiple synuclein proteins suggesting they are able to cooperate but may not necessarily be essential for neurotransmitter release (Abeliovich *et al.*, 2000, Anwar *et al.*, 2011, Yavich *et al.*, 2004). This has lead to the hypothesis that α-synuclein may act as an activity dependent negative regulator of DA neurotransmission. Other studies have also demonstrated changes to the vesicle pool dynamics in α-synuclein null mice instigating the protein in the maintenance of these pools (Cabin *et al.*, 2002, Murphy *et al.*, 2000). Indeed it has also been noted that small increases in the expression of α-synuclein does not lead to overt phenotypes, but does cause disruption to normal neurotransmitter release through the inhibition of normal vesicle reclustering after endocytosis (Nemani *et al.*, 2010). Other *in vitro* work has also demonstrated diminished vesicular release in response to over-expression of human α-synuclein which was considered to be responsible for pathological synaptic changes and the study also showed similar protein changes existed in diseased human brains (Scott *et al.*, 2010).

In mice that lack cysteine string protein alpha (CSP-α), a synaptic co-chaperone for SNAP-25 involved in SNARE complex formation, a progressive neurodegenerative phenotype is observed that leads to premature death. If these animals are crossed with those over-expressing α-synuclein the phenotype is rescued and conversely when they are crossed with α-synuclein null mice their phenotype becomes worse (Chandra *et al.*, 2004b). However this is not true if crossed with γ-synuclein null animals suggesting a specific role of α-synuclein (Ninkina *et al.*, 2012). It has been demonstrated that α-synuclein is able to directly interact with VAMP-2 to promote SNARE complex assembly and so may function in the normal maintenance of these complexes that allow normal neurotransmitter release (Burré *et al.*, 2010). In mice lacking all three synucleins an age dependent impairment of SNARE complex formation was noted; however there is conflicting evidence to this that arose in another study which found this to be an indirect affect of α-synuclein inhibiting SNARE complex formation through the sequestering of arachidonic acid (Darios *et al.*, 2010). Studies carried out in our own lab assessing this impairment of SNARE complex formation in the dorsal striatum of triple synuclein null animals have failed to replicate these results. It is most likely that this is due to the fact that our studies assessed these changes in a specific brain region whereas in the Burré *et al* study the results were established from whole brain samples. This is likely to indicate that the varying types of synapses respond differently to the absence of the synucleins. However despite these contrasting results there have also been alterations described in the brains of PD patients with regards to SNARE protein distribution (Scott *et al.*, 2010). For a

definitive answer on the relevance of α-synucleins role in SNARE complex assembly further studies are required, especially in human samples.

Alpha-Synuclein Gain of Function vs Loss of Function

It is commonly considered that the pathological changes that occur in PD and other synucleinopathies occur as a gain of toxic function of α-synuclein. Although the hypothesis that a loss of normal function of α-synuclein has as yet received little attention, it is likely that this mechanism may contribute to the disease progression. It is a plausible theory that α-synuclein that has become incorporated into large insoluble aggregates such as LBs is unable to fulfil its normal role due to the functional protein being depleted within the cell. As well as this abnormal post-translational modification of α-synuclein may also inhibit its ability to function in its normal capacity at the synapse. This could then lead to further stress within the neuron which adds to the disease process, producing a vicious circle of events. It is evident from previous work discussed above that α-synuclein appears to have an important role in the normal maintenance of neurotransmission at the synapse and is therefore likely that alterations to this may lead to synaptic impairments. In particular the midbrain dopaminergic system that is affected in PD may be more sensitive to these alterations due to the intrinsic toxicity of dopamine and ratios of dopamine active transporter (DAT) and vesicular monoamine transporter (VMAT) that may predispose these neurons to stressful situations, meaning any slight change could be detrimental.

In part the reason that the loss of function hypothesis has gained little ground is due to the fact there has been no truly appropriate model in which to explore the affects of a loss of function of α-synuclein. A number of conventional α-synuclein knockout animal lines have been produced, however these models appeared to be far from ideal as the knockout is constitutive, allowing the brain to establish a compensatory mechanism during critical development periods of high plasticity in the animal nervous system. It is possible that this mechanism explains the resistance seen in α-synuclein null animals when exposed to MPTP. During development neurons that cannot establish a compensatory mechanism are lost but those remaining become more robust due to activation of compensatory mechanisms, making them partially resistant to the toxic effect of MPTP and, possibly, other environmental factors. It is also true that PD is a disease of the aging nervous system and thus models that are reliant on constituent knockout of α-synuclein throughout the animals' development do not recapitulate the situation in the brain of PD patients. It is feasible that depletion of α-synuclein after a period of high nervous system plasticity, particularly in the aged nervous system, will

have a more pronounced effect on neuronal physiology and might mimic certain pathological processes characteristic to PD. Indeed, aged α-synuclein knockout animals displayed more prominent alterations in their DA, DAT and TH levels in the striatum as well as a reduction of TH positive neurons in the SNpc, supporting the view that α-synuclein is important for normal synaptic integrity (Al-Wandi *et al.*, 2010). These results may also suggest that any compensatory mechanism established in these animals during development is not as effective in old age. Mice lacking CSP-α that were crossed with α- and β-synuclein knockout mice developed a more aggressive neurodegenerative phenotype, whereas if they were crossed with animals overexpressing α-synuclein this phenotype was rescued (Chandra *et al.*, 2005). This again highlights the importance of α-synuclein in the normal function of the synapse and shows it to potentially have a protective role against neurodegeneration.

Another problem with the previously created models is that they have been hampered by the role of the remaining synucleins. Due to the high degree of homology within the family it is likely that they can at least in part compensate for the loss of α-synuclein and increases in the remaining members of the synuclein family have been reported in previously produced knockouts (Chandra *et al.*, 2004a, Robertson *et al.*, 2004). As well as this, more prominent pathology has been seen in young double and triple knockout animals in comparison to α-synuclein knockout animals alone, supporting the role of familial functional compensation (Anwar *et al.*, 2011, Chandra *et al.*, 2004b, Senior *et al.*, 2008).

Alpha-Synuclein mouse models

Alpha-Synuclein Transgenic models

Due to the clear evidence of the role of α-synuclein in disease a vast array of α-synuclein transgenic mouse models have been created, which have employed expression of wild-type or mutated forms of the protein under different promoters. This section intends to give a brief introduction to the many transgenic α-synuclein mouse models. Whilst these have been of some use, it is apparent from these models that it is difficult to reproduce a clear PD phenotype or a classical LB in a mouse model.

The earliest attempt to produce a transgenic α-synuclein model was in 2000 when human WT α-synuclein was expressed under the platelet derived growth factor-β (PDGFβ) promoter (Masliah *et al.*, 2000). A number of lines were produced that had various levels of both protein and mRNA levels of human α-synuclein. The highest expressing line displayed the most severe phenotype with clear motor deficits as shown by rotarod analysis. All of the lines displayed

both nuclear and cytoplasmic α-synuclein positive inclusions by two months, which was a progressive phenotype with more being produced as the animals aged. However unlike LBs these inclusions were not as dense and fibrillar and were often located in the nucleus. Both levels and activity of tyrosine hydroxylase were lower in the highest expressing line suggesting there may be a threshold effect for the levels of α-synuclein within the cell. Another group also independently produced transgenic mice expressing either human α-synuclein or human A53T mutant α-synuclein under the PDGFβ promoter (Sharon *et al.*, 2001). It is hard to draw comparisons between results obtained by these two groups as the second group focused their study on the interaction of α-synuclein with fatty acids and although they were able to demonstrate the build up of soluble oligomers in two transgenic lines, phenotypes of these mice or results of any ultra-structural analysis have not been reported (Sharon *et al.*, 2003).

Expression of α-synuclein has been targeted more directly to the central nervous system using the prion protein (PrP) promoter. Several lines have been produced expressing α-synuclein from various origins in a number of different forms including wild type, A53T and A30P. Results of these studies however are not consistent but a general pattern can be obtained indicating that the majority of models expressing mutant α-synuclein under control of the PrP promoter show some pathology whereas models expressing wild type protein commonly do not. For example Giasson *et al* produced mice expressing either wild type or A53T: only those expressing the A53T mutant showed a severe motor phenotype that eventually lead to paralysis and death and displayed intraneuronal α-synuclein accumulation (Giasson *et al.*, 2002). Another group that produced lines of mice overexpressing human wild type, A53T or A30P only saw late adult onset neurodegenerative and motor problems in the A53T lines which also showed inclusions that were immunoreactive for both α-synuclein and ubiquitin (Lee *et al.*, 2002). The study also failed to show any obvious qualitative alterations in tyrosine hydroxalase. However, Gomez-Isla and colleagues were able to demonstrate pathology in a line of high expressing human A30P mutants. This line displayed age related motor decline but despite general neuronal soma staining for α-synuclein no inclusions or other pathological profiles have been observed. The group had another line expressing a lower amount of the mutant protein but this did not demonstrate any pathology, highlighting the importance of high expression in producing these models (Gomez-Isla *et al.*, 2003). Generally speaking in all of the above described cases the nigrostriatal system was still intact with other areas of the brain being more affected.

The Thy-1 promoter, which gives predominantly neuronal expression, has also been frequently used to produce α-synuclein transgenic mouse models. Van der Putten *et al* produced models that expressed A53T and wild type α-synuclein under the Thy-1 promoter and demonstrated that A53T over-expression could produce neuropathology by 3 months of age (van der Putten *et al.*, 2000). These animals had LB-like inclusions and motor impairment as well as synaptic and axonal changes. Interestingly, using this approach over-expression of the wild type α-synuclein protein has been shown to lead to similar pathological changes, therefore supporting the role of non-mutated α-synuclein in sporadic PD. Motor neurons of the brainstem and spinal cord were the most affected in these models, which does not properly recapitulate the pathology seen in PD, but it is widely accepted that Thy-1 driven expression is low in the dopaminergic neurons of the substantia nigra compared to other regions of the central nervous system (Buchman and Ninkina, 2008). However a study comparing expression of human α-synuclein under either the Thy-1 or PDGFβ promoter showed that Thy-1 was more valuable in obtaining expression of the transgene in the substa nigra (Rockenstein *et al.*, 2002). Mice independently produced expressing human wild type, A53T or A30P α-synuclein under the Thy-1 promoter also exhibited a age dependent neurodegenerative phenotype (Chandra *et al.*, 2005). In the case of the A30P line the animals exhibited motor neuron loss associated with insoluble α-synuclein aggregates. The line expressing the human wild type form of α-synuclein was shown to have the least aggressive phenotype with only 15% of the animals developing modest neurodegenerative changes by 16 months. Interestingly the group also produced 5 lines of mice overexpressing wild type mouse α-synuclein which did not show any neurodegenerative phenotype. This may be due to the aggregation dynamics of mouse α-synuclein which has been shown to be less prone to aggregation than the human protein (Uversky, 2007).

Attempts have also been made to produce mice with dopaminergic specific expression of α-synuclein but have in general been unfruitful. Studies that have produced mice expressing wild type human α-synuclein or A53T or A30P variants under the tyrosine hydroxylase promoter have failed to show any pathological changes in the midbrain dopaminergic systems of these animals (Matsuoka *et al.*, 2001, Wakamatsu *et al.*, 2007). Only expression of a mutant form of human α-synuclein bearing both the A30P and A53T substitutions under control of this promoter produced mice that displayed pathology including age related changes in dopamine metabolism and motor coordination (Richfield *et al.*, 2002). It is likely that significant results are not seen using the tyrosine hydroxylase promoter due to its relatively low expression levels meaning that the levels of α-synuclein required for pathology are just not reached.

Models driving the expression of C-termially truncated forms of α-synuclein have also been produced and studied. This form of alpha-synuclein has been shown to be abundant in LBs and shows a clear propensity to aggregate *in vitro* (Baba et al., 1998). Variants of these models include amino acid residues 1-120 and 1-130 truncations. In the case of the 1-130 line of animals the expression of the human truncated protein was driven by the tyrosine hydroxylase promoter. Although no alpha-synuclein positive aggregates were present in the dopmaninergic neurons of this model there was a selective loss of dopaminergic neurons in the substania nigra pars compacta during embryogenesis which was not progressive. The mice also displayed significant reductions in striatal dopamine concentrations along with behavioural dysfunction (Wakamatsu *et al.*, 2008). Another model in which alpha-synuclein 1-120 is expressed under the tyrosine hydroxylase promoter on a background in which endogenous alpha-synuclein expression is absent has also been produced. This model also displayed significant reductions in striatal dopamine concentrations and decreased spontaneous motor activity. In contrast to the previously discussed model these animals displayed aggregates of alpha-synuclein 1-120 in dopaminergic neurons of the subtantia nigra and the olfactory bulb but despite this no loss of dopaminergic neurons was described (Tofaris *et al.*, 2006). A more recent study of this model has also revealed an age dependent redistribution of striatal SNARE proteins and decreased dopamine release (Garcia-Reitbock *et al.*, 2010).

From these models a number of conclusions can be made. The first of these is that it is apparent that the mutated forms of α-synuclein associated with familial forms of PD, as expected, are more aggressive and consistently produce pathology in transgenic animals. Contrary to this wild type α-synuclein appears to require higher expression levels to cause pathology. Secondly none of the mouse lines discussed above are a suitable model as they do not cause progressive neurodegeneration of the nigrostriatal pathway. This is likely to be due in part to the limitations associated with poor expression patterns of available promoters and possibly because dopaminergic neurons of rodents appear to be more robust than those of humans. It is well established that rodents are more resistant to MPTP than humans for example (Gerlach *et al*., 1996, Fornai *et al*., 1997). This may result in the requirement of particularly high expression specifically located in the SNpc dopaminergic neurons. One method that has been used to overcome this has been viral vector mediated expression of α-synuclein allowing injection of the virus directly into the area of interest. These studies have been successful in reproducing the loss of dopaminergic neurons in the SNpc in both rodents and primates and in some cases have shown α-synuclein positive inclusions (Kirik *et al.*, 2002,

Kirik *et al.*, 2003, Yamada *et al.*, 2004). However these studies are highly labour intensive and require careful controls to be taken with each new batch of virus prepared. Another drawback of these studies is that there is no way to assess the copy number per cell making it hard to measure the expression level achieved. Although this model seems ideal in a number of ways, reproducibility is an issue and this is likely due to the variation seen in batch to batch viral production. Thirdly, although pathological accumulation of α-synuclein has been described in some of these models none have shown classical LBs, which are a major hallmark of the human disease. Of course it is likely that over-expression of α-synuclein alone is not enough to cause this type of pathology and in fact a second form of stress may also be required as some sort of trigger in these cells.

Unfortunately these models have not given an answer to how α-synuclein is able to cause damage to subsets of neurons and these results, along with those from knockout animals, may suggest that abnormalities in normal cellular function is important in the pathology of PD and likely culminates through a variety of pathways in cell death. It is indeed possible that changes in the normal dynamics of α-synuclein may be altering dopamine metabolism in patients long before the onset of symptoms, thus making the dopaminergic system more susceptible to later pathology.

Alpha-Synuclein Knockout Models

To date there have been a number of α-synuclein knockout lines produced, all of which have constitutive gene knockout thus possibly allowing for a functional compensation mechanism to be initiated during the nervous system development. This section aims to give an overview of the models so far produced and discuss the results discovered by these studies.

The first α-synuclein knockout model was produced by Abeliovich *et al* and relied on the targeted deletion of the first two exons of the gene (Abeliovich *et al.*, 2000). These animals were viable, with normal brain architecture and under normal conditions synaptic dopamine release was unaffected. Alterations in straital dopamine concentrations were noted with α-synuclein knockout mice harbouring an 18% reduction, whereas DOPAC concentrations remained comparable to wild type (WT) controls. Striatal slices stimulated with paired pulses, to assess Ca^{2+} dependent dopamine release, found that $\alpha^{-/-}$-synuclein mice showed significantly faster rates of recovery compared to their WT counterparts. Whereas the WT slices had reduced dopamine release after the second stimulus in comparison to the first, $\alpha^{-/-}$-synuclein slices failed to show this. Using amphetamine to induce DA release, a method that differs greatly compared to normal electrical impulse release, the study found that DA release by this

manner was normal in these alpha-synuclein knockout animals, indicating that Ca^{2+} independent release was unaffected by the loss of α-synuclein. Taken together with the results of the paired stimuli release dynamics these results may suggest an inhibitory role of α-synuclein in activity-dependent regulation of dopamine neurotransmission. Interestingly alpha-synuclein knockout mice have a significantly reduced locomotor response to D-amphetamine (dAMP) in comparison to WTs. Despite the normal release and reuptake response to amphetamine seen in these animals the alteration in motor reaction may be explained by alterations at the postsynaptic membrane.

Given the lack of pathology and the normal morphology of the dopaminergic neurons in the SNpc it was concluded that α-synuclein was not essential for normal neuronal maintenance or neuronal development. However this study could not dispel the idea that β- or γ-synuclein were acting to functionally compensate for the lack of α-synuclein.

In 2002 Cabin *et al* published their findings on another α-synuclein knockout model which did not entirely confirm the findings of the Abeliovich study (Cabin *et al.*, 2002). This model first differs in the fact that the knockout is based on the loss of exons 4 and 5 of the α-synuclein gene rather than the first two exons. This study also primarily studied the hippocampal neurons, rather than those of the nigrostriatal system as in the previously mentioned study. The gross brain architecture was found to be normal but no consistent results were produced with regards to striatal dopamine concentrations due to wide variations in concentrations between animals, therefore making it difficult to compare to the previous study. Although normal synaptic transmission was observed, it was also identified that these animals had an impaired ability to restore the pool of docked vesicles from the reserve pool as fast as their WT counterparts. Using electron microscopy to asses synaptic ultra-structure of cultured hippocampal neurons it was discovered that alpha-synuclein knockout mice had a 44% decrease in the number of vesicles in the reserve pool and an overall total of 50% decrease in presynaptic vesicles. This may have been the reason for the noted impairment in repetitive stimulation of brain slices taken from the knockout mice. Upon challenging with amphetamine there were no differences found in the response of knockout animals in comparison to wild type controls, unlike the Abeliovich*et al* study. An important consideration when interpreting these two studies is that they were carried out on mice of different backgrounds, which may have been the cause of some of the discrepancies described. This study supports the hypothesis that α-synuclein is required for normal synaptic function.

The fact that these knockout models show no detrimental alterations is supported by the discovery of a line of Harlan mice that have a genomic deletion that includes the entire α-synuclein locus (Specht and Schoepfer, 2001). These animals again show no overt phenotype which may suggest that β- and/or γ-synuclein or an unrelated mechanism may be providing functional compensation in the absence of α-synuclein.

A model produced by Dauer *et al* in which a transcriptional blocking casette containing a stop codon was inserted prior to the start codon of the α-synuclein gene (Dauer *et al.*, 2002). This was sufficient to abolish α-synuclein transcription. Although detailed morphological studies were not made in these mice, which were kept on a 129/Sv background, it was apparent that these mice had a normal phenotype as well as typical morphology within the brain. Importantly this group were the first to test the selective dopaminergic neurotoxin MPTP on α-synuclein knockout mice and showed a striking resistance to the toxin. They studied both acute, in which cell death is necrotic, and chronic, which induces cell death via apoptotic mechanisms, regimens of MPTP dosing. In both cases the alpha-synuclein knockout mice exhibited a robust resistance with similar cell counts to animals injected with saline, whereas the WT controls displayed a 29% and 58% reduction in tyrosine hydroxylase (TH) positive neurons of the SNpc in chronic and acute regimes respectively. This result has been repeated since by numerous groups (Robertson *et al.*, 2004, Schluter *et al.*, 2003). The study was able to show normal DAT and VMAT dynamics in the knockout animals but when primary neuronal culture from them were treated with rotenone, another dopaminergic neurotoxin that is lipophilic and therefore does not require DAT for transport into the cell nor is it sequestered in vesicles, they were shown to be significantly more sensitive to the toxin. These results when considered with those of the MPTP experiment suggest that the resistance seen to MPP^+ in these α-synuclein null mice is controlled through a mechanism prior to the toxin being able to inhibit complex I.

MPTP was also used to challenge another line of α-synuclein knockout animals produced by Schluter *et al* (Schluter et al., 2003). These mice had the targeted deletion of SNCA by the replacement of the first coding exon, exon II, with a neomycin resistance cassette which proved enough to silence the expression of the α-synuclein protein. These animals had no morphological changes in their brain structure and no significant changes were noted in the expression levels of various synaptic proteins including tyrosine hydroxylase. Changes in the striatal levels of β-synuclein were noted to be increased by 11% but this was not deemed significant. Striatal concentrations of dopamine and its metabolites were not noted to be

altered, but this result may differ from that of previous studies due to the background the mice were kept on differing from those used previously. Upon challenging these animals with MPTP, using both acute and sub-chronic regimes, it was concluded that these animals exhibited a partial resistance to the toxin. However this study did not comment on any SNpc cell counts and concluded this protection through the measurement of dopamine concentrations in the striatum. As well as testing MPTP on their own line of animals Schluter *et al* also administered it to the Harlan mice that exhibit a spontaneous deletion of α-synuclein. These animals did not display any protection from the effects of MPTP. These conflicting results could be due to the differing backgrounds of the two models which highlight the importance of having a standard background for all models. Another possible reason could have been that the brains were harvested at different time points following the last MPTP injection; for the Harlan mice the brains were harvested after 10 days whereas in the model the group created this occurred after 5 days. This may have allowed the levels of dopamine to recover as rodents are known to be more resilient to MPTP toxicity than humans and it is considered that they can recover from some of the affects of MPTP administration (Blandini and Armentero, 2012).

The discrepancies between these alpha-synuclein knockout models may arise from the different genetic backgrounds on which they were produced as well as the design of the knockout models (Mourice *et al*., 2004). The knockout animals produced in the Cabin *et al* study were based on the replacement of exons 4 and 5 of the α-synuclein gene with a neomycin resistance cassette, therefore disrupting the genes expression. However this does not alter the start codon of the gene as is the case in other models and may therefore still permit some gene expression, creating a truncated protein, which has never been properly tested or at least reported. This highlights how important model design is in allowing the role of genes to be unravelled.

Another approach to study the function of α-synuclein has been to use various methods to knockdown its *in vivo* expression. A study in rats which injected recombinant adeno-associated virus encoding small interfering RNA (siRNA) to silence α-synuclein expression into the SNpc achieved up to approximately 93% knockdown (Gorbatyuk *et al.*, 2010). This study demonstrated a rapid 82% reduction of TH positive neurons in the SNpc which occurred by 4 weeks post injection but was not progressive. A dramatic decline of striatal dopamine concentrations was also observed. As well as these striking alterations an abnormal response was also noted when these animals were exposed to amphetamine. A unique feature of this

model was that *in vivo* knockdown was able to occur in adult rats in which the nervous system was therefore fully developed. This is of interest as it means that an efficient compensatory mechanism for the functional loss of α-synuclein would not have been implemented as may be the case when the gene is knocked out congenitally. This may explain the dramatic alterations seen in the nigrostriatal systems of these rats. This study demonstrated that α-synuclein is required for normal synaptic function and that in its absence neurodegeneration can occur.

Other knockdown studies have been carried out and have produced inconsistent results. One such study was carried out in the primate SNpc by continual infusion of naked siRNA to knockdown α-synuclein. However this study only managed to ascertain a knockdown of 40-50% and recorded no changes in the number of dopaminergic neurons or in striatal concentrations of dopamine and its metabolites (McCormack *et al.*, 2010). Another study in which cultured primary hippocampal neurons were subjected to α-synuclein antisense oligonucleotides demonstrated approximately 50% knockdown and the study was able to observe a significant difference in the number of distal but not docked vesicles compared to untreated controls (Murphy *et al.*, 2000). This would support a role for α-synuclein in normal synaptic regulation. As yet other described studies have not confirmed this, which may be due to the fact that these were cultured neurons or simply because not all studies have looked at the ultra-structural synaptic morphology of such cultures. It is likely that the variability within these studies is down to the different methods used to deliver the knockdown as well as how efficient the knockdown was. It is also very difficult to compare studies carried out across different species, making it appropriate to further investigate the loss of function of α-synuclein in more models.

Double Synuclein Knockout Mouse Models

As well as the single synuclein knockouts discussed above double knockout models have also been created and investigated with the aim of more clearly defining the roles of the protein family. By producing these models it was hoped that the apparent functional compensation seen in the single knockout lines would be overcome.

Double $\alpha\beta^{-/-}$synuclein knockout animals were produced by Chandra *et al* by crossing previously produced α-synuclein null animals from Schulter *et al*, discussed above, with newly created β-synuclein null mice (Chandra *et al.*, 2004b, Schluter *et al.*, 2003). The resulting animals were viable, fertile and show no overt phenotype. When quantitative analyses of protein levels were assessed in these animals changes were observed in γ-synuclein, which was increased by 50%, as well as a 30% increase in both 14-3-3ε and complexin. The 14-3-3 protein family are

involved in cellular signalling whereas complexins are known to bind to SNARE complexes helping to control synaptic vesicle release. The upregulation seen in both of these proteins may suggest a functional interaction with the synucleins or that they are acting in a compensatory mechanism. Ultra-structural analysis of these animals failed to reveal any alterations in synaptic vesicle density or distribution as well as changes in synaptic markers or synaptic bouton area as assessed in cultured cortical neurons. This along with the fact that no dopaminergic neuron loss in the SNpc was noted suggests that these two synucleins are not required for the survival of this population of neurons. Although dramatic changes were not seen in most parameters that were investigated there was a clear 18% reduction in striatal dopamine levels, but no changes were noted in its metabolites, 3,4-Dihydroxyphenylacetic acid (DOPAC) and Homovanillic acid (HVA), suggesting catabolism of dopamine was not affected. The study also assessed both single α- and β-synuclein null mice and found no alterations in their dopamine concentrations. Further to this when $\alpha\beta^{-/-}$synuclein mice were crossed with CSP-$\alpha^{-/-}$ mice there was enhanced progression of the neurodegenerative phenotype when compared to the CSP-$\alpha^{-/-}$ mice being crossed with only alpha-synuclein knockout mice (Chandra *et al.*, 2005). This lends support to the hypothesis that there is a degree of functional compensation that may occur within the synuclein family and in agreement with this γ-synuclein levels in double knockout animals were found to be increased.

As well as double $\alpha\beta^{-/-}$synuclein mice $\alpha\gamma^{-/-}$ mice have also been produced and studied in detail. The absence of γ-synuclein alone ultimately leads to a change of the normal balance of the synucleins within the cell and in this imbalanced situation α-synuclein may become more toxic to the cell. To test this, mice lacking both α- and γ-synuclein were produced and studied. These animals were produced by crossing γ-synuclein null mice produced by Ninkina *et al* and α-synuclein null mice produced by Abeliovich *et al* which were backcrossed onto a pure C57Bl/6J line (Abeliovich *et al.*, 2000, Robertson *et al.*, 2004, Ninkina *et al.*, 2003). No gross morphological changes were seen in the brain of these animals nor were there any significant alterations in the striatal concentrations of dopamine and its metabolites. There was however an increase in the protein levels of β-synuclein in the $\alpha\gamma^{-/-}$synuclein animals and a significant reduction in the number of tyrosine hydroxylase positive neurons in the SNpc but not the ventral tegmental area in comparison to WT controls. This study also went onto challenge the double knockout animals with a sub-chronic MPTP regime. When SNpc neurons were counted in WT animals from MPTP treated and untreated cohorts there was a significant decrease in the number of neurons in the treated group as would be expected. However in $\alpha\gamma^{-/-}$ synuclein mice there were no significant differences in the number of TH positive cells between the

treated and untreated groups, implying these animals were resistant to the neurotoxic affect of MPTP. This study also revealed that single alpha-synuclein and single gamma-synuclein knockout mice also demonstrated resistance to MPTP toxicity. One possibility for this is that these animals have an initial loss of SNpc neurons, as seen from the tyrosine hydroxylase positive counts, during a critical phase of development and a compensatory mechanism that is initiated to cope with the loss of these two proteins inadvertently makes the remaining neurons more resistant to toxic insults. It is possible that the increased β-synuclein levels play a role in this mechanism as it has previously been demonstrated to have a neuroprotective role as discussed below.

Further studies on the $\alpha\gamma^{-/-}$ synuclein mice revealed that despite their decreased striatal dopamine concentration they had a hyperdopaminergic phenotype in novel environments and also displayed a lower rate of alternation in the T-maze spontaneous alternation task. Further exploration of this phenotype revealed that the double mutant animals, but not single α- or γ-synuclein null animals, had a twofold increase in the extracellular striatal dopamine concentrations after the application of small electrical stimuli as assessed by fast-scan cyclic voltammetry (FCV) (Senior *et al.*, 2008). It was demonstrated that this increase was not due to decreased reuptake of dopamine via the dopamine active transporter, which was consistent with findings by Robertson *et al*, and was considered more likely that the lack of both of these synucleins resulted in an increased probability of release of the neurotransmitter (Robertson *et al.*, 2004).

The $\alpha\gamma^{-/-}$synuclein mice have also been studied using microarray analysis in order to determine any obvious changes that would indicate a specific compensatory mechanism (Kuhn *et al.*, 2007). This revealed that the expression of 215 genes was altered in these animals. However there were no specific contenders that were apparent and these results are hard to compare with other studies due to different strains and ages of animals being used.

From the work carried out with double synuclein knockout animals there has been some intriguing data to suggest that the synucleins are important in some manner in dopamine metabolism. However there are clear problems in these studies with regards to functional compensation that may still be in place from the remaining synuclein. In order to overcome this problem triple synuclein mice have been recently produced with the hope over revealing a more defined role for the protein family (Anwar *et al.*, 2011). This will be discussed in detail in chapter 3.

Beta-Synuclein

Beta-synuclein Structure and Aggregation Properties

Beta-synuclein was first purified from the bovine brain and was originally named as PNP 14 before it was recognised as a member of the synuclein family (Nakajo *et al.*, 1993). It encodes a 134 amino acid protein which differs from the other members of the synuclein family as it lacks the 11 amino acid sequence corresponding to the non-amyloid component (NAC) found in these proteins (Lavedan, 1998). The human gene is located at chromosome 5q35 and is arranged into 6 exons, five of which are coding (Spillantini *et al.*, 1995). The protein shares 61% homology with α-synuclein making it more conserved within the family than γ-synuclein whilst mouse and rat β-synuclein share 97.8% homology with the human protein demonstrating the high level of conservation of this protein between species (Spillantini *et al.*, 1995, Jakes *et al.*, 1994). The distribution of β-synuclein throughout the cell is similar to that of α-synuclein with both proteins being found localised to the synapse. Throughout the brain β-synuclein expression pattern overlaps the regions in which α-synuclein is expressed with no evidence currently existing that shows synapses that are specific for only α- or only β-synuclein (Lincoln *et al.*, 1999).

Unlike α- and γ-synuclein the free state secondary structure of β-synuclein is much less predisposed to forming a helical structure which is most likely due to the proteins lack of the NAC domain which results in the protein having a less compact structure than the other two synucleins. The aggregation properties of β-synuclein are also dramatically altered in comparison to α- and γ-synuclein, β-synuclein is the family member least prone to aggregate. Even when relatively high concentrations of the protein are shaken at 37°C for several weeks the material remains in a soluble form which is not the case for either α- or γ-synuclein (Biere *et al.*, 2000, Uversky *et al.*, 2002). This is most likely explained by β-synuclein lacking the 11 amino acid residue of a hydrophobic NAC region thought to be responsible for the fast aggregation kinetics of α-synuclein. β-synuclein has also been shown to behave in a different manner to α-synuclein by the fact that it is unable to form pore-like oligomeric structures (Park and Lansbury, 2003). Although the general consensus is that β-synuclein is not normally inclined to aggregate, fibrillation of the protein can be initiated in the presence of zinc, iron or copper ions (Yamin *et al.*, 2005). Like γ-synuclein, β-synuclein has been shown to be able to almost completely inhibit α-synuclein aggregation at a 4:1 molar excess (Uversky *et al.*, 2002).

Accordingly, two independent *in vivo* studies have found that over-expression of β-synuclein in mice over-expressing α-synuclein is enough to alleviate some of the associated phenotypes normally found in the α-synuclein transgenic animals (Hashimoto *et al.*, 2001, Fan *et al.*, 2006).

Beta-Synuclein and disease

Beta-synuclein is not generally considered a causative factor in human diseases, having never been clearly linked to the disease process. The protein is not a component of LBs, however there is some documentation of β-synuclein positive abnormal structures but it should be stressed that these are highly uncommon (Galvin *et al.*, 1999). In 2004 two rarely occurring mutations, V70M and P123H, were described in a highly conserved portion of β-synuclein and were shown to be a risk factor for dementia with Lewy bodies. However these findings are only suggestive of β-synuclein having a role in disease and failed to show a clear mechanism by which these alterations may cause disease. One possibility is that β-synuclein is unable to carry out its role in modulating α-synuclein behaviour which is supported by the fact that β-synuclein aggregation was absent in a single patient with the P123H mutation at post-mortem whereas α-synuclein pathology was present as expected. More recently transgenic mice have been produced expressing the P123H variant of β-synuclein under the Thy-1 promoter. Surprisingly these animals revealed β-synuclein positive inclusions, which were not detected in wild type littermates or animals over-expressing wild type β-synuclein, that were particularly concentrated in the striatum and globus pallidus and were observed in an age dependent manner. As well as this the animals also showed cognitive and some minor motor abnormalities. This work would support the hypothesis that P123H β-synuclein is itself pathogenic, which is supported by evidence that when these animals were crossed with α-synuclein null animals no differences were demonstrated in these animals. However when the P123H β-synuclein line was crossed with mice over-expressing α-synuclein their neuropathology was enhanced as was mortality (Fujita *et al.*, 2010). These results show that β-synuclein has the potential to be pathogenic but there is still very little clear evidence directly linking it to human disease.

Beta-synuclein mouse models

Mice lacking the β-synuclein gene have been produced and studied. These animals were created by floxing the first coding exon for the β-synuclein gene, allowing it to be removed when required. However there are no apparent studies on animals which were aged prior to the knockout of β-synuclein occurring. Instead the study opted to knockout the gene and then breed these animals to create a cohort lacking β-synuclein, essentially removing the

conditional mechanism of the knockout and producing mice of constitutive knockout (Chandra *et al.*, 2004b). The resulting animals were shown to be viable and fertile, being comparable with wild type littermates even when aged. No reduction in striatal dopamine concentrations or in synaptic vesicle pool size was found, suggesting some degree of functional redundancy across the synuclein family, supported by the fact that $\alpha\beta^{-/-}$ synuclein animals do have a significant decrease in their striatal dopamine content. When β-synuclein null animals were crossed with mice that over-express mutant human α-synuclein there were no changes detected in the onset or extent of the neurodegenerative phenotype normally observed in these animals, as may have been expected. This suggests that endogenous β-synuclein is not capable of protecting against pathological changes caused by over-expressed α-synuclein.

Animals that over-express β-synuclein have also been produced and have not been reported to suffer any side effects to this over-expression. In a study in which human β-synuclein was expressed under the Thy-1 promoter there were no overt affects. Behavioural analysis revealed them to be normal as did striatal dopaminergic synapse studies. This study clearly demonstrated that the over-expression of β-synuclein was not detrimental to mice and doesn't appear to have any adverse affects on the normal survival of various neuronal populations. In addition to this when double transgenic mice were produced expressing human forms of both α- and β-synuclein it was found that this was enough to ameliorate the motor dysfunction normally associated with α-synuclein expression as well as the accumulation of α-synuclein. On top of this significantly better rotarod performance was demonstrated in bigenic animals in comparison to single α-synuclein transgenic mice. Taken together these results may suggest that β-synuclein may be able to provide a neuroprotective role as a negative regulator of α-synuclein aggregation (Hashimoto *et al.*, 2001). A second study that produced mice over-expressing human β-synuclein under the PrP promoter also found the adult and aging animals to be undistinguishable from their wild type counterparts even in the highest expressing line, where β-synuclein was approximately eight times higher than endogenous levels. When the levels of endogenous α-synuclein were investigated in the cerebral cortex of these animals the protein levels, but not mRNA, were found to be moderately reduced which only reached significance in the line of mice expressing the highest levels of β-synuclein. These animals were crossed with a line of A53T α-synuclein transgenic mice with the resulting animals showing ameliorated phenotypes with a longer survival, a lesser motor phenotype observed using rotarod and a significant reduction of subcortical α-synuclein aggregation (Fan *et al.*, 2006). Both of these studies support that β-synuclein over-expression does not appear to be detrimental and is possibly acting neuroprotectively. This

highlights the importance of having the correct balance of synucleins throughout the nervous system.

Gamma-Synuclein

Gamma-synuclein structure and aggregation properties

Gamma-synuclein was the last of the three proteins in the synuclein family to be identified. The human gene was first identified in *in silico* analysis of breast cancer transcriptomes and was therefore named breast cancer-specific gene 1 (Ji *et al.*, 1997). Mouse cDNA clones were obtained in a subtraction cloning experiment and the encoded protein, at that time named persyn, was identified as a member of the synuclein family with expression mainly in the primary sensory neurons, sympathetic neurons and motor neurons as assessed in mice and rat tissues (Buchman *et al.*, 1998). It was revealed that the gene, SNCG, was located at human chromosome 10q23, and consists of five exons encoding a 127 amino acid protein which was found to be expressed to some extent in the substantia nigra, thalamus, subthalamic nucleus, hippocampus and amygdala (Lavedan *et al.*, 1998, Ninkina *et al.*, 1998, Alimova-Kost *et al.*, 1999). The γ-synuclein protein is the least conserved of the family sharing 55.9% homology with alpha-synuclein and 54.3% with beta-synuclein however it is still an evolutionary conserved protein with the human protein having 66% amino acid identity with the primitive chordate lamprey (Lavedan *et al.*, 1998, Lavedan, 1998). The C-terminus of the protein is the most diverse in comparison to the other family members whereas the N-terminus contains the entire non-amyloid component sequence, like α-synuclein, and 6 copies of the KTKEGV imperfect repeat (Lavedan *et al.*, 1998). The major difference seen in γ-synuclein compared to β- and α-synuclein is its cellular distribution. Unlike α- and β-synuclein, that are located predominantly to the presynaptic terminals, large amounts of γ-synuclein is also found in axons and in some neurons, in the perikaryal cytoplasm (Ninkina *et al.*, 2003).

The structure that γ-synuclein undertakes when lipid bound is more comparable to β-synuclein, whereas in its free-state secondary structure it has the tendency to form ordered helical structures meaning it is more analogous with α-synuclein (Sung and Eliezer, 2007). In cell free conditions it has been shown that compared to α-synuclein, γ-synuclein aggregates less but is still able to form amyloid fibrils (Uversky et al., 2002). γ-synuclein takes three times longer to form fibrils than α-synuclein showing clearly that it is less inclined to spontaneously aggregate (Biere *et al.*, 2000). Despite this, like β-synuclein, γ-synuclein has the ability to

inhibit the formation of α-synuclein fibrils at a ratio of 1:1 with the efficiency of inhibition increasing with the increasing ratio of γ-synuclein to α-synuclein (Uversky *et al.*, 2002).

Gamma-synuclein in disease

Although γ-synuclein has not been shown to have a role in PD it may potentially be implicated in other diseases. No pathogenic mutations have been discovered in γ-synuclein however there are two known polymorphisms in SNCG (Ninkina *et al.*, 1998). More recently a polymorphism in the non-coding region of γ-synuclein, which might affect the expression levels of the gene has been linked with diffuse Lewy body disease (Nishioka *et al.*, 2010). Although it is uncommon, γ-synuclein positive axonal spheroids have been described in diseases such as Hallervorden-Spatz and Lewy body dementia (Galvin *et al.*, 2000, Galvin *et al.*, 1999). An example of a neurodegenerative disorder with strong links to γ-synuclein dysfunction is glaucoma, a disease that leads to permanent blindness due to increased intraocular pressure leading to the loss of retinal ganglion cells (RCGs). In the eye γ-synuclein is known to be specifically expressed in RGCs with the level of this expression being shown to be decrease in patients suffering glaucoma (Surgucheva *et al.*, 2002). A common glaucoma animal model, the DBA/2J mouse, also demonstrates a down regulation of γ-synuclein through the progression of the disease, which correlates with the loss of RGCs(Soto *et al.*, 2008). A recent study has implicated the protein in the response to increased intraocular pressure with it up-regulating Mac-2 at the myelination transition zone and providing some protection in the axons. In animals lacking γ-synuclein no up-regulation of Mac-2 was seen following increased intraocular pressure and a greater degree of axonal loss occurred. In mouse models of glaucoma proteinase K resistant forms of γ-synuclein were identified suggesting that aberrant forms of the protein may have a pathological role (Nguyen *et al.*, 2011).

As mentioned γ-synuclein was first recognised as a breast cancer specific gene, it is apparent that the protein seems to exist at high concentrations specifically in more progressed and malignant tumours and is not expressed in normal breast epithelia (Ji *et al.*, 1997). As well as in breast cancers, elevated expression of γ-synuclein has also been noted in ovarian, as well as other types of tumours (Bruening *et al.*, 2000, Ahmad *et al.*, 2007, Liu *et al.*, 2005). Both *in vitro* and *in vivo* over-expression of γ-synuclein has been demonstrated to have detrimental effect on the metastatic capabilities of tumour cells. When breast cancer cells over-express γ-synuclein they become more motile and invasive but do not grow any faster than non-γ-synuclein expressing controls. Further to this when cells over-expressing γ-synuclein were implanted into nude mice a similar number of tumours grew when compared to implants not

over-expressing γ-synuclein and their growth had a comparative time course. However those positive for γ-synuclein expression caused a significantly greater number of metastases (Jia *et al.*, 1999). It is so far unclear how γ-synuclein initiates these changes and to what extent it alone is responsible for these changes. Moreover, a recent study in our laboratory demonstrated that γ-synuclein is not required for mammary gland tumourigenesis and metastasis.

Recently it has become apparent that γ-synuclein appears to have a role in adipocyte physiology. Increased expression of γ-synuclein has been consistently observed in adipose tissue of obese patients and this can be decreased through dieting (Oort *et al.*, 2008). Further work unveiled the relationship between γ-synuclein and brain lipid composition through the use of γ-synuclein null mice (Guschina *et al.*, 2011). Recent work has confirmed that γ-synuclein is nutritionally regulated in white adipose tissue (Millership *et al.*, 2012). γ-synuclein null mice exposed to a high fat diet were protected from obesity and its associated complications, unlike WT mice fed the same diet. This could not be explained by altered food intake or the amount of lipids adsorbed from their diet. When γ-synuclein was reintroduced into subcutaneous fat pads, via lentiviral expression, the size of adipocytes was seen to increase in transduced cells and not in non-transduced cells or those injected with a control lentivirus. This supports a role for γ-synuclein in the control of adipocyte size *in vivo*. To explore how γ-synuclein may be exerting its control on adipocyte metabolism the levels of SNARE complexes were investigated. SNARE complexes are thought to be required for lipid droplet formation and as described above, α-synuclein has been shown to modulate assembly of neuronal SNARE complexes. A 35% decrease in SNARE complexes was observed in γ-synuclein null mice fed a high fat diet, despite the fact that individual SNARE proteins where not down-regulated, suggesting that γ-synuclein is able to regulate SNARE complex formation during times of nutrient excess. It is suggested that γ-synuclein is able to deliver SNAP-23 during the formation of the SNARE complexes and this allows the increase in lipid droplet volume. Previously α-synuclein have been shown to interact with lipid rafts and lipid droplets and so it is not entirely surprising that γ-synuclein may also cooperate with cellular lipid metabolism (Cole *et al.*, 2002, Fortin *et al.*, 2004).

Gamma-synuclein mouse models

Homozygous mice that over-express mouse γ-synuclein under the Thy-1 promoter were found to develop an age and transgene dose dependent motor deficits and neuropathology, eventually leading to paralysis, as well as early lethality (Ninkina *et al.*, 2009). γ-synuclein

positive inclusions were also apparent in the cytoplasm in subsets of neurons which in some cases were eosinophilic but the majority were spheroids or dystrophic neurites. Fractionation of spinal cords from these animals showed evidence that large proportions of the γ-synuclein was in fact insoluble and forms amyloid-like fibrils. By 12 months of age these animals had lost over 60% of their spinal cord motor neurons when compared to wild type controls. Further investigation of these animals revealed that they appear to recapitulate certain aspects of amyotrophic lateral sclerosis (ALS), including selective damage and loss of certain subsets of upper and lower motor neurons whilst the sensory neurons remain relatively unscathed (Peters *et al.*, 2012). Unpublished work from our lab has shown that in some cases of ALS there appears to be some neuronal inclusions immunopositive for γ-synuclein however this will require further study before any firm conclusions are drawn from it. This model proves that γ-synuclein is capable of causing detrimental effects to neurons of the central nervous system that resemble effects of α-synuclein rather than β-synuclein.

Gamma-synuclein null mice have also been created, using a targeted knockout approach, and studies show that the resulting animals are viable and have a normal phenotype. There was no evidence of any motor or peripheral sensory neuronal cell loss, cell populations that normally express high amounts of γ-synuclein, nor were any ultra-structural changes seen in these cells (Ninkina *et al.*, 2003). These results would suggest that either the protein is a non-essential for normal functioning of these cells or compensation is occurring. A later study was able to demonstrate that β-synuclein expression is slightly up-regulated in animals that lack γ-synuclein and to a larger extent in animals lacking both γ- and α-synuclein (Robertson *et al.*, 2004). When midbrain dopaminergic populations were studied in γ-synuclein null mice it was noted that early post natal and adult animals had a 15-20% decrease in the numbers of dopaminergic neurons in the SNpc but not the VTA. However when these cells had been counted at E18 embryos their numbers had been comparable to wild type animals. Upon challenging these animals with MPTP, unlike wild type controls, there was no significant loss of the SNpc dopaminergic neurons (Robertson *et al.*, 2004). A possible explanation for this apparent resistance to the neurotoxin could be that during a critical development stage a compensation mechanism is activated to ensure the survival of these neurons in the absence of γ-synuclein resulting in a population of more robust cells which can resist the toxic insult of MPTP. A possible role of upregulated β-synuclein in this process should not be overlooked.

As is clear from the discussions above the biological function(s) of the synucleins are still far from clear and further work and more appropriate models will be required to help reveal this.

One clear issue is that animal models can never fully recapitulate the human disease as there is often unrealistic levels of expression or inappropriate localised expression of the proteins. A second major problem is that many studies concentrate on the changes triggered during early development and therefore not in an aged nervous system which is highly relevant to the study of age related diseases. With improved models that represent a more physiologically relevant view it is likely that more can be revealed about these elusive proteins. It is hoped that the work in this thesis is able to contribute some further insight into the synuclein family.

Aims

1. To characterise the midbrain dopaminergic system of triple synuclein knockout mice in order to better understand the importance of the synuclein family in this subset of neurons in the absence of any possible familial compensation.

    a. Assess the survival of VTA and SNpc dopaminergic neurons in the absence of three synucleins
    b. Assess synaptic structure and condition including neurotransmitter concentrations
    c. Assess any behavioural phenotypes

2. To investigate the survival of midbrain dopaminergic neurons of triple synuclein null animals following exposure to MPTP

3. Produce a conditional tamoxifen inducible alpha-synuclein knockout mouse model and undertake initial characterisation of the model

Chapter 2

Methods

Animals

Generation of TKO mice

Triple synuclein knockout (TKO) mice were produced as described by Anwar et al (Anwar et al., 2011). Briefly, animals already possessed by our lab lacking both α- and γ-synuclein on a pure C57Bl/6 Charles River background, as described in Robertson et al., 2004, were crossed with beta-synuclein null animals previously produced by Chandra et al (Chandra et al., 2004, Robertson et al., 2004). Animals homozygous for knockout of all synucleins were then further backcrossed for 6 generations with Charles River C57Bl/6 animals to produce cohorts of triple synuclein knockout and wild type litter mate animals. Unless specifically stated otherwise male animals of 4-5 months were used in all experiments.

Husbandry

All animal husbandry was carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986). Animals were housed individually or in cages of 5 or less with a 12 hour light cycle followed by a 12 hour dark cycle. All animals had free access to food and water.

Histology

Fixation

Animals were sacrificed using a schedule 1 method and tissues rapidly harvested and placed into cassettes ready to be immersed in Carnoy's fixative overnight. Following this the samples were dehydrated as below and were then infiltrated with paraffin wax (RA Lamb) at 60°C for 3 hours prior to being embedded in fresh wax and left to set on a chilled ice plate.

Carnoy's Fixative:

60% Ethanol

30% Chloroform

10% Glacial Acetic Acid

Dehydration following overnight Carnoy's fixation at 4°C:

95% Ethanol	5 mins (x3)
100% Ethanol	10 mins
100% Ethanol	30 mins
1:1 Ethanol:Chloroform	30 mins
Chloroform	60 mins
Chloroform 4°C	Overnight

Sectioning

Once embedded the samples were then ready to section. Sectioning samples in preparation for histological examination occurred using a Microm HM310 microtome set to section 8µm sections using a razor-cutting blade. The sections were then floated in a water bath filled with dH_20 and warmed to 40°C. Sections were collected appropriately onto poly-L-lysine coated slides (Fisher) and allowed to dry overnight at room temperature.

Immunostaining

Samples were first dewaxed in xylene, followed by a graded rehydration series through decreasing strengths of ethanol before being brought to water. Antigen retrieval occurred by microwaving slides at 750W for 10 minutes in 10mM sodium citrate (pH 6.0). After the samples were cooled any endogenous peroxidase activity was quenched using a solution of 3% H_2O_2 in methanol for 30 minutes at 4 °C. Following this samples were washed using PBS after which a blocking solution of 10 % appropriate serum (e.g. horse serum) in 0.4% T-PBS was applied, followed by a further wash in PBS. Samples were then incubated with the primary antibody at the appropriate dilution. The antibody was prepared in blocking solution and incubated for one hour at room temperature. This was washed off using PBS before applying the a secondary biotinylated antibody at a dilution of 1:1000 prepared in 0.4% T-PBS and incubated for one and a half hours at room temperature. Samples were then washed with PBS followed by application of ABC solution (Vector Laboratories). This was left for 30 minutes and then washed with PBS. 3,3'-Diaminobenzidine (DAB) (Sigma) was then added to the samples in order to visualise the antibody and left for a further 5-15 minutes until sufficient staining was

apparent. Slides were then washed using dH_20 twice and the samples were serially dehydrated and taken to xylene before glass coverslips were mounted using DPX (RA Lamb).

Quantification of dopaminergic neurons of the SNpc and VTA

Tyrosine hydroxylase stained slides were used to count the dopaminergic cell populations of the SNpc and VTA. Prior to staining SNpc and VTA were serial sectioned and mounted to slides in a manner that produced two duplicate sets of slides as demonstrated below in figure 2.1. This meant one set could be stained whilst the other remained for replication or for further analysis where required. Mouse anti-tyrosine hydroxalase monoclonal antibody (TH-2, Sigma) at a dilution of 1:1000 were used to identify dopaminergic cell bodies. The criteria for deciding if a cell should be counted depended on there being a clear space in the middle of the cell body, indicating the cells nucleus. The criteria for counting are clearly demonstrated in appendix 1. All counts were carried out blindly and every tenth section on the slides was counted at 40x magnification and recorded separately for both left and right regions of the VTA and SNpc. Following this the mean nuclear diameter was measured using AnalySIS imaging software for 30 cells per animals. Total cell populations of each structure were then calculated using the Abercrombie correction technique as shown below.

$$P = (1/f) \times A(M/D+M)$$

Where P = the corrected cell count; F = frequency of cell count (i.e 1/10 = 0.1); M = section thickness (µm); D = average nuclear diameter.

Figure 2.1 **Layout of Slides for Stereological Counts**

Demonstrating the method in which serial sections were laid out over slides allowing duplicate sets to be produced. This meant that one set could be used for analysis by tyrosine hydroxylase counts and the other for repeats or any other required purposes.

Quench Solution

Hydrogen Peroxide	3%
Methanol	

Sodium Citrate buffer (pH 6.0)

Sodium citrate tribasic salt dehydrate	10 mM
dH_2O	
Adjuist pH to 6.0 using HCl	

Table 2.1 List of Primary Antibodies used for Immunohistochemistry

Target	**Additional Information**	**Supplier**	**Working Dilution**
Tyrosine Hydroxylase	Clone TH-2 Mouse Monoclonal	Sigma	1:1000
Dopamine Active Transporter	MAB369 Rat Monoclonal	Chemicon	1:500
Alpha-synuclein	Clone 42 Mouse Monoclonal	BD Transduction Laboratories	1:500
Beta-synuclein	Clone 8 Mouse Monoclonal	BD Transduction Laboratories	1:5000
Gamma-synuclein	SK23 Rabbit Polyclonal	(Buchman *et al.*, 1998)	1:1000

Secondary Antibody	Supplier	Concentration
Biotinylated Secondary antibodies	Vector	1:1000

Assessment of Protein and Neurotransmitter Levels

Western Blotting

To asses protein levels from various samples Western blot analysis was carried out. The Western blotting technique is a convenient method to visualise a specific protein from an array of others in a semi-quantitative manner and is reliant on the protein macromolecules being separated depending on their size and charge.

Snap frozen or fresh tissue/cell samples were homogenised in Laemilli buffer and boiled at 100^{o}C for 10 minutes. Where required total protein content of a sample was measured using a commercially available kit (Biorad) that is based on the Bradford assay to allow all samples to be normalised and loaded with an equal amount of total protein. These samples were then run through a two phase gel, the upper part being a 6% "stacking gel" and the lower a higher percentage "resolving gel" at 200V for the appropriate amount of time to separate desired protein bands. The resolving gel contained an appropriate percentage of acrylamide to satisfactorily separate proteins required for assessment i.e. a high percentage for lower molecular weight proteins and a low percentage for those of a high molecular weight. A pre-stained protein ladder was also loaded on each gel to allow easy identification of protein size (Fermentas). The percentage of polyacrylamide in the resolving gel was typically between 8-16%.

6% stacking gel

H_20	3.3ml
30% Acrylamide/BisAcrylamide solution	1.0ml
1.25 M Tris pH 6.8	620µl
10% SDS	50µl
10% APS	50µl

Resolving Gel – varying percentages

	10%	**16%**
H_20	3.97ml	2.00ml
30% Acrylamide/BisAcrylamide solution	3.33ml	5.30ml
1.25 M Tris pH 8.8	2.50ml	2.50ml
10% SDS	100µl	100µl
10% APS	100µl	100µl

10µl of TEMED was added to each gel to start the polymerisation reaction.

Following electrophoresis the gel was assembled into a transfer stack using a dry blot approach (Techware, Sigma). In this manner proteins were transferred to a PVDF membrane at a constant of 6-9 mA for 1.30 hours after which the membrane was retrieved and washed in TBS-T before being blocked for 1 hour in 4% non fat milk (Marvel). Following this the membrane was incubated overnight at +4 ^{o}C with the appropriate primary antibody in 4% milk. The membrane was then washed repeatedly with TBS-T and placed in 4% milk containing the appropriate secondary HRP conjugated antibody (Amersham) at a concentration of 1:3000 for 1 hour. After the membrane was washed in TBS-T the protein bands were visualised with either ECL or ECL+ chemiluminescent detection system in accordance with the manufacturer's instructions (Amersham). Should the membrane be required for reprobing it was again washed in TBS-T, reblocked and then appropriate primary and secondary antibodies applied before visualising.

2x LB Laemmli buffer:

100 mM Tris-HCl pH 6.8

20% Glycerol

0.2% Bromophenol blue

200 mM 2-mercaptoethanol

4% SDS

10x Running Buffer

250 mM Tris Base

2.0 M Gycine

1% SDS

Diluted to 1x working solution with dH_20

10x Transfer Buffer

250 mM Tris Base

1.5 M Glycine

Diluted to 1x working solution with dH_20 and the addition of 20% methanol

TBS-T

TBS (Sigma) dissolved in 990ml ddH_20

10% Electran Tween20 (VWR) 10ml

Table 2.2 **List of Primary Antibodies used for Immunoblotting**

Target	**Additional Information**	**Supplier**	**Working Dilution**
VMAT-2	Rabbit Polyclonal	Santa Cruz	1:500
SNAP-25	Clone 20 Mouse Monoclonal	BD Transduction Laboratories	1:1000
VAMP2	Rabbit Polyclonal	Sigma	1:1000
Alpha-synuclein	Clone 42 Mouse Monoclonal	BD Transduction Laboratories	1:500
Tyrosine Hydroxylase	Clone TH-2 Mouse Monoclonal	Sigma	1:5000
Synaptophysin	Clone 2 Mouse Monoclonal	BD Transduction Laboratories	1:25000
Synaptotagmin	Clone ASV48 Mouse Monoclonal	QED	1:5000
Amphiphysin	Clone 15 Mouse Monoclonal	BD Transduction Laboratories	1:10000
Complexin 2	Clone 48 Mouse Monoclonal	BD Transduction Laboratories	1:500
Synapsin IIa	Clone 1 Mouse Monoclonal	BD Transduction Laboratories	1:10000
CSP	Rabbit Polyclonal	Santa Cruz	1:1000
β-actin	Clone AC-15 Mouse Monoclonal	Sigma	1:10000
GAPDH	Clone 6C5 Mouse Monoclonal	Santa Cruz	1:1000

Secondary Antibody	Supplier	Concentration
HRP conjugated secondary antibodies	Amersham	1:3000

High-Performance Liquid Chromatography (HPLC)

In order to accurately assess striatal levels of dopamine and its metabolites HPLC was employed. Samples were prepared using approximately 5mg of freshly dissected dorsal striatum which were homogenised in 0.5ml of 0.06M $HClO_4$ and the resulting homogenate was centrifuged at 16000g for 15 minutes at +4^{o}C. Following this the supernatant was carefully removed and kept on ice until it was injected into the HPLC column. A 4.6 x 150mm electrochemical detection microsorb C18 reverse-phase column (Varian) and Decade II ECD with a Glassy carbon-working electrode set to +0.7 V, with a Ag/AgCl reference electrode (Antec Leyden). To measure the concentrations of dopamine and its metabolites the mobile phase consisted of 12% methanol (v/v), 0.1 M monosodium phosphate, 0.68 mM EDTA and 2.4mM 1-octane sulfonicacid (OSA), pH 3.1.

NSD-1015 Administration

To measure the rate of dopamine synthesis *in vitro* animals were given a 100mg/kg intraperitoneal injection of NSD-1015. Animals were killed 40 minutes following the injection and their dorsal striata collected to ascertain L-DOPA accumulation using HPLC analysis.

Isolation of Synaptic Vesicle

All procedures were carried out at +4 ^{o}C. The dorsal striata of 6 mice were homogenised in 0.5 ml of lysis buffer using a glass homogeniser. Following this the samples were centrifuged at 1000g for 10 minutes to sediment the nuclei and debris. The supernatant was carefully collected and then centrifuged at 20000g for a further 20 minutes to obtain the cytosolic supernatant and a pellet containing the crude synaptosome fraction. . For synaptic vesicle isolation the pellet was resuspended in 0.5 ml 0.32M sucrose, diluted to 2 ml with ddH_20, homogenised in a glass-teflon homogeniser and left for 10 minutes on ice before the addition of 0.3 ml of 250 mM HEPES, pH 7.4 and 0.3 ml of 1 M potassium tartrate. This was then centrifuged at 20000g for 20 minutes in order to pellet synaptic membranes. Following this the supernatant was taken to a fresh ultracentrifuge-capable eppindorf tube and centrifuged at 120000g for 40 minutes to obtain a pellet of synaptic vesicles. This pellet was then resuspended in 1 ml of vesicular uptake assay buffer using a 25 gauge needle.

Lysis Buffer

0.32 M Sucrose

5 mM HEPES pH 7.4

Protease inhibitors (Complete Mini, Roche) – 1 tablet per 10ml of buffer

Vesicular uptake assay buffer

25 mM HEPES pH 7.4

100 mM potassium tartrate

0.1 mM EDTA

0.05 mM EGTA

1.7 mM ascorbic acid

2 mM ATP

Recombinant β-Synuclein

The pCS19 β-synuclein cDNA containing vector had previously been produced in our lab. The vector contains the coding region of the human β-synuclein gene and was used to transform KU98 *E.coli*. Bacterial expression of the β-synuclein protein was induced when the bacterial culture, growing at 37^{o}C, reached 0.6 OD_{600} using 0.5mM IPTG. Following IPTG induction the bacterial cultures were grown for a further 4 hours at 26 ^{o}C. The recombinant protein was then purified as described previously (Jakes *et al.*, 1994).

Behavioural Testing

Rotarod

The rotarod is designed to assess the balance and endurance of the mice. It is a horizontal beam placed above a trip switch which has an incorporated timer meaning that the time to fall is recorded for each animal. The Ugo Basil 7650 Rotarod was set up in order to accelerate from 4 to 40 rpm in 30 second intervals over a 5 minute test period. Prior to the test being performed the animals were moved in their home cage to the procedure room for 60 minutes to acclimatise to the new environment. Animals were then given a practise session on the apparatus set to a constant speed of 24 rpm followed by a 60 minute rest period before the

test began. Mice were tested 3 times with a minimum rest period of 40 minutes between tests and their mean latency to fall time was recorded (Robertson., *et al* 2004).

Inverted Grid Test

This test can be used to asses both the strength and coordination of a mouse. The grid is a 30x30cm square mesh made up of 5mm squares from 0.05mm diameter wire. The mouse is placed on the grid and the grid is then slowly rotated 180 degrees bringing it into an inverted position. This is held 30cm above a thick layer of bedding material. The mouse is then tested for 1 minute and the latency to fall was noted of any animals that didn't complete an entire minute. The mouse was then returned to rest in its home cage and the test repeated allowing the mean of three attempts to be compiled.

Home-like Cage Activity

This test was used to measure the locomotor activity levels of the mice and also assess how they react in a new environment. Prior to the test beginning the animals were moved to the test room in which the light cycle is the same as in the animals holding room. They were allowed a 30 minute acclimatisation period in the test room before the test was started. The animals were placed in a clear Perspex box measuring 40x40cm and were left for the appropriate amount of time. In the case of testing locomotor activity in novel environments the test lasted a total of 26 hours. However, when animals were exposed to pyschostimulants they were placed in the home-like cage as described below. The apparatus is equipped with infrared beams and is able to record when these are broken. The number of beam breaks was recorded every 4 minutes over the test period.

MPTP and Psychostimulant Administration

Subchronic MPTP treatment

Male mice aged to 4 months were given one intraperitoneal injection of 30mg/kg MPTP (Sigma) or PBS a day for 5 consecutive days whilst being carefully monitored for any severe changes in their health. Twenty one days following the last MPTP injection animals were euthanized by phenobarbital overdose and brains were dissected for fixation or biochemical analysis.

Psychostimulant treatment

In all cases drugs were prepared and dissolved in sterile 0.9% saline. All drugs were sourced from Sigma and in the case of both cocaine and D-amphetamine (dAMPH) animals received a single 10mg/kg or a 4mg/kg intraperitoneal injection respectively after being placed in the activity camera apparatus for 30 minutes in order to allow acclimatisation. Following the injection the animals were returned to the recording apparatus for an additional 90 minutes of recording. In the case of methyl L-3, 4-dihydroxyphenylalanine (L-DOPA), animals were allowed 20 minutes to acclimatise to the testing room before being administered a single intraperitoneal injection of 50mg/kg and returned to the home cage. After a 20 minute interval the animal was then placed in the recording equipment and dAMPH injections were administered as described above.

Cloning

PCR

Isolation of genomic DNA (gDNA) was undertaken using a Wizard SV gDNA purification system (Promega). After the optimisation of each set of primers gDNA was then either used to create nested fragments using 5µl gDNA and 45µl of amplification mastermix (table 2.3) and amplified for 45 cycles or used to create blunt ended fragments ready for immediate cloning. In this case 1.5µl of gDNA plus 0.5µl of each forward and reverse primer was added directly to the Accuprime Pfx SuperMix (Invitrogen) and cycled at appropriate conditions for the fragment required. In the case of nested primers after the first reaction in a normal mastermix but with fewer cycles a further 15 cycles using the resultant template DNA (1.5µl) was carried out using the Accuprime system with the non-nested primers to ensure blunt ends for subsequent cloning. All primers used can be found in appendix 3.

Amplification Mastermix	Concentration	Typical Cycling Conditions
dNTP	0.2 mM	94°C – 15 sec
Primers (each)	0.25 µM	60°C – 30 Sec
KCl	50 mM	72°C – 40 Sec
Tris-HCl pH 9.0	10 mM	
$MgCl_2$	1.5 mM	
Taq Polymerase	5 units	
Triton X-100	0.1%	

Table2.3 **PCR Mastermix**

Components of the amplification mastermix, including buffer and concentrations along with typical cycling conditions used unless stated otherwise.

Agarose Gel Electrophoresis

Typically a 1% agarose gel was prepared using 1xTAE, ethidium bromide (Sigma) and agarose (Eurogenetec). 5µl of DNA ladder and 10µl of sample were loaded and ran to visualise fragment size. In some cases it was necessary to produce low melting point (LMP) agarose gels (Metaphore) in order to purify fragments from the gel, in which case the entirety of the appropriate sample was loaded.

TAE 50X 1L

242 g of Tris Base

57.1 ml Glacial Acetic Acid

100 ml 0.5 M EDTA (pH 8.0)

Diluted to 1x working solution

Gel Loading Buffer

100 mM Tris-Cl

4 % (w/v) SDS

0.2 % (w/v) Bromophenol Blue

20 % (v/v) Glycerol

Agarose gel purification of DNA fragments

Some PCR reactions could not be optimised to produce only one clear band of correct sized DNA. In this case, following the PCR reaction the resultant DNA was ran on an agarose gel and visualised on the UV lamp at 312 nm. Using a clean scalpel the correct sized fragment was excised from the gel. The selected portion of the gel was then weighed and the DNA purified from it using the QIAquick gel extraction kit (Qiagen). This was done following the supplied manufactures instructions. This method was also required once correct fragments for cloning into the construct had been identified and therefore needed to be extracted from the pCR-Blunt II-TOPO vector after enzymatic digests. In this case approximately 10 µg of DNA would first be digested and the resulting fragments were purified ready for insertion into the vector.

Transformation of Competent Cells

Correctly sized fragments from AccuPrime™ *Pfx* DNA Polymerase (Invitrogen) PCR reactions underwent ligation into the pCR-Blunt II-TOPO vector (appendix 2 shows plasmid map). A mastermix containing 2µl fresh PCR product, 0.5µl salt solution and 0.5µl of pCR II-Blunt-TOPO vector and made to 6µl with dH_20 were mixed according to the manufactures protocol (Invitrogen). Following this 3µl of the mastermix was added directly to chemically competent cells, with the appropriate efficiency: most commonly one shot max efficiency DH5α-$T1^R$

(Invitrogen) otherwise one Shot Stbl3 chemically competent E. coli (Invitrogen) or DH5αTH high efficiency cells (New England Biolabs). These were defrosted on ice and 2-4µl of the appropriate plasmid in the case of retransforming or TOPO blunt cloning reaction was added before incubating on ice for a further 15 minutes. The cells then underwent heat shock at 42°C for 30 seconds to allow the bacterial membranes to become permeable, allowing the passage of plasmid DNA across them. The cells were then placed back on ice for 2 minutes to allow the membranes to recover. SOC medium was then added to the competent cells and incubated for 1 hour at 37°C in a shaking incubator to allow the expression of antibiotic resistance genes before the cells were spun for 5 minutes at 5000g and most of the media aspirated. The cells were then gently resuspended and spread on appropriate selective agar plates and incubated overnight at 37°C.

Preparation of Agar Plates and Luria Broth

Luria broth was produced by adding 25g of Luria Broth Base (Invitogen) per 1L of dH_2O. The addition of select agar (Invitrogen) to a final concentration of 2% in 300ml of Luria broth was used to generate agar. These were then both autoclaved before use and then stored until required. Selective antibiotics were added to both agar and Luria broth at 50µg/ml concentrations.

Selection of bacterial colonies

After plates were incubated at 37^{o}C overnight they were then used to select single bacterial colonies in order to further amplify recombinant plasmid DNA. Single colonies were picked from plates using sterile pipette tips and placed in 5ml selective Luria broth overnight at 37^{o}C whilst being rocked in order to aerate the suspension.

Purification of Bacterial Plasmid DNA

Overnight inoculations were spun at 5000g for 5 minutes and the resulting suspension discarded to leave only a pellet. The bacterial pellet was then re-suspended and the DNA purified using the manufacturer's instructions for the Qiagen qiaprep miniprep column purification system. This system relies on alkaline lysis of the bacterial cells followed by adsorption of the DNA onto a silica membrane under high salt conditions. The resulting DNA was eluted in 50-100µl dH_20 and used for further analysis of the clone.

DNA extraction using Phenol Chloroform

Mouse tissues were lysed for 4-16 h at 55^{o}C in digestion solution containing Proteinase K. In the case of ES cells, medium was aspirated, plates were frozen at -20^{o}C until required following which digestion solution was added and cells collected by scraping. Following incubation the lysed material was transferred to a fresh eppendorf tube, an equal volume of phenol (pH 8.0) was added and after gentle but thorough mixing spun for 10 minutes at max speed. The supernatant was then transferred to a new eppindorf tube and again an equal volume of phenol was added and contents mixed before spinning at max speed for 10 minutes. This was repeated with phenol-chloroform 1:1 before the final supernatant was removed to a fresh eppindorf tube. Precipitation of the DNA followed with the addition of 2 M NaAc at 10% total volume and 2.5 volumes of 100% ethanol. If a DNA clot was visible it was transferred to a new eppindorf tube containing 70% ethanol. If no clot was visible the sample was spun for 2 minutes at max speed. Following this both spun down pellets and DNA clots were washed 3 times in 70% ethanol. After the final rinse excess ethanol was removed and the sample left at room temperature until any remaining ethanol had evaporated. The resulting DNA was eluted in dH20 to an appropriate volume and left at 4^{o}C overnight until properly dissolved.

Digestion solution:

100 mM NaCl

50 mM Tris (pH 8.0)

2 mM EDTA

1% SDS

2 mg/ml Proteinase K

Restriction Enzyme Analysis

Restriction enzyme digests were employed to check the presence or absence of the correct fragment within a vector and also for Southern analysis of targeted ES clones. In both cases a mastermix containing DNA, the appropriate enzyme at 1 unit/µg DNA and the correct buffer at 10% of the total volume was incubated at 37^{o}C (unless stated otherwise by the manufacturer's

guidelines) for a minimum of one hour. For genomic DNA being digested for Southern blot analysis the digests were left overnight and used excess restriction enzyme in order to ensure complete digest. The reaction was then run on an agarose gel to assess fragment size.

Sequencing

To ensure that all cloned fragments were correct they were sequenced using a variety of primers (appendix 3). In the case of any fragments within the pCR-Blunt II-TOPO vector these were sequenced using either T7 or SP6 primers. All sequencing was carried out by the Cardiff University sequencing core. Analysis of the sequences was carried out using ApE (A plasmid Editor v1.17) software.

DNA Quantification

DNA concentration was estimated by measuring solution absorbance at 260nm on the Nanodrop (Labtech International).

Ligation Reactions

When fragments with the correct sequence were identified they could then be used for further cloning. Once fragment and vector had been digested and purified their aliquots were run on a gel alongside each other in order for an estimation of vector/insert ratio to be made. A ligation mastermix was prepared containing vector and insert at the estimated molar ratio of 1:5, ligase buffer and T4 DNA ligase. Once mixed it was placed into a water bath at 17^{o}C, which was then placed in the cold room overnight to create a falling temperature gradient. Following this 2µl of the reaction was used for transformation of high efficiency competent cells.

Cell Culture

In order to produce cells that could be used for blastocyst injection and ultimately produce a line of mice the targeting procedure required a suitable line of embryonic stem (ES) cells. In this instance the JM8A3N ES cell line was selected as it is derived from the C57Bl/6N mouse strain, meaning this would limit the number of backcrosses required to produce a pure C57Bl/6 line over other conventional ES lines produced on the more traditional S129 background. Importantly this line also displays high germline competency which is an important consideration when producing a new animal model (Pettitt *et al.*, 2009).

Culturing of JM8 Feeder Free ES Cells

Cells were cultured on 0.1% gelatin coated plates (Nunc) in full media at 37^{o}C and 5% CO_2. The plate surface was covered with 0.1% gelatin solution for 5 minutes before it was aspirated and the plates were then left to dry for 1 hour prior to cells being applied. Once cells reached 70-80% confluence they were passaged. Media was aspirated, cells washed with pre-warmed D-PBS and trypsin applied for 5 minutes at 37^{o}C (table 2.4 demonstrates appropriate volumes). To inactivate trypsin 5-10 volumes of full media was added and 1/6th of the cell suspension split to each new plate. Unless stated otherwise all cell culture components were purchased from Invitrogen.

Dish Diameter (cm)	Volume of Gelatin/D-PBS (ml)	Volume of Trypsin (ml)	Volume of full Media (ml)	Volume of full media & DMSO (ml)	Number of Freezing Vials
3	3	0.5	5	1	2
10	5	1	10	2.5	5

Table 2.4 **Volumes for Cell Culture**

Examples of appropriate volumes of media, trypsin, gelatine, D-PBS and freezing components for different dish sizes of JM8 cells.

Titration of Leukaemia Inhibitory Factor (LIF)

Stocks of murine LIF purified previously in the lab were tested against a commercially available comparison, ESGRO (Millipore), used as directed by manufacturers specifications. Cells were cultured in varying concentrations of each LIF and scored over 14 days for the amount of differentiated and migrating cells. Following this the most appropriate LIF was decided on and used in the media at 1ml per 500ml of full media. LIF was present in all media except that used the day before blastocyst injections.

Freezing JM8 Cells for Liquid Nitrogen Storage and Defrosting

Freezing was carried out using freezing medium and table 2.4 indicates appropriate volumes. Media was aspirated and cells washed with pre-warmed D-PBS before adding pre-warmed trypsin for 5 minutes at 37^{o}C. Trypsin was inactivated with full media and the cell containing suspension transferred to a falcon and spun for 3 minutes at 300g. Media was aspirated and the pellet re-suspended in freezing medium which acts as a cryoprotectant, reducing ice crystal formation. The cell suspension was transferred to cryovials and placed at -80 ^{o}C overnight before transferring to liquid nitrogen.

To defrost cells the vial was thawed as quickly as possible at 37 ^{o}C and with gentle agitation. Once thawed the cell suspension was added to full media and spun for 2 minutes. Media was Aspirated and the pellet re-suspended in full media and transferred to a gelatinised plate of the appropriate size.

Titration of Antibiotics

Working concentrations of selective antibiotics were titrated using naïve cells. The criterion for the correct concentration was that it should kill all cells in 3-5 days of application. G418 was tested at 105 mg/ml and Gancyclovir at 2.2 mM, concentrations previously used for another ES line and it was concluded that these concentrations are also suitable for the JM8A3N line and therefore were used in future experiments. In the case of puromycin 10µg/ml, 5µg/ml, 3µg/ml and 1µg/ml were tested. It was decided that 3µg/ml concentration would be used for further experiments.

Lipofectamine Transfection of Positive ES Cell Clones

Cells were first split the day before transfection took place allowing the cells to reach approximately 60% confluency the following day. For a 10cm dish 2 eppendorfs were prepared, one containing ~6µg DNA and the other 40µl Lipofectamine 2000 (Invitrogen) which

were diluted in media in the absence of antibiotics and FBS. This was allowed 10 minutes at room temperature before they were combined and left for an additional 20 minutes at room temperature in order for the DNA and Lipofectamine 2000 to produce adequate complexes. Following this further KODMEM minus antibiotics and serum were added to make 7ml of medium. Cells had media aspirated and were rinsed in D-PBS before the media containing the transfection reagent and DNA complexes was supplied. Following this the cells were incubated at 37°C at 5% CO_2 for 3 hours before having the transfection media aspirated. The cells were then washed in D-PBS and normal full media was replaced.

Selection of ES Cell Clones following transfection

The day after transfection cells were exposed to selective antibiotics, in the case of the pCAGGs-Flpe vector this was puromycin at 3μg/ml. This was continued for approximately 10 days until colonies could be identified and picked as described below. Once the cells had been split across two wells one was exposed to G418 in order to confirm the loss of the neomycin cassette.

Full Media

500ml KO-DMEM

50ml ES certified FBS

5ml 100x Penicillin-Streptomycin

5ml 100x L-Glutamine

3.2% β-mercaptoethanol (Sigma)

1ml LIF

Antibiotics added only when required for targeting experiments

Freezing Medium

Full medium with 20% FBS and 10%DMSO (Sigma)

Freshly prepared when required

0.1% Gelatin Solution

2.5ml prewarmed 2% Gelatin (Sigma)

50 ml D-PBS

Trypsin

500ml D-PBS

0.1 g EDTA (Sigma)

0.5 g D-glucose (Sigma)

0.22µm filter sterilise before addition of the following

5ml Checken Serum

20ml 2.5% trypsin

Solution was aliquoted into appropriate volumes and frozen

Targeting

DNA preparation

Construct DNA was purified and linearized by complete digest with NotI (New England Biolabs). After running a sample on an agarose gel to ensure complete digest the digested DNA was recovered using ethanol precipitation. After the DNA was purified it was kept under 70% ethanol at -20 ^{o}C until required. Once the DNA was required the DNA pellet was spun, ethanol aspirated and the pellet was left to dry in a sterile tissue culture hood. DNA was diluted in sterile ddH_20 water in order to provide a final concentration of 30µg in 30µl.

ES Cell Preperation and Electroporation

Cells were cultured on 5 gelatinised 10cm plates until 70-80% confluency was reached and washed in D-PBS before being trypsinised for 5 minutes. Following this the cells were spun for 5 minutes at 300g and resuspended in 800µl D-PBS. 30µl of DNA solution was added to the resuspended cells and transferred as quickly as possible to a sterile electroporation cuvette (Biorad). This was placed into the electroporator and pulsed at 240 V/500µF. The cuvette was then placed back in the tissue culture hood and left to stand at room temperature for 20 minutes. During this time 10 new plates were prepared with gelatine. The cells were carefully removed from the cuvette and resuspended in 50ml of full media without selective antibiotics. The cells were then split across the plates as shown in table 2.5.to give plates of varying cell concentrations.

Dish	Amount of Full Media (ml)	Amount of cell re-suspension (ml)
1	1	9
2	2	8
3	3	7
4	4	6
5	5	5
6	6	4
7	7	3
8	8	2
9	9	1
10	10	Rinse out tube

Table 2.5 **Volumes for ES Cell Resuspension Following Electroporation**

Volumes of media and cell resuspension for plating following electroporation.

After overnight incubation the medium was changed and 48 hours after electroporation changed again to medium containing the selective antibiotic G418 at 105 mg/ml. 72 hours after the electroporation gancyclovier was also added to the medium to a final concentration of 2.2 mM. The plates were then incubated and medium changed daily until approximately day 10 when clones began to appear.

Once the clones were clearly visible they were carefully picked using a different sterile tip for each colony and placed into a bubble of trypsin for 5 minutes, following which the trypsin suspension was plated onto one well of a pre-gelatinised 4 well plate filled with the G418-containing medium. Once confluent, cells from this well were split onto one 3cm dish and two separate 4 well plates as demonstrated in figure 2.2. When each 4 well plate was nearly confluent freezing medium was applied and the dishes were placed in sealed polysterine boxes, to ensure slow freezing, before being stored at -80^{o}C. At all times the selective antibiotics were present in the normal ES medium unless stated otherwise. Once the 3 cm plate was confluent the medium was removed and the plate rinsed with 1x PBS before being frozen at -20 ^{o}C until required for Southern blot analysis.

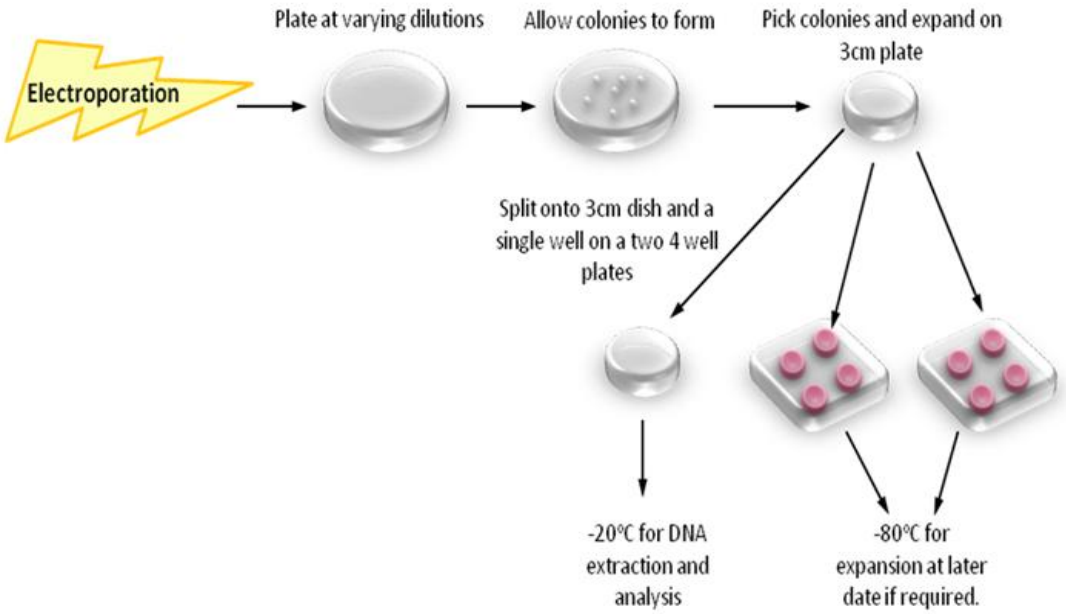


Figure 2.2 **The Targeting Process**

Schematic representation of ES cells taken from electroporation to analysis and freezing.

Analysis of clones by Southern blots

Southern blotting is a highly sensitive technique allowing one copy of a gene to be detected (Southern, 1975). This is important when producing targeted animal models as the presence or absence of the targeted DNA needs to be confirmed. The technique uses specific probes that can hybridise to known DNA sequences that have been digested with restriction enzymes.

This allows the recognition of either the targeted or wild type gene through the difference in size of the digested DNA fragment.

DNA was isolated from individual ES cell clones grown on 3 cm dishes using phenol-chloroform as described above. The resulting concentrated DNA for each clone then underwent initial Southern screens using BamHI digests. If a clone then appeared to have been correctly targeted further restriction enzyme digests and a second hybridisation probe were used to confirm this.

Digested DNA of ES clones were run on a 0.9% agarose gel at a low voltage to begin while samples cleared the wells. After this the voltage was increased and ran until the DNA was sufficiently separated. The gel was imaged before being cut to an appropriate size using a clean razor blade and inverted so that the flattest side of the gel would be exposed to the membrane ensuring maximal transfer of DNA. The gel was then placed in depurination 0.25M HCl solution for 20 minutes due to the large nature of the expected fragments and this was then replaced with fresh for a further 20 minutes. The gel was then rinsed twice with dH_20 before denaturation buffer was added. This was left gently rocking for 20 minutes before the buffer was drained and fresh was added for a further 20 minutes. Following this the gel was rinsed in dH_20 and placed into neutralisation buffer for 20 minutes after which this solution was replaced with fresh.

Blotting membrane was prepared by cutting hybond N+ nylon membrane (Amersham) with clean scissors to the same size of the gel. This was then soaked in boiling ddH_20 before being placed briefly in 2x SSC. DNA from the gel was transferred to this membrane overnight using standard capillary blotting in 10xSSC. The following morning the membrane was removed using clean forceps and allowed to dry. Once dry the membrane was placed on a UV lamp to check the transfer and to highlight any visible landmarks e.g. ladder and position of lanes. The membrane was then baked at 80^oC for 2 hours before being rinsed in 2xSSC.

To prepare the desired probe for Southern hybridisation, the production of which is described in chapter 5, approximately 20µg of DNA was EcoRI digested after which the DNA was run and an eukaryotic insert was eluted from a 1% agarose gel. 100 ng of eluted DNA was then labelled with 0.1mCi of α^{32}P-dCTP in a nick translation reaction using Nick-translation kit (GE Healthcare). The labelled DNA was separated from unincorporated dNTPs by passing through Sephadex G-50 Nick-column (GE Healthcare). Labelled DNA was denatured by incubation at 100^oC for 5 minutes, followed by immediately mixing it with ice cold hybridisation buffer. The

probe was then hybridised to the Sothern membrane to allow visualisation of the transferred DNA fragments. The membrane was placed into a hybridisation tube with 5 ml of prehybridisation buffer. This was then left rolling at 67^{o}C for 4 hours after which the prehybridisation buffer was removed and replaced with prehybridisation buffer containing ^{32}P-labelled probe. The hybridisation reaction was left rolling at 67^{o}C for 16-20 hours before the membrane was removed and washed 3 times, 15 minutes each, in 2xSSC with 0.2% SDS at 67^{o}C. The membrane was then wrapped in cling film and X-ray film placed on top in order to allow visualisation of the hybridised DNA. The cassette containing the membrane and X-ray film were placed at -80 ^{o}C for 1-12days to allow the best exposure before the film was developed.

Depurination Solution

0.25 M HCl

Denaturation Solution

1.5 M NaCl

0.5 M NaOH

Neutralisation Buffer

1 M Tris-Cl (pH 7.4)

3 M NaCl

10x SSC

1.5 M NaCl

0.15 M Sodium citrate

Pre-hybridisation Buffer

4x SSC

0.5% SDS

5x Denchardt's solution

100 µg/ml denatured salmon testis DNA

Production of Chimeric Mice

Preparation of Cells for Blastocyst Injections

Cells were defrosted 3 days prior to the injections occurring in order for them to reach subconfluency but not require passaging, thus allowing them the best opportunity to remain pluripotent. The day before the injections were due to take place medium was aspirated and cells rinsed in pre-warmed D-PBS. Medium was replaced with full medium without LIF and selective agents to prepare the cells for injection. On the day of injections cells were washed in pre-warmed D-PBS, this was aspirated and trypsin applied as described previously. Trypsin was inactivated with full medium without LIF and selective agents. The cell suspension was transferred to a Falcon tube and spun 300g for 3 minutes, before media was aspirated and cells washed with D-PBS. Cells were spun once more and then re-suspended in 1 ml M2 medium (Sigma) and spun again. Following this cells were re-suspended in 100µl M2 and kept on ice until injection.

Blastocyst Injections

C57Bl/6 breeding pairs were set up 4 days prior to blastocyst harvesting and vaginal plugs checked the next morning. On the day of blastocyst injections uteri were collected from plugged females and flushed with M2 medium. Any blastocysts or morulas were collected and transferred to fresh M2 medium and kept at 37 ^{o}C until required. An aliquot of ES cells suspension was put in a drop of M2 medium in a depression slide under mineral oil along with several collected blastocysts. The slide was placed onto a microscope stage and prepared for injections. The holding pipette was used to hold the blastocyst in position and round; similar sized ES cells were selected with the injection needle. Between 7 and 12 ES cells per blastocyst were injected by Bridget Allen or Orosia Asby of the Cardiff University Transgenic mouse Facility.

Uterine Transfer

To produce recipient female CD1 mice were housed with vasectomised males 3 days prior to injection day, vaginal plugs were checked the next day and plugged mice were recorded as pseudo-pregnant recipients. On the day of transfer they were prepared for surgery by anesthetizing with avertin (0.25-0.5mg/g). Once the mice were under anesthesia they were placed on a heat pad and their fur cleaned using 70% ethanol prior to making an incision across the lower back, in line with the first lumbar vertebra. The ovary, oviduct and some

uterus were exposed, using the fat pad of the ovary to pull them out of the peritoneal cavity. Using a 27 gauge needle a hole was made in the uterus and the tip of the transfer pipette carefully placed into the pre-made hole and up to 12 blastocysts were gently transferred into each side of the uterus. The uterus, oviduct and ovary were returned back into the peritoneal cavity and the incision closed with a wound clip, which was later easily removed. The animal was given pain relief and placed into a clean cage, kept warm and observed until recovered from the anesthesia. These procedures were carried out by Bridget Allen or Orosia Asby of Cardiff University Transgenic mouse Facility.

Statistics

Statistical analysis was performed to confirm that differences between groups of data were significant or not. All statistical analysis was carried out using Minitab software with guidance described in the *Practical Data Analysis Workbook for Biologists* (Bowker and Randerson., 2010).

Chapter 3

Characterisation of Triple Synuclein Knockout Mice

Introduction

The existence of the synuclein family has long been known and the role of α-synuclein in disease has been studied for over a decade. However as yet studies designed to unveil the role of these abundant presynaptic proteins have proved unfruitful. Studies carried out previously on single and double synuclein KO animals have given inconclusive results with regards to the role of these proteins (summarised in chapter 1). One clear confounding factor is that members of the synuclein family are highly homologous and therefore it has often been considered that the remaining member or members of the family in knockout models have been able to functionally compensate for the loss of the missing protein. This is supported by the fact that the remaining synuclein in both $\alpha\gamma^{-/-}$ and $\alpha\beta^{-/-}$ synuclein knockout animals is upregulated suggesting at least some functional overlap in the synuclein family (Chandra et al., 2004, Robertson et al., 2004). Another result that supports this hypothesis is that the pathology of animals lacking cysteine string protein alpha (CSP-α) is heightened in the absence of both α- and β-synuclein rather than either alone (Chandra et al., 2005).

As it is well accepted that there is a clear association between the pathology of the midbrain dopaminergic system in Parkinson's disease and α-synuclein, it is important that the proteins normal function is correctly understood in order to unravel how dysfunction can lead to disease. The only way to clearly resolve the predicament of familial redundancy was to produce and study triple synuclein knockout mice. Both β- and γ- synuclein are also highly expressed in the SNpc and unbalance of the synucleins may add to pathological processes. For this reason the nigrostriatal system of these TKO animals was explored in the hope of revealing a function for the synucleins.

Results

The production of the triple knockout animals used in this chapter is described in Anwar et al., 2011. Briefly, heterozygous β-synuclein KO animals produced by Chandra *et al.* (2004) were backcrossed onto a pure C57Bl/6J background before being crossed with $\alpha\gamma^{-/-}$ synuclein double knockout animals on the same background, already possessed by our lab. Therefore the resulting animals were on a pure background.

Unless stated otherwise all animals used were male in order to limit any impact that hormonal cycles in female animals may have on the brain and were between the ages of 4-5 months of age. The absence of all three synucleins in the brain of TKO mice was confirmed by immunohistochemistry as illustrated in figure 3.1.

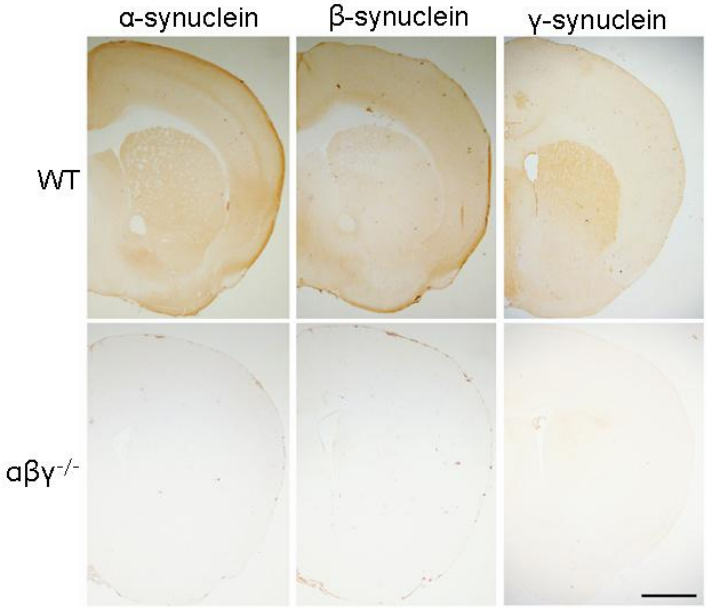


Figure 3.3 **Lack of Synuclein Expression in TKO Mice**

Detection of the synucleins in both WT and triple synuclein null mice brains. Coronal sections at the Bregma 0.38mm level stained with antibodies against either α-, β- or γ-synuclein. Normal distribution of each synuclein is shown in the WT brain whereas in the triple synuclein null animal the expression of all three synucleins is absent. Scale bar represents 1mm.

Survival of Dopaminergic Neurons

The synucleins, in particular α-synuclein have been linked to dopaminergic neuronal survival. In order to assess the effect of the loss of all three synucleins on the midbrain dopaminergic neurons the number of TH positive cells of the venteral tegmental area (VTA) and the SNpc were stereologically counted in 4 month old male mice (figure 3.2). A total of 18 WT and 28 $\alpha\beta\gamma^{-/-}$ synuclein mice were used and both sides of the VTA and SNpc were assessed as described in Materials and Methods. As can be seen in figure 3.2 these counts revealed no significant differences in the number of TH positive cells in either the VTA or SNpc of $\alpha\beta\gamma^{-/-}$ synuclein animals in comparison to their WT counterparts. Aged animals of 24 months also showed no alternations in their number of TH cells in the VTA or SNpc (figure 3.2).

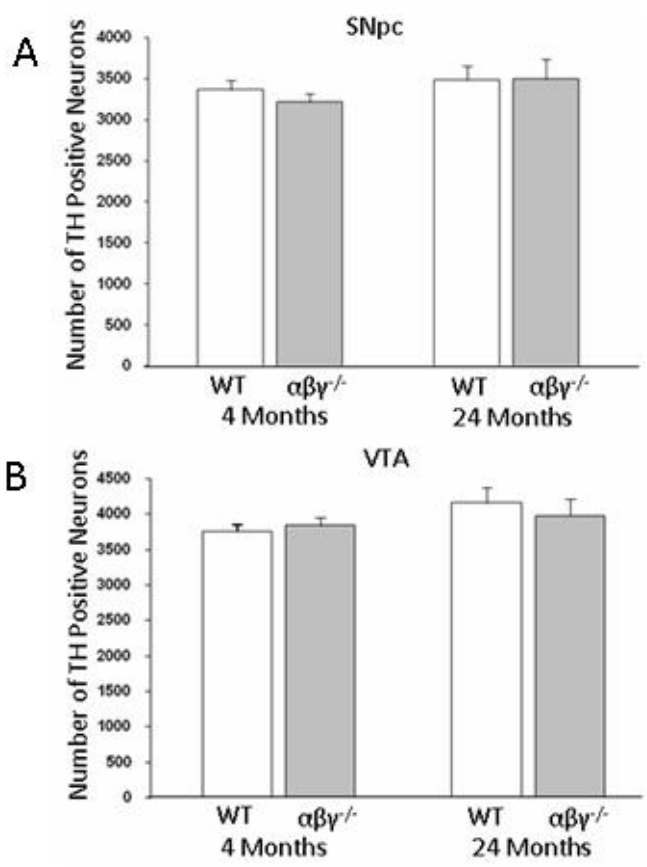


Figure 3.4 **Midbrain Dopaminergic Cell Counts for TKO Mice**

(A) Total number of TH-positive neurons in SNpc of 4-month old animals, wild type (n=18) and TKO (n=28), and 24 month old WT (n=9) and $\alpha\beta\gamma^{-/-}$ (n=18). (B) Total number of TH-positive neurons in the VTA of 4-month old mice, wild type (n=18) and TKO (n=28) mice and 24 month old WT (n=9) and $\alpha\beta\gamma^{-/-}$ (n=18).

Synaptic Marker Expression and Distribution

Figure 3.3 A and B demonstrates that there were no alterations in the levels of various synaptic markers as shown by quantitative Western blot. To assess normal gross distribution of striatal synapses coronal sections were taken of both WT and $\alpha\beta\gamma^{-/-}$ synuclein 4 month old animals and immunostained for TH and the dopamine active transporter (DAT). Again there were no alterations in the gross morphology of the striatum of TKO animals in comparison to WT controls (figure 3.3, C).

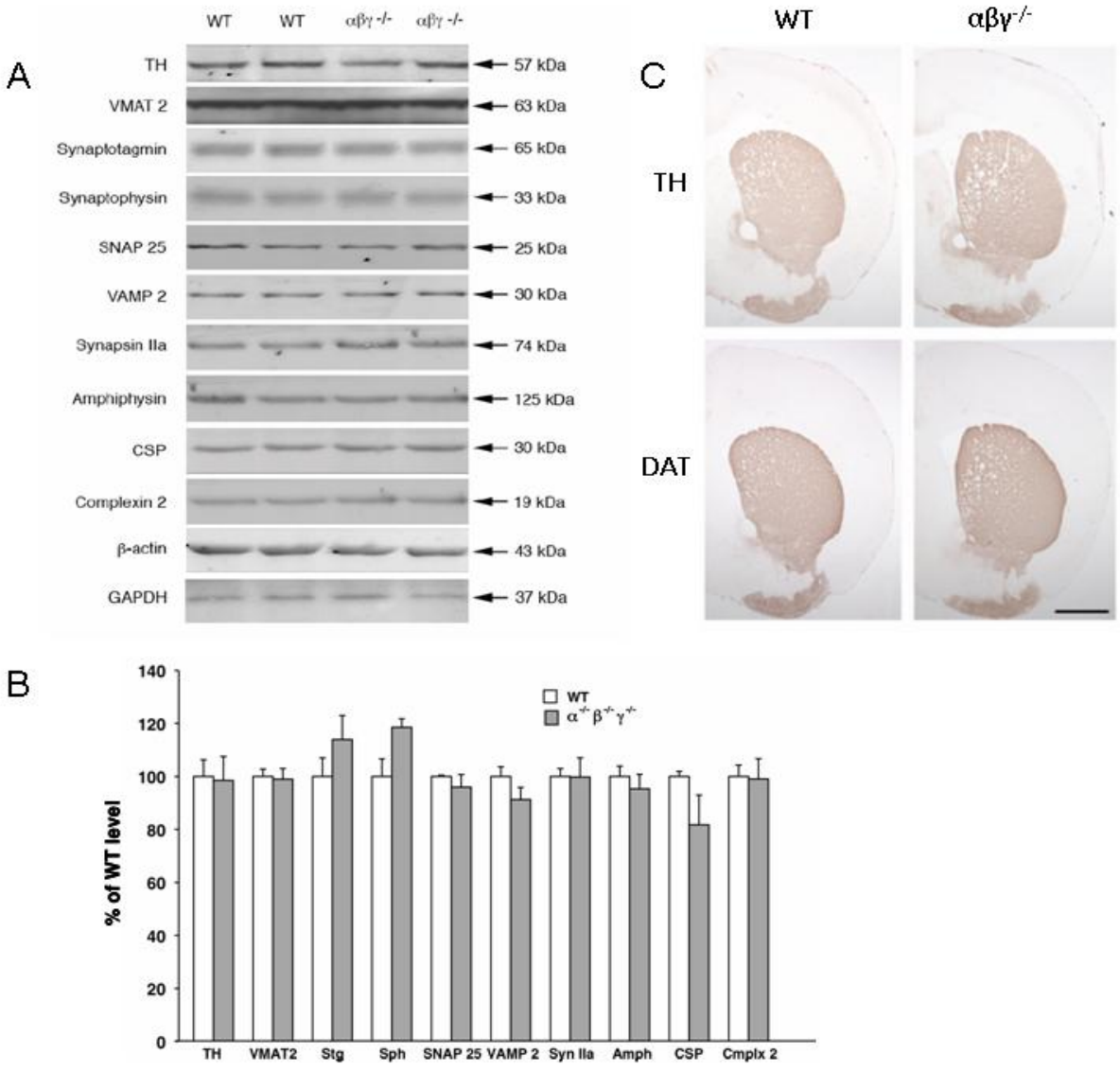


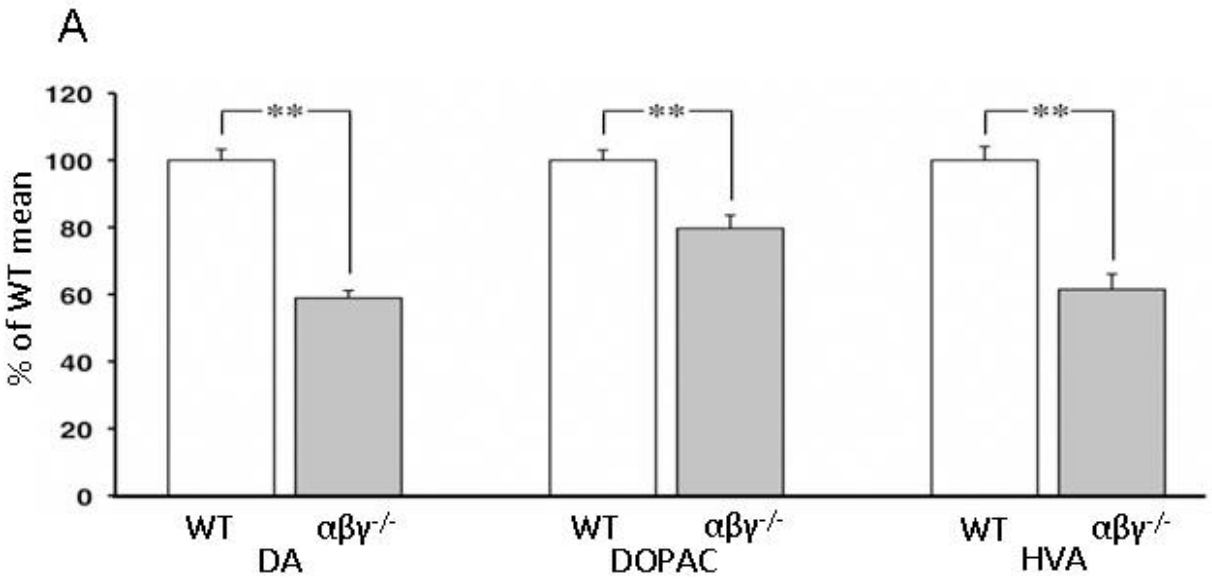
Figure 3.5 **Synaptic Marker Expression Analysis**

(A) Representative Western blots showing synaptic markers in the striatum of both WT and $\alpha\beta\gamma^{-/-}$ mice. Striatum samples from 3-4 individual animals per genotype were used to assess

the mean expression level of synaptic markers. Fluorescently labeled secondary antibodies were used to calculate band density ratio between target protein and β-actin. Bar chart shows means ±SEM of these ratios normalised to the mean ratio for WT animal samples as 100% (B). (C) Coronal sections of WT and $\alpha\beta\gamma^{-/-}$ 4 month old animals. Images represent Bregma 0.38mm level and have a 1mm scale bar applicable to all images.

Concentrations of Striatal Dopamine and its Metabolites

The work described in this paragraph was carried out in collaboration with Steven Millership. Although it was apparent that the $\alpha\beta\gamma^{-/-}$ synuclein animals had a full complement of their dopaminergic neurons in the SNpc it was important to investigate if these cells were functioning correctly with regard to dopamine production at their terminals in the striatum. Previous synuclein knockout models have shown alterations in the normal levels of striatal dopamine, pointing to it as an important factor to investigate. Dorsal striatum of 4 month old male TKO and WT animals were collected, monoamines extracted and their concentrations were analysed by HPLC with electrochemical detection. As seen in figure 3.4 there was a significant decrease in the amount of striatal dopamine in the TKO mice compared to WT animals. Significant decreases in both DOPAC and HVA, dopamine (DA) metabolites, were also seen in the TKO mice, however these were less affected than the levels of dopamine. Due to the alterations in the normal equilibrium between dopamine and its metabolites, these TKO animals have a significantly higher DOPAC/dopamine ratio than WT animals, and while we observed a trend towards decreasing HVA/dopamine ratios in TKO mice, the difference does not reach statistical significance (Table 3.3, B).



B

Genotype	DOPAC/dopamine (mean±SEM)	HVA/dopamine (mean±SEM)
WT	0.107 ± 0.006	0.106 ± 0.004
αβγ-/-	0.127 ± 0.005**	0.117 ± 0.008

Figure 3.6 **Striatal Dopamine Concentrations**

Striatal concentrations (pmol/mg protein) of dopamine (DA) and its metabolites, DOPAC and HVA, were normalised to the mean value for wild type animals (100%). Means±SEM of data obtained from 15 wild type (WT) and 14 $\alpha\beta\gamma^{-/-}$ mouse samples are shown (**p<0.01; Kolmogorov-Smirnov test). (B) Metabolite to dopamine concentration ratios of WT and $\alpha\beta\gamma^{-/-}$ from striatal samples. Statistical significance was tested by Kolmogorov-Smirnov two sample non parametric test where ** represents p<0.01.

TKO Mice Display Normal Striatal Tyrosine Hydroxylase Activity

As it was evident that the TKO animals displayed a reduction in striatal dopamine concentrations the activity of TH, the rate limiting enzyme in dopamine synthesis, was assessed. Mice were treated with NSD-1015, an inhibitor of aromatic L-amino acid decarboxylase (AADC). AADC is the enzyme directly downstream of TH and therefore accumulation of L-DOPA could be assessed by HPLC. As shown in figure 3.5 there were no

significant changes in L-DOPA concentrations following the inhibition of AADC confirming that TKO mice had normal functioning TH.

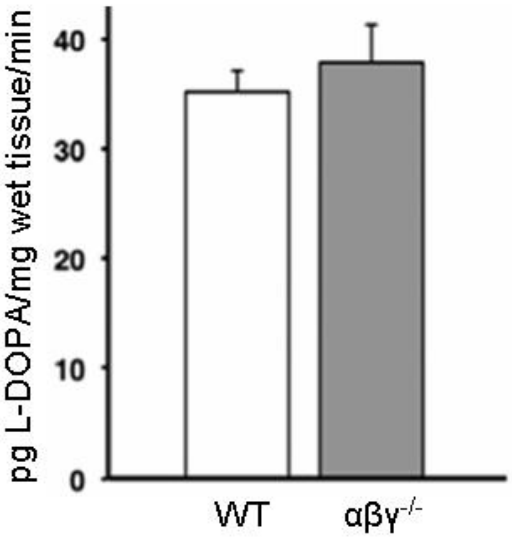


Figure 3.7 **Tyrosine Hydroxylase Activity**

The rate of L-DOPA accumulation in the striatum of both WT (n=5) and TKO (n=5) mice following inhibition of AADC.

Behavioural Analysis

Work described in this section was done in collaboration with Owen Peters. Having shown significant alterations in striatal dopamine concentrations in the $\alpha\beta\gamma^{-/-}$ synuclein mice it was apparent that any behavioural deficits should also be investigated. Endurance, co-ordination and balance were tested using the accelerating rotarod test. By 4 months $\alpha\beta\gamma^{-/-}$ synuclein animals already showed a significant loss in their ability to stay on the accelerating rotarod for the duration of the test in comparison to age matched WT animals (figure 3.6, B). The mice were also tested using the inverted grid test, in which co-ordination and balance are assessed. As can be seen in figure 3.6 (A) TKO animals are able to complete a 60 sec test up until 12 months of age but showed a decreasing ability to complete this task with age. By 24 months there was a significant reduction in the time that TKO animals could stay on the inverted grid compared to their WT counterparts.

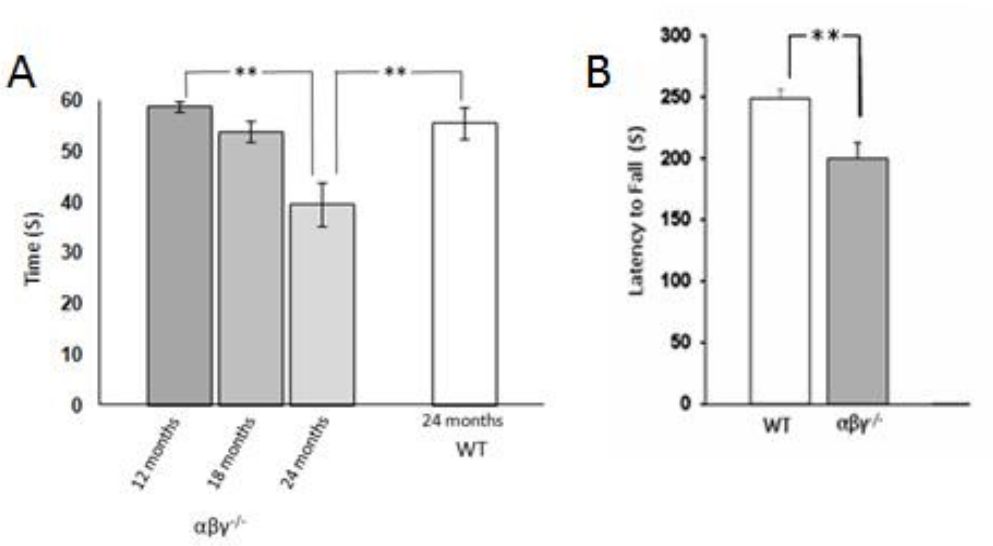


Figure 3.8 **Assessment of TKO Mice on the Inverted Grid and Rotarod**

Means ±SEM of experimental 12 month (n=24), 18 month (n= 17) and 24 month (n=10) aged TKO and 24 month (n=16) old WT animals were tested using the inverted grid test (A). (B) 4 month old $\alpha\beta\gamma^{-/-}$ (n=30) were compared with aged matched WT (n=27) animals with accelerating rotarod and their latency to fall was recorded. The best result for each animal was used for calculation of the group mean. Statistical testing by the Kolmogorov–Smirnov test is represented as p=<0.01 **.

Four month old animals were also tested for locomotor activity by placing them in a home-like cage environment for a 28 hour period. During this time the number of infrared beam breaks were recorded and it was apparent that the TKO animals responded to a change of environment with increased locomotor activity in comparison to WT animals. For the first 4 hours in the new cage the TKO animals showed a significant increase in motor activity compared to WT animals (figure 3.7, A, B). Following acclimatisation to these changes their levels of activity again matched that of the WT animals and only altered when the lights in the room where turned off as part of the normal light dark cycle maintained in the animal house. This is a sign of a hyperdopaminergic phenotype, which was also noticed for TKO mice in parallel experiments carried out by our collaborators in the University of Oxford using different behavioral tests.

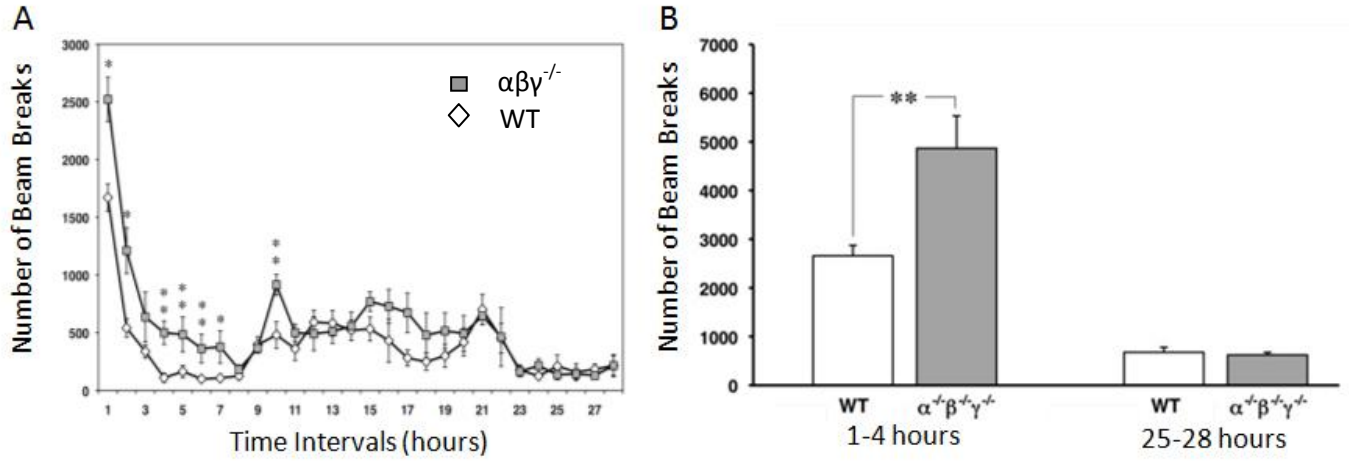


***Figure 3.9* Assesment of Activity Levels in TKO Mice**

(A) The locomotor activity of $\alpha\beta\gamma^{-/-}$ (n=13; grey diamonds) and WT (n= 13; white diamonds) animals measured by the number of breaks of infrared beams over of a 28 hour period in a home-like cage. Note that the rise in activity in hour 10 corresponds with the light in the test room being turned off. The number of beam breaks in the first and last 4 hour intervals is shown in B which both correspond to the period between 10.00am – 2.00pm. Statistical testing by the Kolmogorov–Smirnov test is represented as p=<0.05 * and p=<0.01 **.

Response of TKO Mice to Psychostimulants

Due to the apparent DA deficit paired with the hyperdopaminergic phenotype DA dynamics were further explored in these TKO mice using various psychostimulants. Animals were injected with D-amphetamine (dAMP) which displaces dopamine stored in the synaptic vesicles into the synaptic cleft. TKO mice injected with 4mg/kg dAMP displayed a lesser locomotor activity response and this response was also developed later in comparison to age-matched WT animals (figure 3.8, B).

TKO mice were also treated with 10mg/kg cocaine, which acts by blocking dopamine reuptake from the synaptic cleft. In contrast to the results of the dAMP TKO mice responded to cocaine with the same dynamics and amplitude of locomotion increase as WT animals figure 3.8, A)

L-DOPA is a widely used drug in the treatment of PD which works to overcome the rate limiting step in the DA synthesis pathway. This dopamine precursor is able to cross the blood brain barrier and enter the central nervous system (CNS) where it is then converted by AADC into DA to help increase the diminished concentrations of DA in PD patients. 50mg/kg methyl L-DOPA

was administered to TKO mice 20 minutes prior to dAMP injections and was found to restore the locomotor response of dAMP treated TKO animals to that of their WT counterparts (figure 3.8, C).

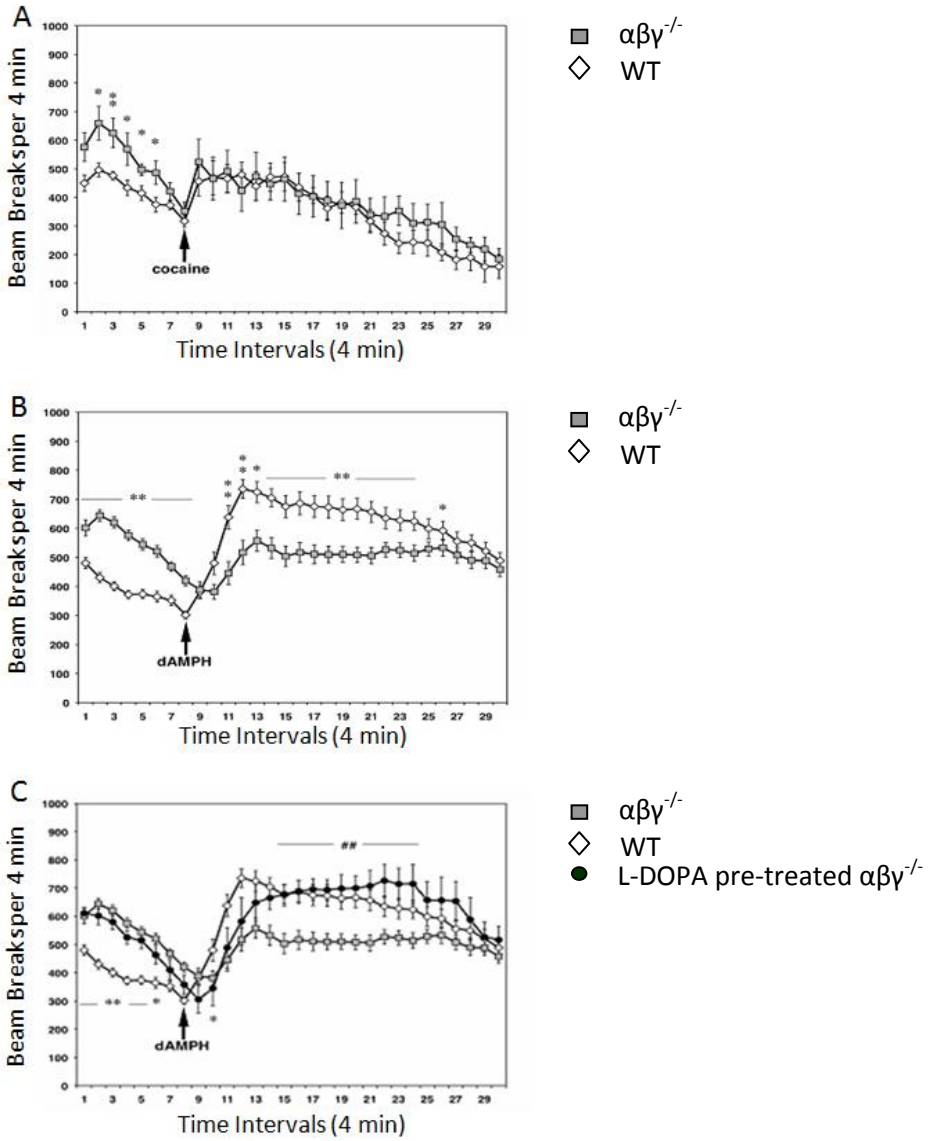


Figure 3.10 **Psychostimulent Assessment of TKO Mice**

For all tests measuring locomotor activity in response to treatment with psychostimulants WT animals are represented by white diamonds and $\alpha\beta\gamma^{-/-}$ animals by grey squares. Statistical analysis between groups for each 4 minute interval by Kolmogorov-Smirnov is represented at **,## for p= < 0.01 and * for p= <0.05. (A) Animals were subjected to 10mg/kg cocaine after a 30 minute acclimatisation period in the home-like cage. Following the injection the animal was returned to the same cage and activity recorded for a further 90 min. (B) Mice were given 4mg/kg dAMP following the same procedure as described in D. (C) 20 minutes prior to being

placed in the recording apparatus animals were pre-treated with 50mg/kg L-DOPA and were consequently injected with dAMP. Animals pretreated with L-DOPA are represented by black circles which overlay the same graph as B. Statistical differences are represented with ## between L-DOPA treated and naive $\alpha\beta\gamma^{-/-}$ and ** between L-DOPA treated mice and wild-type mice

Discussion

As shown above, triple synuclein null mice have proved a useful model in revealing some insight to the role of the synucleins in the midbrain dopaminergic neurons. It would appear that this family of proteins is required for normal dopamine handling, bolstering previous studies also hinting at this. However it is also clear that the synucleins are not required for the normal development and survival of dopaminergic neurons.

To investigate the effect caused to the nigrostriatal system by the removal of all three synucleins TH positive cell bodies of both the VTA and SNpc were counted in a stereological manner. TKO animals had no significant alterations in either of these structures when compared to WT mice at both 4 and 24 month time points. This is consistent with results of another recent study which showed no change in the number of neurons in the CA3 and CA1 subfields of the hippocampus (Greten-Harrison et al., 2010). However, it should be noted that a previous study looking at single $\alpha^{-/-}$ and $\gamma^{-/-}$ synuclein animals as well as double $\alpha\gamma^{-/-}$ synuclein mice did show a small (15%) reduction of dopaminergic neurons, which was not progressive, in the SNpc but not in the VTA (Robertson et al., 2004). This may be due to a compensatory mechanism only being partially activated in these animals, an idea which will be discussed later. The fact that the TKO mice have a full complement of dopaminergic neurons in both the SNpc and VTA suggests that losing all three synucleins is not detrimental to cell development or survival.

Although there were no gross morphological changes in these animals it was also important to explore any obvious changes in synaptic marker expression and distribution as an indicator for synaptic dysfunction. Using an array of synaptic markers, striatal samples were assessed in both WT and TKO mice but revealed no changes in protein expression levels by Western blot analysis. Gross morphological examinations of the striatum using coronal sections immunostained with DAT and TH, both specific markers of dopaminergic nerve terminals, also highlighted that there were no differences morphologically between the two genotypes. This is in agreement with other single and double synuclein animals that have been previously

characterized. However changes to the levels of CSP-α and VAMP2 have been noted in aged TKO mice produced independently to our own animals, which we have not been able to verify. Burré *et al* noted that there was a significant decrease in the levels of VAMP2 and a significant increase in CSP-α levels in their TKO mice which in turn lead them to describe a decrease in SNARE-complex assembly in these animals (Burré et al., 2010). Changes in the levels of VAMP2 were also shown in aged TKO animals, in which the animals are derived in the same manner (Greten-Harrison et al., 2010). However we were unable to replicate these results in our own line of TKO animals and these discrepancies may be down to the fact that different neuronal populations were studied. In both aforementioned papers protein markers were assessed in total brain samples while we studied a specific region of the brain, dorsal striatum. Moreover, the results presented in this chapter were obtained for 4-month old but not ageing TKO mice and for this reason are not directly comparable. It should also be noted that these TKO mice described by Burre *et al*. and Greten-Harrison *et al*. have a distinct age-related motor phenotype and diminished survival neither of which have been demonstrated in the TKO animals produced in our lab (Anwar et al., 2011). This may be down to differences between mouse strains - our animals are on a pure C57Bl/6J whereas those described by Burre *et al.* and Greten-Harrison *et al.* are not (Burré *et al.*, 2010, Greten-Harrison *et al.*, 2010).

Following on from the synaptic characterisation the levels of striatal dopamine and its metabolites HVA and DOPAC were quantified using HPLC. Despite there being no morphological changes in these TKO mice there was a clear dopamine deficit in the dorsal striatum at 4 months of approximately 40% in comparison to WT counterparts. Although HVA and DOPAC showed significant reductions of 38.5% and 20.3%respectively, they were to less of an extent than DA (41.1%) therefore resulting in increased metabolite/DA ratios, which for DOPAC/DA reached statistical significance, suggesting a high turnover of DA. One possible reason that DA levels may have been lower than WT animals is due to reduced activity of TH, the rate limiting enzyme in the production of DA. However this was dismissed by inhibiting AADC, the enzyme downstream of TH, and then measuring the levels of L-DOPA using HPLC. This showed that there were no differences in the amount of L-DOPA, the precursor of dopamine, produced between TKO and WT animals.

A dopamine deficit has been noted in some previous studies of single and double synuclein null mice, but to a lesser extent than that seen in TKO animals. Both $\alpha\beta^{-/-}$ and a line of $\alpha^{-/-}$ synuclein mice have been shown to harbor an 18% reduction in their striatal DA content (Abeliovich et al., 2000, Chandra et al., 2004). However, $\alpha\gamma^{-/-}$ synuclein mice were not

described as having any dopamine deficit. This may suggest that β-synuclein may be more readily able to act in a compensatory mechanism. The only study which has shown a similar level of DA loss involved aged (more than 2 years of age) $\alpha^{-/-}$ synuclein mice. This study was able to demonstrate that in these aged animals there was a DA deficit of 36% (Al-Wandi et al., 2010). This may be explained by a possible compensatory mechanism not being able to sufficiently cope in old age when other challenges may be occurring in the affected neurons.

Despite displaying a clear reduction in dopamine concentrations these TKO animals displayed a hyperdopaminergic phenotype, as shown by the fact that they react to changes in their environment with a much larger increase in locomotive activity than that observed in WT mice. However once the TKO animals have become habituated to these changes in their environment their level of activity returns to similar levels of their WT counterparts. Work done by our collaborators also supports this notion: they were able to show that TKO mice were more active in the nonanxiogenic open field test and also in the hole board test, demonstrating a significantly higher number of nose pokes for TKO mice compared to WT. Increased levels of synucleins have been associated with anxiety and so to ensure anxiety was not the cause of this increased activity in novel environments TKO mice were also tested in the bright open field and the elevated plus maze (Graham and Sidhu, 2010, George *et al.*, 2008). TKO mice showed no differences in their response in these tests compared to WT animals, suggesting that loss of all three synucleins does not affect anxiety levels, in agreement with a study of α-synuclein knockout animals tested for anxiety (Anwar *et al.*, 2011, Pena-Oliver *et al.*, 2010).

To further explore apparent abnormalities in dopamine neurotransmission the use of psychostimulants were employed. dAMP is able to displace DA from synaptic vesicles by entering them via the vesicular monoamine transporter (VMAT2) in exchange for dopamine. The resulting cytosolic increase in dopamine concentration is able to initiate the reversal of DAT which in turn allows dopamine efflux into the synaptic cleft (Sulzer *et al.*, 1995). The effect of dAMP is dependent on the size of the presynaptic pool of dopamine and its effects are not dependent on regulated exocytosis. When mice were injected with 4mg/kg dAMP TKO animals responded at a slower rate and to less of an extent than WT mice. This is not surprising; due to the 40% reduction in DA there is less overall DA to displace. However, when TKO mice were pre-treated with an injection of L-DOPA, a drug that overcomes the rate limiting step in DA synthesis, prior to injecting with dAMP the response was restored to that of WT levels. When TKO mice were exposed to cocaine, which works by blocking the reuptake of

DA from the presynaptic cleft, the response was similar to that of WT mice. The responses seen in the TKO animals when challenged with drugs that alter normal DA neurotransmission suggest that these animals have less DA stored in their synaptic vesicles. The number and intrasynaptic distribution of synaptic vesicles had been shown by our collaborators to not be significantly altered compared to that of WT animals and this has also been confirmed in hippocampal neurons of TKO mice produced independently of those used here (Anwar et al., 2011, Greten-Harrison et al., 2010). Thus it is unlikely that the diminished response to dAMP is due to a smaller pool of synaptic vesicles, a more plausible explanation is that these vesicles are less loaded with the neurotransmitter.

The results above appear to be contradictory, i.e. TKO mice have a hyperdopaminergic phenotype in novel environments but have significantly decreased levels of DA which appears to arise due to problems in DA synaptic vesicle storage. Our collaborators used fast-scan cyclic voltametry to investigate the release and reuptake dynamics of dopamine in striatal slices and found that although TKO mice have less dopamine they had a >2 fold increase in the realeasabilty of dopamine in the caudate-putamen (CPu), but not in the nucleus accumbens (NAc). They also found that the DA loss was specific to the CPu and that the concentration of DA in the NAc was similar to WT animals, possibly suggesting that SNpc dopaminergic neurons that send their synapses to the CPu are a more sensitive population to the loss of all three synucleins than VTA dopaminergic neurons that send their synapses to the NAc (Anwar et al., 2011). This increased releasability helps explain the hyperdopaminergic phenotype seen in the TKO animals.

When TKO animals were assessed on the accelerating rotarod they already displayed a significant decline in the time that they could stay on the apparatus by 4 months, compared to WT animals. This signifies that these TKO mice are suffering with their ability to balance and coordinate movement effectively. TKO mice also show a progressive loss in their ability to perform the inverted grid test with age compared to WT animals. One possible explanation to this is that there is a compensatory mechanism that is put in place in these TKO mice during a critical development period to overcome the loss of all three synucleins at the synapse. This may mean that although these animals manage to survive and have a reasonably non-overt phenotype that the mechanism put in place is not as effective at replacing the synucleins once the animals start to age and other insults in the neurons begin to occur. This compensation mechanism is likely set up prior to birth so that the animals are able to develop as normal despite having lost a group of highly abundant proteins at their synapses.

From the above it is apparent that, although the synucleins are not required for the survival of dopaminergic neurons of the midbrain, it is evident that they do contribute to the normal handling of dopamine. It is clear that the loss of all three synucleins loads this dopaminergic system with a greater burden than when either one or two members of the family are removed. It is also evident that although there may be some extent of functional redundancy within the synuclein family, it is unlikely that this alone that has masked any overt phenotypes being revealed in the previous single and double knockout animals. Intriguingly some of the results above may suggest that another mechanism may be able to functionally compensate for the lack of all three synucleins, which appears to be less effective with age. Most importantly this work has revealed details on the extent of the synucleins role in the normal dynamics of dopamine at the synapse. It will be important that these mechanisms are further explored to allow a more pinpointed role for each member of the synuclein family to be revealed.

Chapter 4

The Effects of MPTP on Triple Synuclein Knockout Animals

Introduction

The discovery and mechanism of action of MPTP has been previously discussed in chapter 1. This specific dopaminergic neurotoxin has become a very important model for the study of PD due to its ability to replicate SNpc cell loss and is often referred to as the gold standard of toxin induced models of PD. Importantly this model has received validation in human subjects through the unfortunate contamination of heroin which was later injected by addicts (Langston *et al.*, 1983). MPTP's structural similarity to dopamine, illustrated in figure 4.1, is considered important in allowing its characteristic targeting of dopaminergic neurons.

It has been shown in previous studies that mice lacking α-, γ- or both $\alpha\gamma^{-/-}$ synuclein knockouts have various degrees of resistance to the dopaminergic neurotoxin MPTP (Dauer *et al.*, 2002, Drolet *et al.*, 2004, Robertson *et al.*, 2004). This has led the field to hypothesise that these two synucleins are involved in the MPTP toxicity mechanism and that they are required for the loss of SNpc dopaminergic cells by such a mechanism. A clear way in which to further explore this relationship between the synuclein family and MPTP was to expose TKO mice to the neurotoxin. TKO mice were administered a sub-chronic regime of MPTP and their midbrain dopaminergic architecture investigated. It was hypothesised that animals lacking all members of the synuclein family should display at least an equal level of resistance to MPTP as α-, γ- or both $\alpha\gamma^{-/-}$ synuclein null animals.

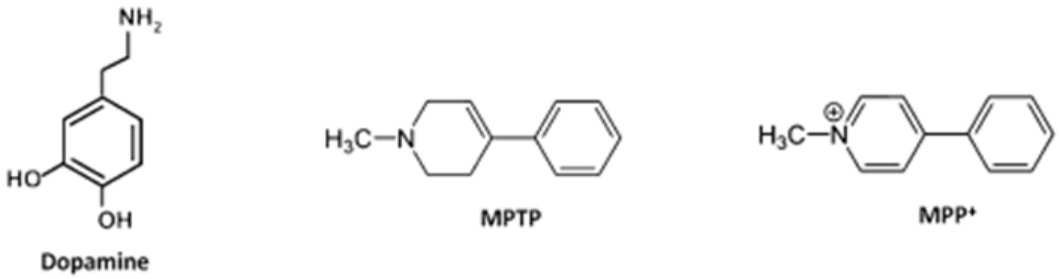


Figure 4.11 **Dopamine, MPTP, MPP^+ Structures**

The molecular structures of dopamine, MPTP and MPTPs active metabolite MPP^+. Image adapted from (Blandini and Armentero, 2012).

Results

MPTP Sensitivity of TKO Mice

Sub-chronic MPTP administration was given as has been previously described (Robertson et al., 2004). Briefly, male mice between 8-10 weeks of age were injected intreperitoneally with either PBS or 30mg/kg MPTP at 24 hour intervals for 5 consecutive days. Tissues were then harvested 21 days following the final injection. Both VTA and SNpc TH positive neurons were stereotactically counted for all genotypes mentioned (figure 4.2). As expected WT mice were sensitive to MPTP showing a 35.4% reduction in SNpc dopaminergic neurons (figure 4.2 I). A greater degree of sensitivity to MPTP treatment was revealed for SNpc TH positive neurons of TKO mice. These animals had a 56.0% decrease in the number of their TH positive neurons which was approximately 21% more than seen in WT mice. A significant, 22.3% loss of VTA neurons in TKO animals was also Identified whilst in the WT animals treated with MPTP the VTA was found to be resistant to the effects of MPTP toxicity as would be expected (figure 4.2, J).

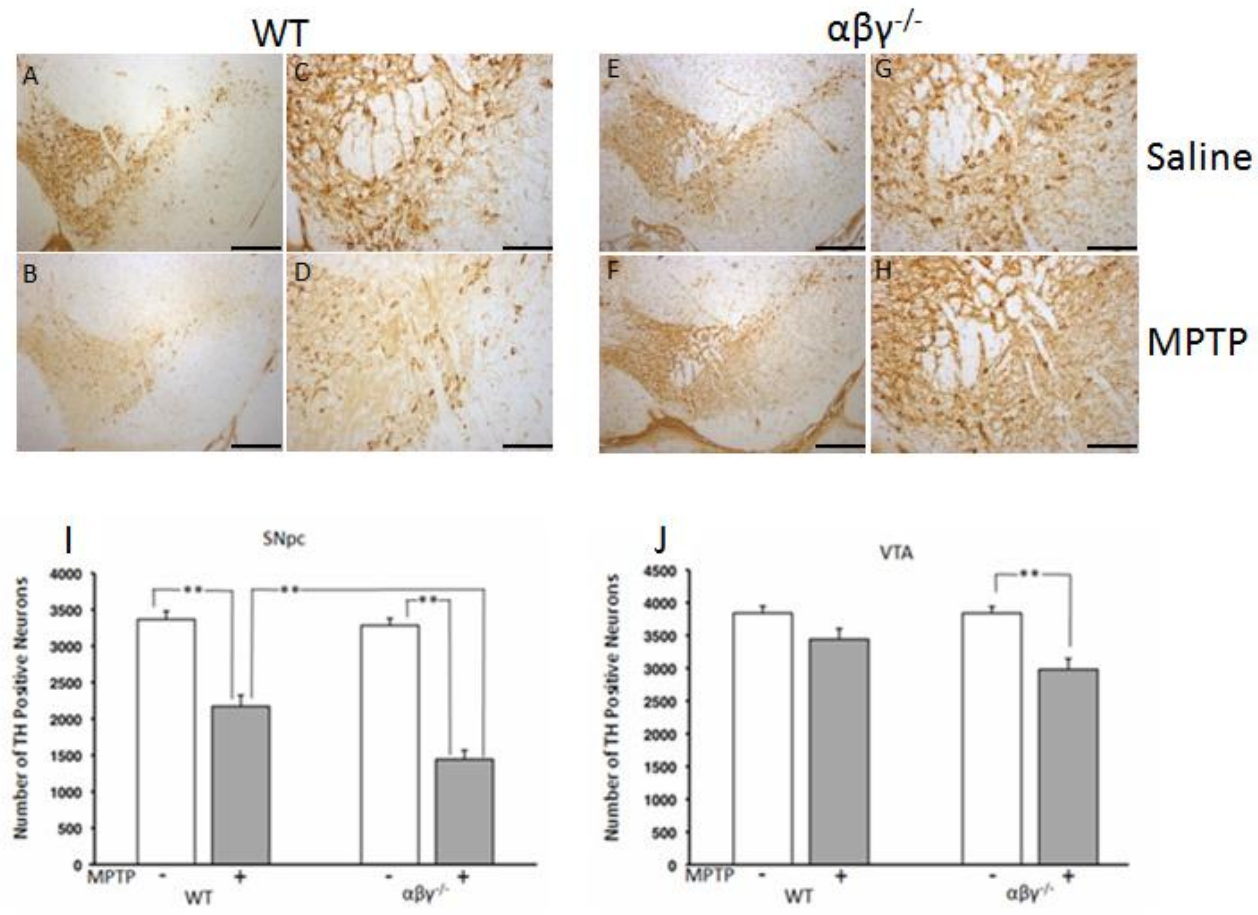


Figure 4.12 **Assessment of SNpc and VTA structures following MPTP treatment in TKO Mice**

A-H representative images of MPTP treated (B, D, F, H) and untreated (A, C, E, G) WT and triple synuclein knockout animals. Images show both VTA and SNpc structures immunostained with tyrosine hydroxylase. Scales bars represent 250µm (A, B, E, F) and 100µm (C. D, G, H). (I) Total mean number ± SEM of SNpc TH positive neurons of MPTP treated and untreated WT and TKO animals. (J) Total of VTA TH positive neurons in both treated and untreated WT and TKO animals. In both cases statistical significance was assessed using the nonparametric Kolmogorov-Smirnov test and is demonstrated using ** where p=<0.01.

MPTP Sensitivity in Mice lacking β-Synuclein

Further to this, knockout mice lacking β-synuclein, which have never been assessed for their sensitivity to MPTP, were investigated in an attempt to shed more light on the role of this synuclein in MPTP toxicity. Following the same sub-chronic MPTP injection regime brain sections from mice lacking either β-synuclein only, or in combination with $\alpha\beta^{-/-}$ or $\beta\gamma^{-/-}$ synuclein, were immunostained with anti-TH antibody and TH-positive neurons were counted

both in VTA and SNpc. In β-synuclein knockout mice 44.0% of DA neuron loss was revealed in SNpc, in $\beta\gamma^{-/-}$ mice - 30.6% and in $\alpha\beta^{-/-}$ mice – 34.3%, all statistically not different from 35.4% loss in WT mice (figure 4.3). This suggests that SNpc DA neurons of all three studied groups of knockout animals are as sensitive to MPTP toxicity as correspondent neurons of WT animals. DA neurons of VTA were resistant to MPTP toxicity with the exception of neurons of $\alpha\beta^{-/-}$ mice that showed slight but statistically significant 16.2% loss.

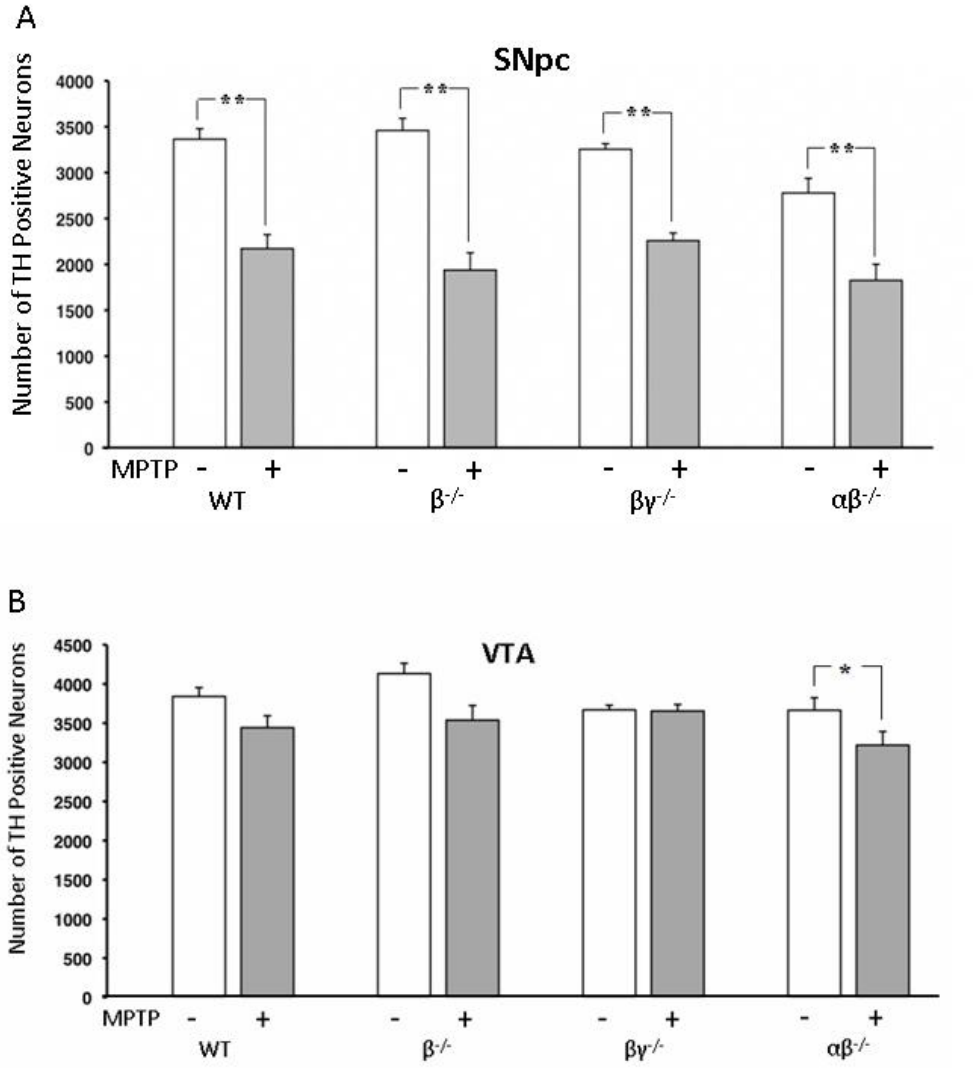


Figure 4.13 **Affect of MPTP Treatment on the SNpc and VTA in Mice Lacking β-Synuclein**

Total TH positive cell counts of the SNpc in MPTP treated and untreated groups of various β-synuclein knockout combinations (A). (B) Total TH positive cell counts of the VTA in MPTP treated and untreated groups of various β-synuclein knockout combinations. In all cases 9-11 animals were used per treatment per genotype and statistical significance was determined by

nonparametric Kolmogorov-Smirnov test demonstrated using ** where p=<0.01 and * where p= <0.05.

Synaptic Vesicle Uptake

In order for neurons to limit the damage obtained by MPP^+, MPTPs active metabolite, it must be sequestered in synaptic vesicles in a VMAT2 dependent manner (Liu et al., 1992). As TKO animals show higher sensitivity to MPTP than WT or other combinations of synuclein knockouts a possible mechanism may lie in the TKO animals not being able to sequester MPP^+ as effectively as other synuclein knockout combinations. As the sequestering process of MPP^+ is similar to the process of vesicular DA uptake and is VMAT2-dependent the amount of VMAT2 protein in the synaptic vesicle fractions of both WT and TKO animals was first assessed and found to be equal (figure 4.4). To further explore this hypothesis synaptic vesicles were collected from the striata of 4 month old male TKO and WT animals and uptake of ^3H-dopamine at a concentration of 10nM was measured as described in Materials and Methods. Results from this experiment, seen in figure 4.4 (B), showed that TKO animals had a 38.5 ± 4.71% reduction in tetrabenazine-sensitive dopamine uptake.

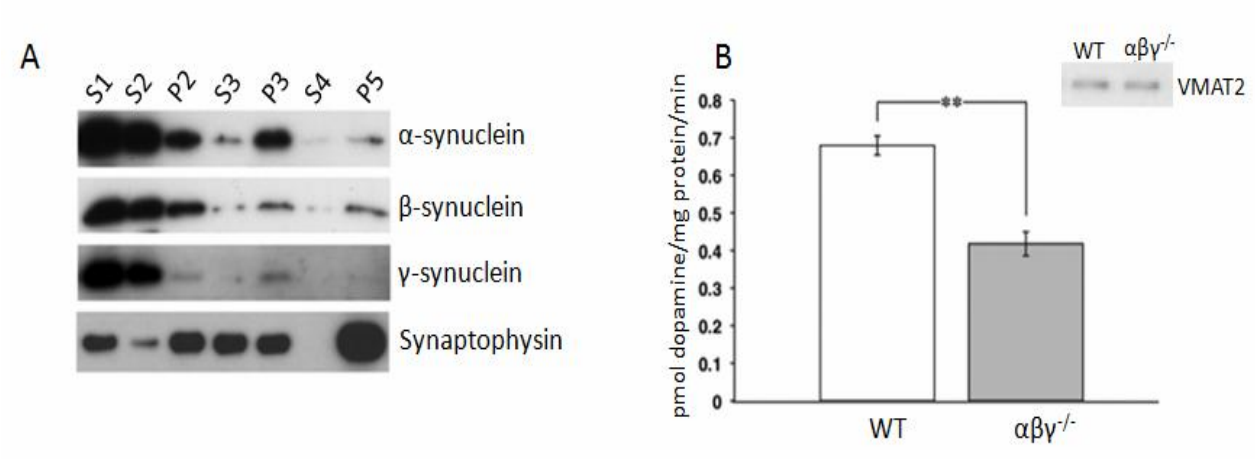


Figure 4.14 **Vesicular Dopamine Uptake in TKO Mice**

(A) Western blot showing protein fractions from WT mouse striatum. S1 was obtained by centrifugation at 5,000 x g. for 10 minutes of crude homogenate. The resulting supernatant was centrifuged at 20,000 x g for 20 minutes and represents the post nuclear supernatant. S2. The consequential pellet contained crude synaptosomes; P2. Following this P2 was resuspended and centrifuged at 20,000g for 20 minutes giving P3 containing synaptic membranes and S3 which was centrifuged at 120,000g for a further 40 minutes to pellet any synaptic membranes. This resulted in S4 and the final pellet, P5, which contained synaptic vesicles for further analysis. As can be seen all three synucleins are present in the vesicle

fraction. (B) Graph representing the mean ± SEM of dopamine uptake of synaptic vesicles purified from the striatum of WT and αβγ$^{-/-}$ synuclein animals. In both groups 15 animals were used split across 3 separate experiments. A representative Western blot shows equal amounts of VMAT2 in vesicular fractions of both genotypes (inset). Statistical significance was determined using the non-parametric Kolmogorov-Smirnov test and ** represents p=<0.01.

Synaptic Vesicle Uptake in the Presence of β-Synuclein

As it was apparent from the results of the MPTP experiments that dopaminergic neurons lacking β-synuclein as well as other synuclein(s) appear to be more sensitive to the toxin than dopaminergic neurons possessing β-synuclein it was important to further evaluate a possible role for β-synuclein in the ability of DA neurons to ameliorate MPTP toxicity in the absence of other synucleins. Therefore, recombinant β-synuclein was used to see if the protein could rescue the vesicular dopamine uptake deficit seen in TKO mice. First, recombinant β-synuclein was preincubated with isolated synaptic vesicles for 15 minutes prior to the introduction of ^{3}H-dopamine. No significant effect on dopamine uptake was observed in these experiments (figure 4.5, A). In another set of experiments recombinant β-synuclein was preincubated at the same concentration and for the same time with supernatant S3 before further purification of vesicles from this fraction by high-speed centrifugation. When resulting vesicles were used in the uptake reaction, a small but statistically significant increase in ^{3}H-dopamine uptake was observed (figure 4.5, B).

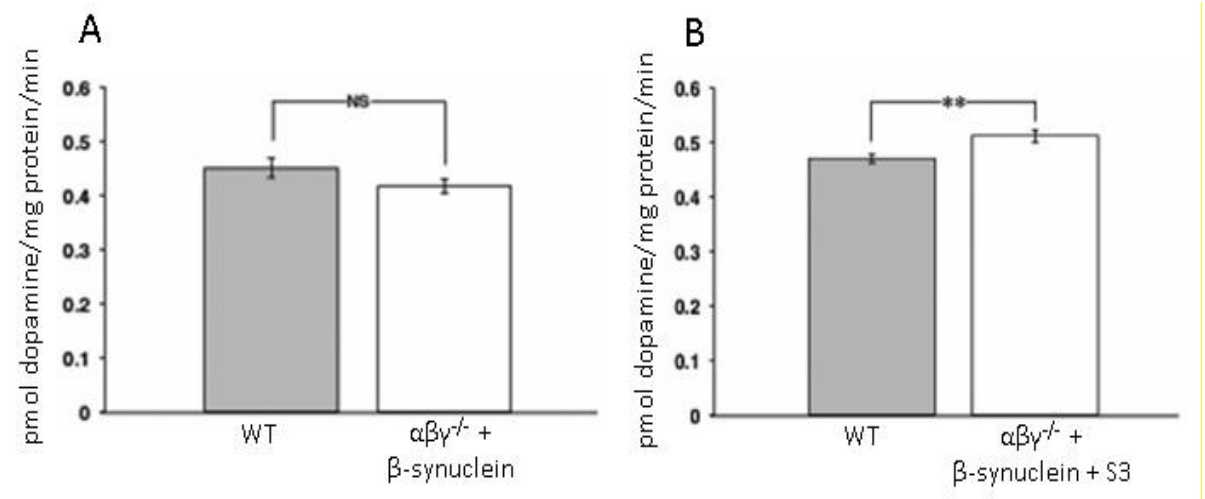


***Figure 4.15 TKO* Vesicular Dopamine Uptake in the Presence of β-Synuclein**

(A) Graph representing the mean ± SEM of vesicular dopamine uptake in the presence of 20ug/ml recombinant β-synuclein only and (B) in the presence of both β-synuclein and the S3 fraction in vesicles purified from αβγ$^{-/-}$ synuclein mice. In both cases statistical significance was

tested using the non-parametric Kolmogorov-Smirnov test and ** represents p=<0.01 and non significant (NS) where p=>0.05.

Discussion

Previous studies carried out by a number of groups have shown that α-synuclein null mice harbour a clear resistance to MPTP toxicity (Klivenyi et al., 2006, Schluter et al., 2003, Dauer et al., 2002, Drolet et al., 2004, Robertson et al., 2004). As previously stated, double $\alpha\gamma^{-/-}$ synuclein mice have also shown a degree of resistance to this neurotoxin with 85.9 ± 6.6% of SNpc TH positive neurons remaining following the same MPTP treatment as described here (Robertson et al., 2004). It was therefore sensible to hypothesise that TKO animals would also be resistant to the affects of MPTP. However our results presented in this chapter clearly demonstrate that the TKO animals show a greater degree of sensitivity to MPTP in SNpc dopaminergic neurons than WT animals. The sensitivity of WT animals to the drug in our experiments was comparable to the sensitivity of SNpc neurons observed in other studies that used a similar protocol of MPTP administration (Robertson et al., 2004, Klivenyi et al., 2006, Dauer et al., 2003). It is also apparent from these experiments that β-synuclein seems to play a crucial role in being able to alleviate MPTP toxicity in the absence of other synucleins. Animals lacking β-synuclein either on its own or in combination i.e. $\alpha\beta^{-/-}$, $\beta\gamma^{-/-}$ synuclein or as TKOs show apparent sensitivity to MPTP.

On top of this the TKO mice and to lesser extent $\alpha\beta^{-/-}$ mice display sensitivity of the dopaminergic population of the VTA which has not been noted in any other previously studied synuclein knockout combination or in WT animals when exposed to MPTP (Robertson et al., 2004). Normally VTA neurons are completely resistant to MPTP and therefore they are often used as an internal control in such studies. It is generally considered that the reason for this selective loss is due to the differences in the ratios of DAT to VMAT2 that are expressed in the two neuronal populations. MPP^+ requires DAT to enter the neuron, following which it either inhibits complex one of the mitochondria or is stored in synaptic vesicles in a VMAT2 dependent manner. This latter process seems to allow the cell to protect itself from certain cell death should the selective toxin reach the mitochondria. Therefore if a cell had a higher concentration of DAT to VMAT2 then more MPP^+ will gain access to the cytosol but will be inefficiently sequestered into vesicles by VMAT2 therefore causing a large degree of toxicity. The opposite of this is the more desired option when dealing with MPP^+ toxicity, as less of the toxin will enter the cell and that that does is properly stored in the synaptic vesicles, thus limiting the potential damage. Mouse models provide evidence for this hypothesis with DAT

knockout mice showing resistance to MPTP toxicity as MPP^+ is unable to be transported into the cells whereas mice that are heterozygous VMAT2 knockout have been shown to lose more than twice the amount of dopaminergic neurons compared to WT animals when treated with MPTP (Gainetdinov et al., 1997, Takahashi et al., 1997). It is also known that in brains of PD patients the putamen displays a large degree of damage and that this brain region has a high ratio of DAT to VMAT2, which may in turn mean that DA is not well compartmentalised within the cell leading to a string of toxicity problems (Miller et al., 1999). It is possible that the TKO and $\alpha\beta^{-/-}$ animals display a more sensitive response to MPTP in the VTA as they are unable to cope with the complete lack of synucleins. This is indeed supported by the apparent role of β-synuclein in participation of DA sequestering in the synaptic terminals. With the loss of all synucleins it is possible that this function cannot be adequately replaced.

A parallel study has shown that TKO mice have normal DAT function (Anwar et al., 2011) and so, knowing that levels of VMAT2 are also important for the effective sequestering of MPP^+ within the cell, the concentration of VMAT2 was compared in the synaptic vesicle fraction of striatal tissue from both TKO and WT animals. VAMT2 concentrations of TKO mice were shown to be comparable to WT animals suggesting that the MPP^+ could be sequestered as normal within the synaptic vesicles providing it is functioning normally. To ensure this was the case normal DA uptake dynamics were explored. It is only due to the structure of MPP^+ being similar to DA that it can enter the neuron via DAT and then be sequestered into synaptic vesicles in a VMAT2 dependent manner; looking at the normal physiological functioning of these cells it would allow some estimation of what was happening to MPP^+.

As expected all 3 synucleins were purified in the cytosolic fraction and they were also shown to co-purify with the synaptic vesicle fraction (figure 4.4, A). This is unsurprising due to the host of literature showing that the synucleins are capable of binding to a number of biological and synthetic membranes (Jensen *et al.*, 1998, Eliezer *et al.*, 2001, Davidson *et al.*, 1998). The next step was to see if synucleins affect the transport of dopamine into the synaptic vesicles. Purified synaptic vesicles from both 4 month old WT and TKO mice had their ability to uptake ^{3}H-dopamine at a physiological concentration measured. This showed a significant reduction in the ability of synaptic vesicles purified from TKO mice to uptake dopamine. This was also shown to be a tetrabenazine (a VMAT2 inhibitor) sensitive process, clearly demonstrating that the uptake deficit was in some manner related to VMAT2 function. One possible explanation for this reduction in DA uptake would be a decrease in the number of synaptic vesicles in the dopaminergic terminals of SNpc neurons. However this has been shown not to be the case in

two separate electron microscopy studies looking at the ultrastructural changes in the synapses of TKO mice (Greten-Harrison et al., 2010, Anwar et al., 2011). This decreased ability to uptake DA efficiently into synaptic vesicles may also explain why the TKO animals have a high DOPAC/DA ratio, which is likely to help prevent the toxic build up of free cytosolic DA in the cell and in turn also results in decreased DA levels in the dorsal striatum. Another result of this diminished DA uptake into the synaptic vesicles would be the attenuated response to amphetamine seen in TKO mice as shown previously.

Synuclein KO animals lacking β-synuclein show an increased sensitivity to MPTP compared to other genotypes. It is possible that the synucleins are somehow involved in the process of vesicular uptake and that certain members of this family are simply more efficient at this process than others. If this were the case it could be assumed that β-synuclein may be the best candidate for this task and when it substitutes lost members of the family, for example in $\alpha^{-/-}$ or $\alpha\gamma^{-/-}$ synuclein knockout mice, efficiency of vesicular uptake increases leading to increased ability to sequester MPP^+ and, consequently increased resistance to MPTP toxicity. Indeed, this is supported by the fact that β-synuclein protein levels are increased in $\alpha\gamma^{-/-}$ and $\alpha^{-/-}$ synuclein animals (Robertson et al., 2004). In contrast, when β-synuclein is removed from the process, as in TKO mice, neurons become more sensitive to the drug, as they cannot sequester MPP^+ into synaptic vesicles with the same competency anymore. In order to assess if β-synuclein can indeed directly affect vesicular monoamine uptake, synaptic vesicles were purified from TKO animals and were preincubated with β-synuclein. When purified synaptic vesicles alone were used there was no alteration on uptake, however when the vesicle-containing S3 fraction (figure 4.5, B) was preincubated with β-synuclein there was a significant increase in uptake. This therefore suggests that β-synuclein alone cannot affect VMAT2 transporter function. However when β-synuclein has the availability of cytosolic factors, i.e. those found in S3, it is able to have an effect on this transport, probably by acting as a chaperone and providing some cytosolic factor that in turn can help induce vesicular transport.

Recent unpublished work carried out by our collaborators in Russia also supports the notion that β-synuclein is able to restore vesicle uptake in TKO animals *in vivo.* TKO animals were given unilateral stereotaxic injections of a lentiviral vector that expressed β-synuclein into the SNpc region. This restored β-synuclein expression and it was transported to the synaptic terminals of the dorsal striatum. When synaptic vesicles were isolated there was a significant increase of DA uptake in the vesicles from the injected side in comparison to the non-injected striatum.

From the work carried out in this chapter it is apparent that animals lacking all three synucleins are indeed sensitive to the effects of MPTP and appear more sensitive than any other mouse lines with various combinations of synuclein knockouts. Strikingly, TKO animals show sensitivity in their dopaminergic VTA populations. This is likely due to their decreased ability to efficiently sequester dopamine into their synaptic vesicles and as a result of this also display poor MPP^+ compartmentalisation. Although it is apparent that β-synuclein can restore this defective sequestering ability it is also evident that it cannot do this alone and requires a cytosolic factor to enable this mechanism. It is likely that all the synucleins have this ability, but to varying degrees of effectiveness and this is something that should be explored. As well as this, further investigation to reveal the mechanism by which β-synuclein is able to allow normal dopamine sequestering will be of interest in allowing a clearer insight into the normal roles of the synucleins.

Chapter 5

Production of a novel conditional α-synuclein knockout mouse model to investigate an adult onset loss of function in synaptic pathology

Introduction

As has been made clear throughout this thesis the role of the synucleins, in particular that of α-synuclein, for a long time remained elusive. Alpha-synuclein has clearly been linked to diseases such as PD through genetic mutations and GWAS and therefore the unveiling of its normal role is incredibly important in order to better understand the disease and possibly provide new therapies. Recent advances in this field clearly demonstrated the importance of α-synuclein in modulation of presynaptic processes involved in neurotransmission. However the role of α-synuclein loss of function in pathogenesis of synucleinopathies has not yet been properly addressed. It is plausible that in the adult or ageing nervous system abnormal post-translational modifications, aggregation and gradual accumulation of insoluble α-synucleins in LBs leave little of the normally functioning protein left at the synapse. This is likely to have an adverse impact on normal synaptic function which in turn may add to the pathogenesis of the disease. One of the reasons that this has not yet been studied is due to the lack of an ideal model in which to do so. It is critical that this is studied to assess how this mechanism may impact on the progression of PD.

Another problem that prevails in the study of the synucleins is that of familial redundancy. However, results from the TKO mice show that although there is some extent of familial redundancy it is most likely not the most important mechanism of compensation. Results arising from TKO mice, such as the aging decline in the ability to perform the inverted grid test, have suggested that there may be a compensatory mechanism at play which is initiated in critical development periods in these animals, which cannot be due to familial redundancy. It is possible that this compensatory mechanism is able to provide long term functional replacement of the synucleins, thereby masking their normal role. However during aging these mechanisms become less efficient and so cannot completely compensate for the loss of synucleins. The result of this incomplete compensation is changes at the synapse which may then lead to synaptic dysfunction, as seen with the TKO animals.

Currently no model is able to satisfactorily overcome possible compensation mechanisms initiated during development, making it impossible to study the loss of function impact of α-synuclein at the synapse clearly. Previous conventional knockout models rely on a congenital knockout of α-synuclein. It is during embryonic and early postnatal development that the brain is at its most plastic and is therefore most likely to instigate a compensatory mechanism.

In order to properly investigate a role of α-synuclein loss of function in the adult it is first apparent that any compensatory mechanism must be overcome. It is therefore a requirement that α-synuclein can be knocked out at any time point during the animals lifespan. In doing this it also has the advantage of allowing the assessment of this loss of function in an ageing nervous system, creating a more realistic model of a degenerative disease process. Importantly, this should also ensure that plasticity at a neuronal level is less apparent than it may otherwise be in a younger animal and therefore allow us to study the role of α-synuclein properly.

For the reasons described above a conditional α-synuclein knockout model has been produced. This model will simulate the loss of function of α-synuclein that is likely seen in patients with PD, which may cause synaptic pathological changes. It is highly likely that some degree of loss of function is working in balance with a gain of function mechanism in PD. This novel model aims to bring valuable new insight into the synucleinopathy field. The production of this model is described in the following chapter and an overview of the work required for the creation of this model is illustrated in figure 5.1.

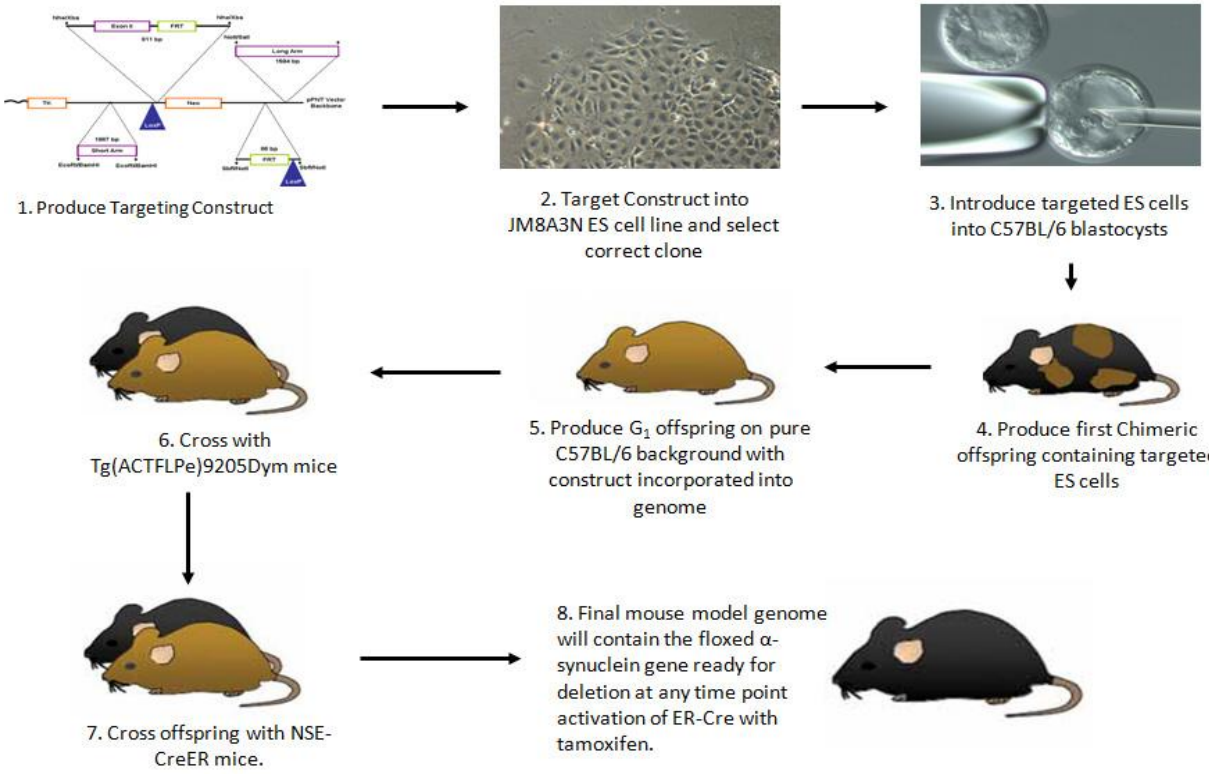


Figure 5.16 Schematic Representation of the Production of a Conditional Knockout Model

Schematic showing the path from targeting construct production through to obtaining the final mouse model.

Design of the α-synuclein conditional knockout targeting construct

A targeting construct is a carefully designed and created sequence of recombinant DNA fragments in a plasmid vector that can be used to replace and manipulate a specific region of the target chromosomal locus. There are some minimal requirements of the sequence which include the arms of homology, in which the sequence is entirely homologous to that found in the genomic locus to be manipulated, thus allowing homologous recombination. It is also important to have selection components, both positive and negative, in the construct as transfection efficiency and proper homologous recombination events are infrequent and so enrichment of these events is necessary. Enclosed between the arms of homology is the region of the target vector which replaces the desired segment of a gene, in our case, the α-synuclein gene within the target locus. The final important consideration is the requirement of a unique restriction site outside of the arms of homology to allow the linearisation of the DNA prior to targeting. This section aims to introduce a detailed overview of how the target construct was created and used to produce the α-synuclein conditional knockout mouse model.

In order to allow the knockout of α-synuclein *in vivo* site specific recombination systems were required. These systems have revolutionised our ability to produce models in which the genome can be easily manipulated. They permit the insertion, inversion, deletion or translocation of the DNA associated with these systems. The Cre-*lox*P system is one example of a site specific recombination system that was first described in 1981 (Sternberg and Hamilton, 1981). It was found within the bacteriaphage P1 genome where it enables the circularisation of its linear genome after infection of E.Coli (Segev and Cohen, 1981). Loci of crossing-over in P1 (*Lox*P) sites act as the recombination hotspots within the bacteriaphage genome with each *Lox*P site consisting of a 34bp sequence that includes two 13bp inverted repeats with an 8bp spacer region separating them (figure 5.2) (Hoess *et al.*, 1982). Cre recombinase (cyclisation recombination protein) is a 343 amino acid protein that catalyses the recombination process that occurs between the *Lox*P sites (Hoess and Abremski, 1984). Since this system has been recognised it has been exploited to allow the modification of specific DNA sequences at precise time points and in specified tissues. One method by which a modification can be controlled in a temporal manner is through the use of a tamoxifen inducible Cre recombinase. This system relies on the fusion of Cre recombinase to the mutated ligand binding domain of the human oestrogen receptor (ER) (Feil *et al.*, 1996). This mutation ensures that the receptor can only be activated by tamoxifen and not endogenous estrogen. In the absence of tamoxifen the fusion protein is kept within the cytoplasm but upon exposure

to the steroid the receptor dissociates from the Cre recombinase protein thus allowing the enzyme access to the nucleus and therefore exerting its activity (Jullien *et al.*, 2008).

Although the Cre-*Lox*P recombination system is very widely used there are other established systems that can be enrolled. This includes the highly similar Flp-*FRT* system, discovered shortly after the Cre-*Lox*P system was described, which is also implemented in this model. It was identified in the 2-µm plasmid that is common to a number of yeast strains and has since been extensively studied (Vetter *et al.*, 1983). The enzyme, flippase (Flp), is the recombinase which recognises FLP Recombination Target (*FRT)* sites responsible for the initiation of the recombination process (Babineau *et al.*, 1985). Like the *Lox*P sites the *FRT* sequences consist of two 13bp inverted repeats with an 8bp spacer region (figure 5.2) (Senecoff *et al.*, 1985). The original sequence for the FRT site does have a third repeat, however this was found to be unnecessary for recombination. This system has also proved very simple and efficient to use in the manipulation of DNA.

In both of these systems the recombination event occurs with absolute fidelity, meaning overall no nucleotide is lost or gained. Importantly no cofactors are required for the cleavage or ligation of the recombination reaction making the system widely exploitable. The system also lends itself well to the genetic manipulation of model organisms by the fact that the recognition sequences are short, thus not interfering with the gene expression, but highly unlikely to occur within the host genome at random. The orientation of two 34bp target sites relative to each other on the desired section of DNA is important for directing the type of modification that will be undertaken by the recombinase enzyme. If the two sites lie in the same orientation the intervening DNA will be excised but if the two sites are in the opposite orientation to each other, i.e. facing each other, the intervening DNA is inverted (Kilby *et al.*, 1993).

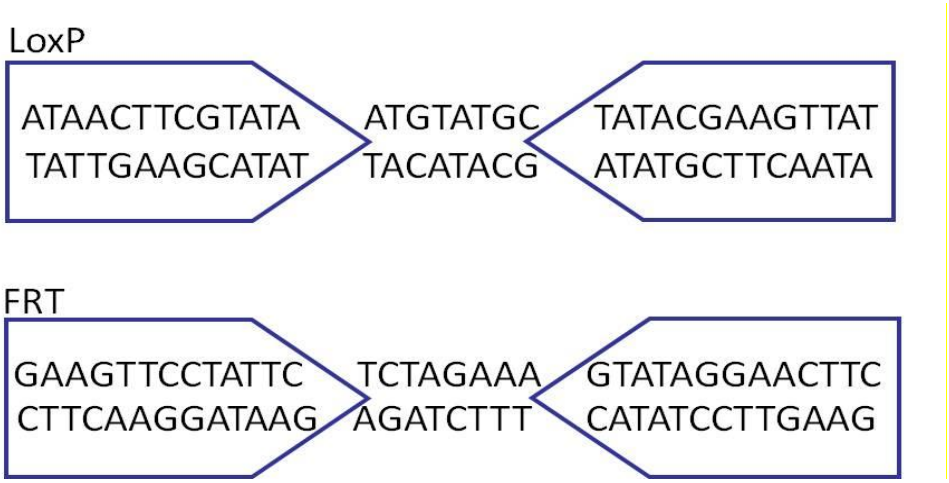


Figure 5.17 **Nucleotide Sequence of LoxP and FRT**

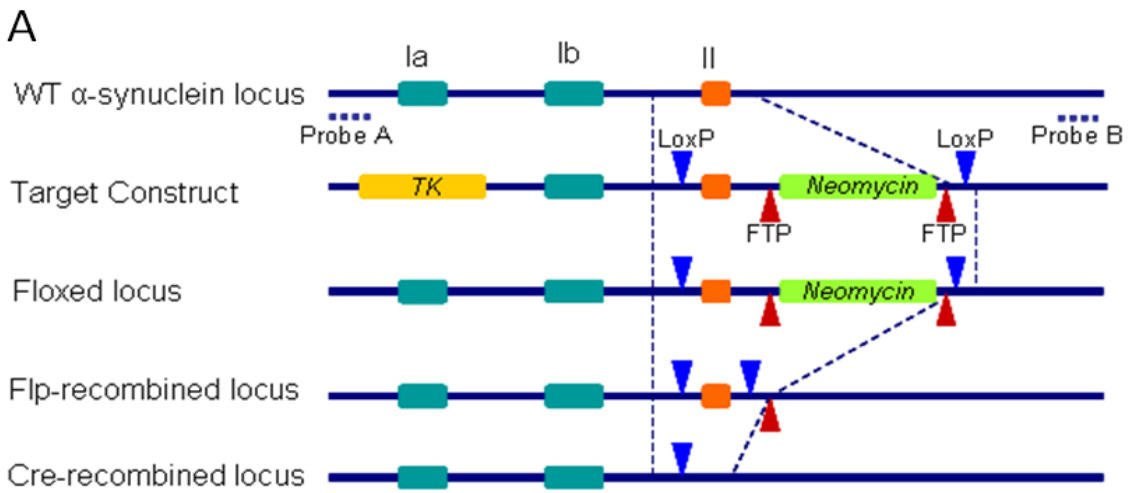
Diagram of recombinase recognition sites. The inverted 13bp symmetry elements of the recognition site are represented in the blue box. The remaining 8bp spacer region provides the cleavage site for the recombinase enzyme. It is the 8bp spacer region that imparts directionality of the sequence, meaning two directly orientated regions within a sequence allow the excision of the intervening DNA, whilst two inverted sites allow the inversion of the intervening DNA.

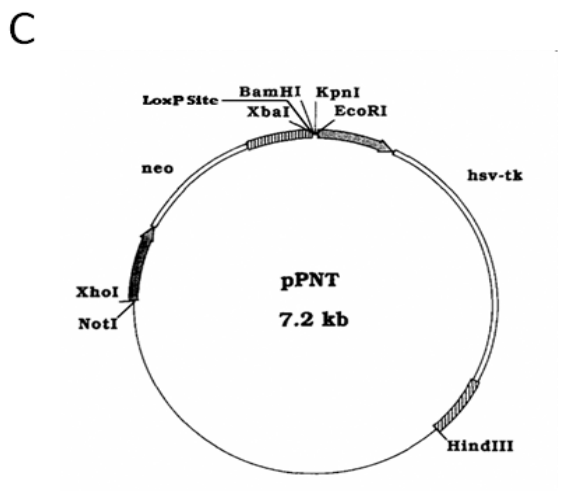
These recombination systems are indispensible in the design of the α-synuclein conditional knockout mouse model. The first coding exon of α-synuclein, exon II, was chosen to be floxed with *Lox*P sites thus allowing it to be knocked out in a Cre dependent manner at any time point. The design of the target construct is shown in detail in figure 5.4, which also shows how homologous recombination occurred in order to target the recombinant DNA into the genome. It is known from previous α-synuclein conventional knockout models that the loss of exon II is sufficient to stop mRNA and protein production of α-synuclein (Abeliovich *et al.*, 2000). In order to allow the targeting process to be enriched positive-negative selective agents were also incorporated into the targeting construct. Despite having arms of homologous sequence on either side of the target construct targeted DNA undergoes random integration more frequently than homologous recombination. The ratio of random to homologous recombination is hard to control experimentally as it depends on things such as the location of the target gene within the genome. Estimates suggest that using a negative selection marker

can help enrichment by decreasing the number of clones with random integration by between 2-20 fold (Hasty *et al.*, 2000). As well as this the length of the arms of homology are also known to affect the degree of successful homologous recombination with the longer the homologous sequences the higher the efficiency of homologous recombination (Thomas and Capecchi, 1987).

The targeting construct was designed with the use of the pPNT plasmid as the backbone. This plasmid already has the incorporation of a neomycin resistance cassette and the Herpes simplex virus thymidine kinase cassette (HSK-tk) as well as a single LoxP site (see plasmid map in figure 5.4). The HSK-tk cassette acts as the negative selection marker as it sits outside the arms of homology in the targeting construct; if the construct has undergone correct homologous recombination the cassette is lost. However if the targeting construct is randomly integrated into the ES cell genome the HSK-tk cassette will persist and under ganciclovir selection kill the cell. Ganciclovir acts as a prodrug that is converted into a toxic triphosphate nucleotide analog by HSK-tk which is then incorporated into the cells DNA inducing cell death (Mar *et al.*, 1985). The neomycin cassette encodes the resistance to G418 and is therefore used as a positive selection marker. Following the electroporation of ES cells with the targeting construct DNA both ganciclovir and G418 were added to the media to allow selection.

Having the neomycin cassette is ideal for the targeting process; however it is important that the cassette can be easily removed from the final targeted genome. It is possible that the neomycin cassette promoter, in this case phosphoglycerate kinase 1 (PGK-1) which is expressed at many genomic locations, may interfere with expression of other genes which is not desirable. Although this is rare it would create a potentially unusable model and so the neomycin cassette is flanked by two *FRT* sites to allow its removal in a FLP dependent manner. This will stop any potential interference with other genes and ensure normal expression of α-synuclein prior to it being knocked out.





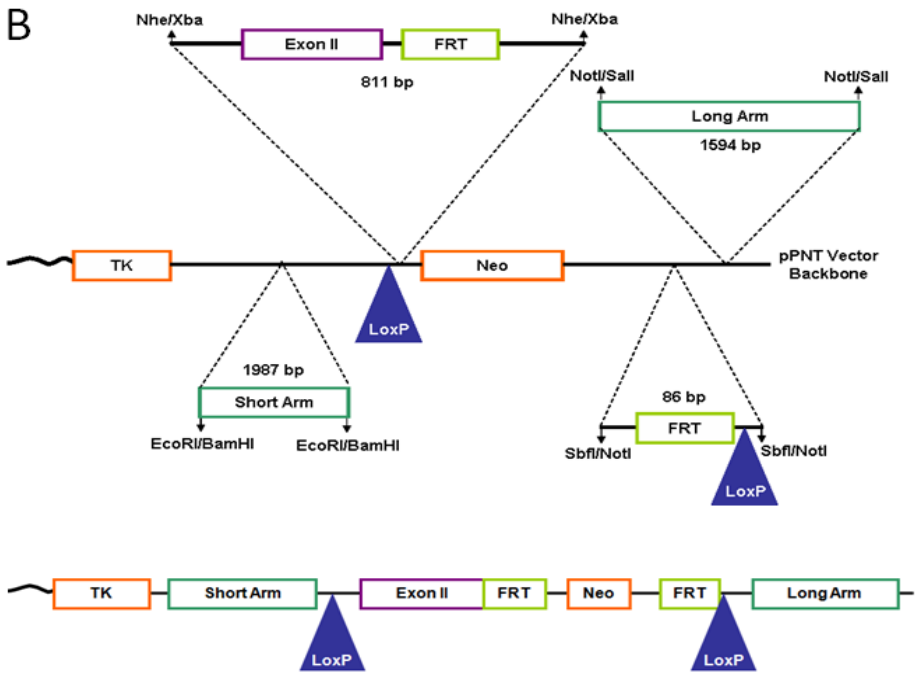


Figure 5.18 **Targeting Construct Design**

A diagrammatic representation of the target construct undergoing homologous recombination with the wild type (WT) alpha-synuclein locus. Note that the thymidine kinase (TK) cassette is lost in the floxed locus as this region of the construct falls outside of the arms of homology, so when homologous recombination correctly occurs it is lost. Following this the floxed locus is shown to undergo Flp recombination therefore loosing the neomycin cassette. Finally through Cre recombination exon II is removed meaning that there is no further expression of alpha-synuclein. The probes used for Southern blot analysis of ES clones are also shown lying outside of the targeting construct. (B) A schematic representation of the targeting construct with each separately created part being highlighted. The restriction enzyme sites used to ligate the separate DNA fragments into the pPNT vector are also shown. Notice that one LoxP site was already present in the pPNT plasmid and only one had to be inserted through the cloning process. (C) A map of the pPNT vector that was used to create the targeting construct showing the neomycin and thymidine kinase cassettes as well as a LoxP site.

Results

Targeting Construct Production

The targeting construct was produced using the pPNT vector as a plasmid backbone. As mentioned above this vector contains a *Lox*P site, neomycin resistance cassette and HSK-tk cassette within its sequence, meaning they could be exploited in the design of the targeting construct which is shown schematically in figure 5.4 (B). Sequences of all primers described in this chapter can be found in appendix 3. Primers were first optimised in all cases with a standard taq polymerase and only when conditions were correctly established was the AccuPrime™ *Pfx* DNA Polymerase mastermix (Invitrogen) used in order to provide blunt ended fragments ready for cloning.

For the production of the 811bp fragment that contained the sequence for α-synuclein exon II and the first *FRT* site that flanks the neomycin resistance cassette nested PCR had to be undertaken due to the length of the reverse primer containing the *FRT* sequence. To produce the first 770bp template "alpha exonII forward NheI" was used in combination with "alpha exon II nested reverse 2". An annealing temperature of 58°C and a synthesis time of 1 minute were required while all other conditions were kept as described in table 2.3. After this fragment had been cloned in to the pCR-Blunt II-TOPO vector and the sequence checked it was then used as template DNA with primers "exon II forward NheI" and "alpha exon II reverse FRT NheI" with an extension of 30 seconds. This resulted in the full 811bp fragment that after NheI digestion was cloned into the XbaI site of the pPNT vector, therefore inserting it just after the *Lox*P site contained within the vector. After selecting clones carrying the insert, the orientation of its sequence then had to be checked by digesting plasmid DNA with BamHI and Xba (figure 5.1, A). In the correct orientation fragments of 828bp and 7362bp were produced, however in the wrong orientation fragments altered to 67bp and 8123bp, making it apparent which clones were correct.

The next fragment to be synthesised was the 1987bp arm of homology. This required the use of the "alpha short arm forward EcoRI" and "alpha short arm BamHI" primers. The annealing temperature required was 60°C followed by 40 seconds at 72°C for extension. This was cloned, checked for its presence in the TOPO vector with simple EcoRI digest and the positive clones sent for sequencing. After a positive clone was selected and the fragment digested out of the TOPO vector using EcoRI and BamHI it was then ligated into the EcoRI/BamHI-digested

pPNT-loxP-exII-FRT plasmid. Colonies were then checked by EcoRI and BamHI for insert and sequenced where appropriate.

Following this the 86bp fragment containing the second *FRT* and *Lox*P sites was created. In order to do this a template DNA oligonucleotide sequence (Sbf FRT LoxP Sal) was ordered from Sigma. 15ng of this template were used along with the "Sbf FRT forward" and "Not Sal LoxP reverse" primers with 42°C annealing temperature and an extension period of 30 seconds at 68°C. TOPO clones were then checked for the presence of the insert by EcoRI digest and sequenced. Once a correctly sequenced clone was identified, the fragment was excised by SbfI and NotI digestion and used for ligation to create the pPNT-Short Arm-loxP-exII-FRT-neo-FRT-LoxP containing vector as shown in figure 5.2.

Finally the 1594bp arm of homology was produced using the "alpha long arm forward SalI" and "alpha long arm reverse NotI" primers. These required an annealing temperature of 56°C and an extension of 2 minutes at 72°C. Following cloning into the pCR-Blunt II-TOPO and a correct clone being identified, a fragment was excised by SalI and NotI digestion and purified. This was then ligated into SalI and NotI digested pPNT-Short Arm-loxP-exII-FRT-neo-FRT-LoxP to produce the final targeting construct figure 5.2, C.

Sequencing of the Final Targeting Construct

Once the construct was completed it then had to be sequenced in order to ensure all the individual components were correct as well as checking that all the ligated ends were as expected. This was done using a variety of primers to read across the whole length of the construct (see appendix 4 for further details as well as the full sequence of the targeting construct).

Production of probes for use in Southern Hybridisation

In order to analyse ES clones produced from the targeting experiments for correct homologous recombination two probes were produced that could be used for Southern hybridisation. One probe was produced that could hybridise downstream of the long arm of homology and another upstream of the short arm. These could be used to assess the fragments produced by various restriction enzyme digests and confirm the position of the targeting construct within the α-synuclein locus was as expected.

The probe upstream of the short arm of homology was produced using the "SAprobeUP" and "SAprobeDOWN" primers to amplify a 751bp fragment using the normal PCR conditions. For

the probe downstream of the long arm the primers “LAprobeUP” and “LAprobeDOWN” were used to create a 1322bp fragment using an extension time of 2 minutes whilst leaving all other PCR conditions as normal. These products were cloned into the pCR-Blunt II-TOPO vector and sequences confirmed. When these probes were required DNA was EcoRI digested out of the vector and purified from the agarose gel (figure 5.2, B). The probes were then labelled as needed with ^{32}P in order to allow visualisation of DNA analysed by Southern hybridisation.

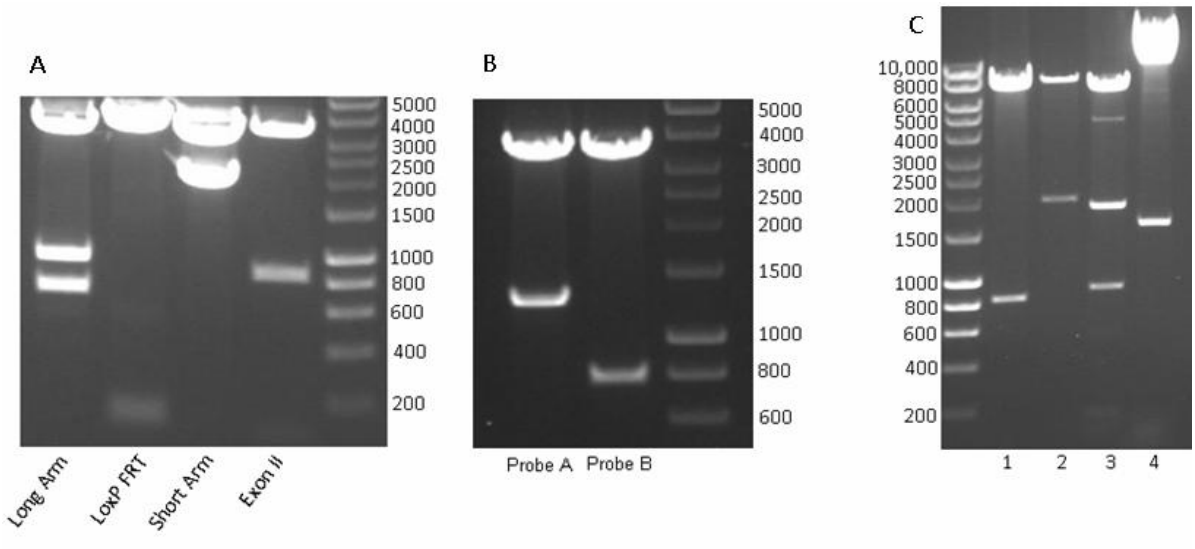


Figure 5.19 **The Production of the Targeting Construct and Southern Probe**

(A) Digestions demonstrating expected fragment size for each construct piece as cloned in the pCR-Blunt II-TOPO vector. The presence of the long arm of homology was confirmed by EcoRI digest giving fragements of 906bp and 680bp along with the backbone of the pCR-Blunt II-TOPO vector. The LoxP FRT fragment was also confirmed by EcoRI digest to reveal a fragment approximately 90bp in size. The presence of the correct short arm section was also checked using EcoRI to reveal a 1987bp fragment. In the case of the excision of the exon II fragment from the pCR-Blunt II-TOPO vector BamHI and XbaI were used to reveal an 811bp fragment. (B) EcoRI digested fragments from TOPO cloned Southern hybridisation probes. Probe A represents a 1322bp fragment used as the probe that is found outside the long arm of homology, whilst Probe B shows the 751bp fragment of the probe found outside of the short arm of homology. (C) Step by step correct insertion of construct fragments in the pPNT vector. Lane 1 shows the insertion of the exon II fragment into the pPNT vector analysed by digestion of the plasmid DNA with BamHI and XbaI to give fragments of 7362bp and 828bp as expected

for the correct orientation. EcoRI and BamHI digest reveals the insertion of the short arm of homology into the pPNT plus exon II vector with fragments of 8169bp and 1982bp: lane 2. Following this the insertion of the second LoxP and FRT sites occurred with XbaI digestion showing fragments of 7187bp, 1856bp, 902bp, 168bp and 111bp in lane 3. The smaller fragments are faint but just visible. The final lane, lane 4, demonstrates the ligation of the final insert, the long arm of homology, into the vector already containing all other components of the targeting construct by digestion with NotI and SalI to reveal fragments of 10214bp and 1574bp. In all case above 1% agarose gels were used.

Establishment of conditions for successful chimera production

Following the successful production of the targeting construct the next step was to insert it into the genome of the chosen JM8A3N feeder free ES cell line derived from the C57Bl/6N mouse strain (Pettitt *et al.*, 2009). These cells were specifically picked due to their C57Bl/6 background meaning animals produced from them would not require numerous back crosses to establish a pure C57Bl/6 line as would have been the case should a more traditional ES cell line derived from mice of 129 strain have been used. It is necessary that the new conditional α-synuclein model be on a C57Bl/6 background as this strain of mice are one of the best characterised inbred strains and importantly are the most commonly used line for studying models of neurobiology, making this new model easily comparable to previously existing ones. This line of cells also has a high germline competency which is an obvious important consideration when producing a new animal model.

The JM8A3N ES cell line is relatively new and had not been used previously in our laboratory. This meant that ideal concentrations of our own recombinant leukaemia inhibitory factor (LIF) had to be titrated as well as the correct G418 conditions being established. LIF is required to culture mouse ES cells, in the absence of a feeder layer, to limit differentiation whilst allowing the cells to continue to proliferate. LIF does this by activating the STAT3 transcription factor (Matsuda *et al.*, 1999). Serial dilution of several purified recombinant LIF batches obtained by expression of recombinant protein in COS7 cells were added to the medium used for cultivation of JM8A3N ES cells, which were visually assessed over a course of 10 days. A commercially available LIF (ESGRO, Millipore) used as directed by manufacturers instructions acted as a control. The cells were assessed for the degree of differentiation and migration (figure5.5). Although it is important to maintain undifferentiated cells in culture these cells must remain pluripotent ready for their injection into blastocysts. It is therefore essential that

the concentration of LIF is not too high within the culture, allowing the cells to maintain the ability to differentiate once injected.

A requirement of the targeting experiments was the selection of clones resistant to G418 which indicated that the target construct had been incorporated into the genome of the clone. It was therefore necessary to titrate the concentration of G418 that effectively killed cells that had not undergone homologous recombination within 5-7 days of selection. It was decided that a concentration of 105mg/ml was sufficient. It was not possible to titrate gancyclovir in the same manner as the target construct had to be present in order for the drug to cause cell death. For this reason a concentration was used that is recommended for other ES cell lines.

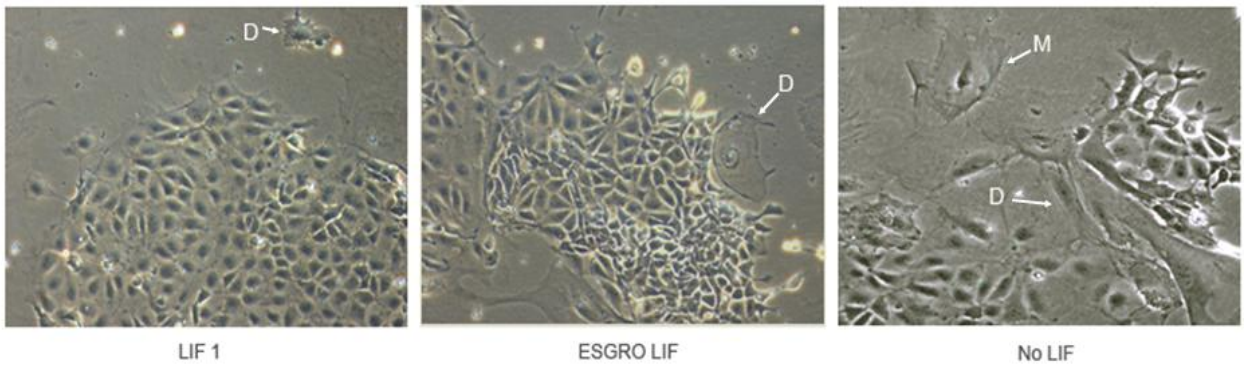


Figure 5.20 **LIF Comparisons**

Comparisons of different LIF conditions, including culture with no LIF, showing some cell migration (M) and differentiation (D). LIF1 (from our own stocks) shows a rounded colony with little differentiation. The culture in ESGRO LIF is fairly similar to LIF1 but with more differentiation. As can be seen in the culture with no LIF there is a lot more differentiation and migration.

Targeting

The targeting construct was first linearised with NotI, purified using ethanol precipitation and the resulting pellet left in the laminar flow hood to allow any remaining ethanol to evaporate. Following this the DNA was dissolved in dH_20 and added to the ES cell suspension. The suspension was then electroporated at 240 V/500µF for 6.4µsec. The cells were then split over plates at various concentrations and left overnight in full media minus selective antibiotics. Twenty-four hours post electroporation cells were put under selective conditions by the addition of both G418 and gancyclovir. After 10 days under selective conditions individual

colonies became visible and these were picked and expanded for further analysis as described in the Methods section.

Screening ES cell clones for homologous recombination by Southern hybrydisation

DNA was extracted from 183 targeted, G418 resistant, JM8A3N clones using phenol-chloroform and BamHI digests were performed for the initial Southern hybridisation screen. BamHI digest and hybridisation with the probe lying outside of the short arm of homology identified a band at 16448bp for the targeting construct due to an internal BamHI site within the target construct sequence as well as a 32600bp band for the wild type allele (see example in figure 5.6, A). Using this combination of DNA digest and hybridisation probe it was possible to confirm the presence of 3 positive clones. These clones were then further analysed using BamHI digest in combination with the probe lying outside of the long arm of homology and also using NcoI digest with the probe on the short arm side of the mouse genome to confirm the finding of the original screen. In the case of NcoI digest and hybridisation the expected band was 3525bp confirming the presence of the targeted DNA as well as the 3815bp band of the wild type allele. BamHI digested DNA hybridised with the probe outside of the long arm of homology revealed a targeted band of 18153bp and 32600bp band of the WT allele (figure 5.6).

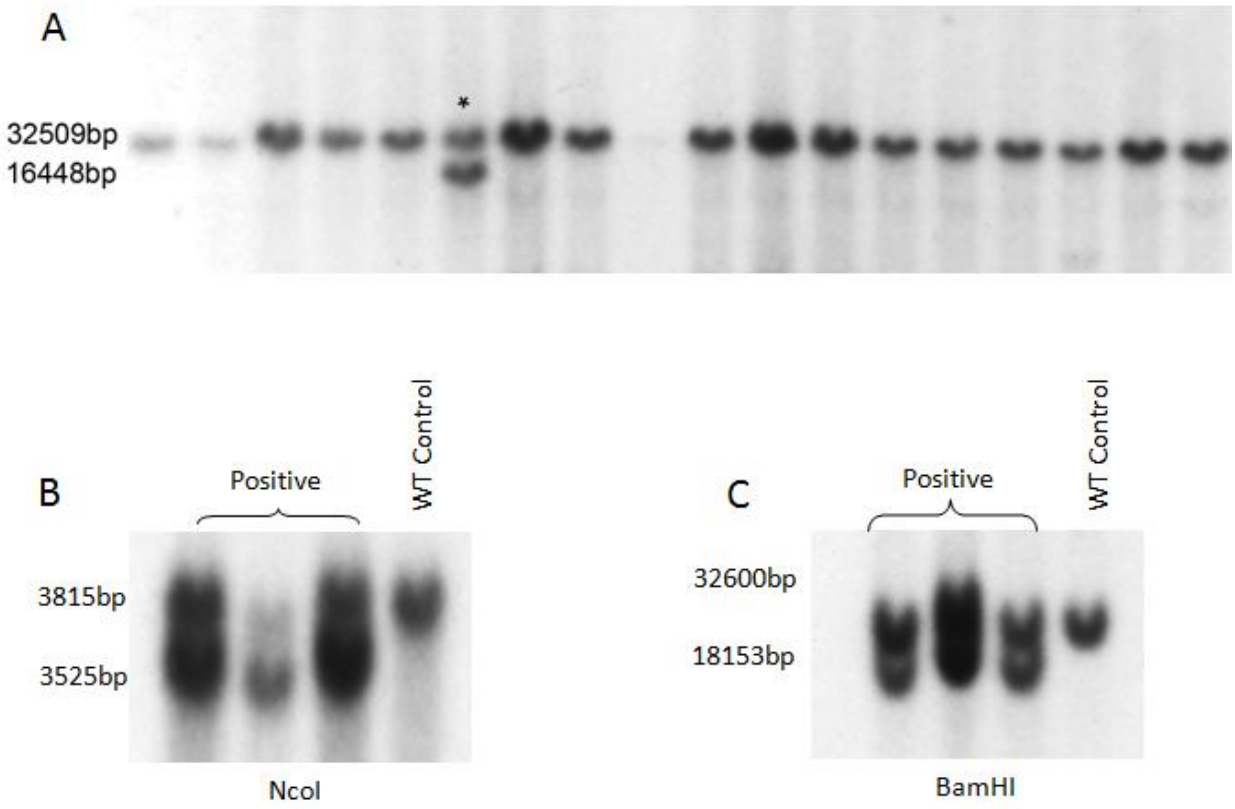


Figure 5.21 **Southern Blot Analysis of Targeted Clones**

(A) Representative Southern blot showing BamHI digested ES cell DNA which was hybridised to a probe located adjacent to the short arm of the targeting construct. Note the positive ES clone (*) showing both the 32509bp band expected from the normal C57Bl6 genome as well as the 16448bp fragment only present when correct homologous recombination of the target construct has occurred and thus inserted another BamHI site responsible for the smaller fragment size. (B) Southern blot showing the 3 positive ES clones digested with NcoI and hybridised with the probe adjacent to the short arm of homology. (C) BamHI digested positive clones along with WT DNA hybridised with the long arm of homology probe to give a band positive for homologous recombination at 18153bp and a WT band at 32600bp.

Analysis of positive clones by PCR

Following Southern screening DNA of clones that appeared positive were used for PCR amplification of fragments encompassing all *FRT* and *Lox*P sites. The following primers were used:

alphaLAfor	5' CATGAGTACTTGTGGCTCAC 3'
alphaint1for	5' TGCTGGGCACAGTGTTGATTG 3'
alphaint1rev	5' AAAGGCTGGGCTTCAAGCAG 3'
Exon1revnew	5' GACATGTATGGCAGTAAGCC 3'
NeoC	5' CTACCGGTGGATGTGGAATG 3'
NeoA	5' ATGGAAGGATTGGAGCTACG 3'

The amplified fragments were gel-purified, if required, and cloned in to the pCR-Blunt II-TOPO vector. Figure 5.7 (A) shows results of the digestion of resulting plasmids with EcoRI that excises cloned PCR sequences. It should be noted that a plasmid analysed on this gel for a fragment amplified from DNA of ES clone 134 using the "alphaint1for" and "alphaint1rev" combination of primers carries an insert originated from amplification of a WT allele. However another, not shown here plasmid carried a fragment amplified from the mutant locus. Eukaryotic inserts of plasmids were sequenced to ensure the presence and correct position of these elements in the genome of ES clones. "AlphaLAfor" was used in combination with "NeoC" to produce a 234bp fragment, with which the sequence confirmed the presence and correct orientation of both the *FRT* and *Lox*P site between the neomycin cassette and the long arm fragment of the targeting construct. The "neoA" and "exon1revnew" primer pair was used to amplify a 416bp fragment and its sequence demonstrated that the *FRT* site was correctly inserted at the end of exon II in the targeted locus. Finally "alphaint1for" and "alphaint1rev" were used to amplify a 406bp sequence containing the final *Lox*P site from the original pPNT plasmid backbone as well as a 354bp WT allele band.

Blastocyst injections of positive ES clones

Once positive ES clones had been selected by Southern hybridisation and the sequences of all *Lox*P and *FRT* sites confirmed it was then possible to select one to use for the first attempt of blastocyst injections. The ES clone with the best undifferentiated morphology on the day of injection was selected, which happened to be clone 126. Cells were prepared and kept in M2 medium on ice until required. 15 blastocysts and 2 morulas were flushed with M2 medium from uteri of C57BL/6 donor females who had been mated with males 4 days prior to the injection day. Seven to twelve ES cells were injected into the blastocoel cavity of each blastocyst and the same into the centre of the morulas. Two CD1 hosts were used with one being injected with 9 and the other 8 blastocyst/morulas unilaterally into the uterus whilst under anaesthesia. The animals were then allowed to recover and given pain relief. Both females produced litters on 17^{th} day after the embryo transfer.

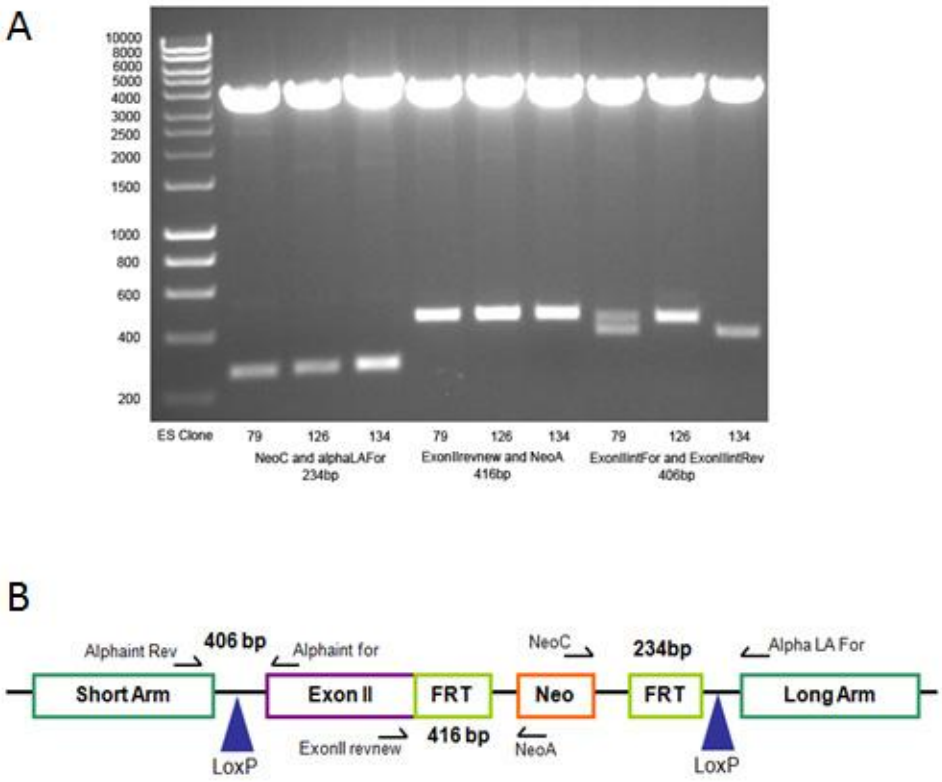


Figure 5.22 **PCR Analysis of Positive ES Clones**

(A) 1% agarose electrophoresis gel showing analysis of fragments amplified from DNA of mouse ES cell clones 79, 126 and 134, and cloned into the pCR-Blunt II-TOPO vector. Plasmids were digested with EcoRI. The primer combination used for amplification of mouse genomic fragments and expected insert size are shown below each lane. (B) Schematic map of the targeting construct showing the position of the primers described for use in A. Using these 3 combinations it was possible to check the sequences of all the *FRT* and *Lox*P sites within the targeted locus.

Chimeric animals and establishment of germ line transfer

From these two litters three chimeric animals were produced, 2 males and 1 female. Once these mice had reached 6 weeks old they were paired with C57Bl6 females to assess their germ line transfer capabilities. Offspring were produced from all three animals but only one male transferred the ES cell genotype to the next generation as was judged by the brown colouration of some pups in the resulting litters (see figure 5.8,.A). This was confirmed by genotyping of all pups using "alphaintfor" and the "alphaintrev" primers. Figure 5.8 (B) shows that animal 64 is the only one in the analysed litter of nine that carries the same targeted locus as the ES cell clone used for chimera production.

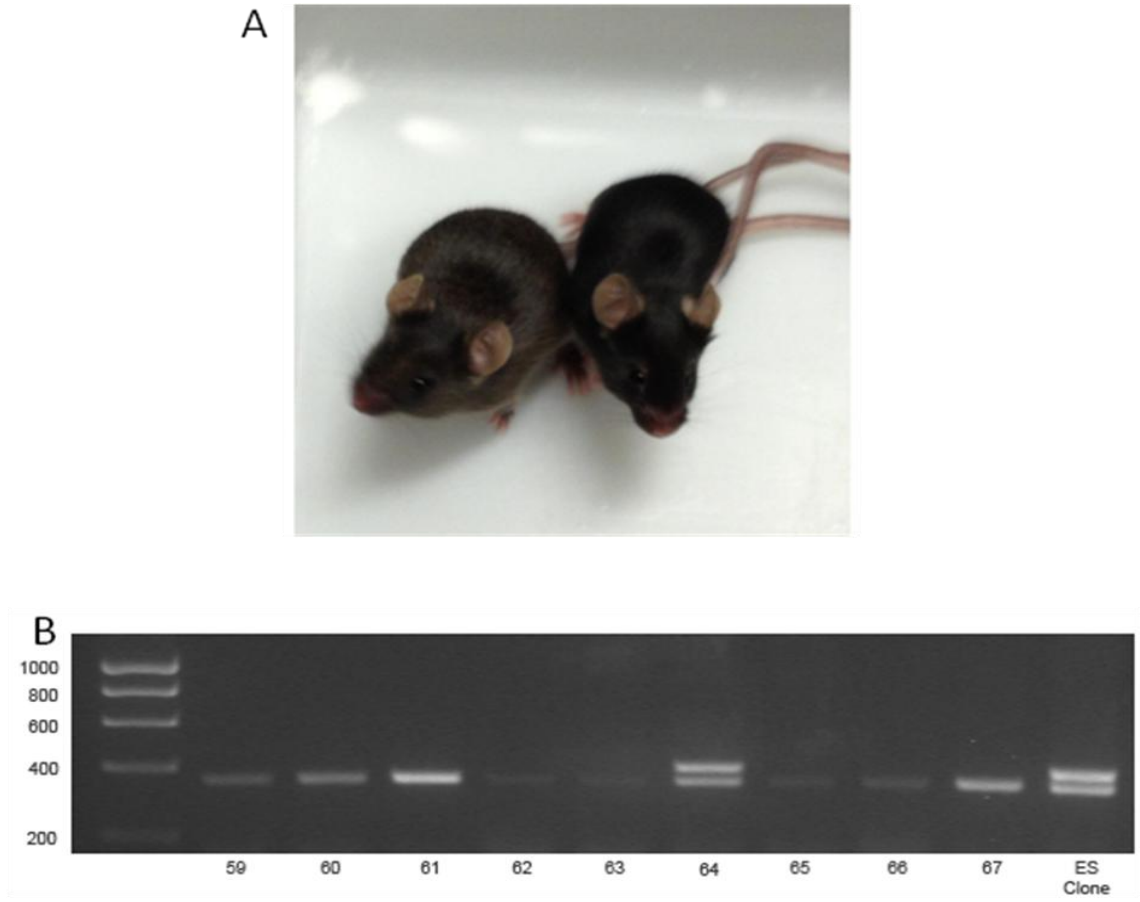


Figure 5.23 **Production of Chimeric Animals and Assessment of Germline Transfer**

(A) Chimeric mouse which appears dark brown (left) next to WT litter mate (right) (B) 2% agarose gel of PCR showing germline transfer from a 126 ES clone chimera. Primers "alphaint for" and "alphaint rev" were used to genotype these animals. In the case of germline transfer a 406bp band would be apparent alongside the 354bp band from the WT allele. Targeted ES clone DNA is shown as a positive control.

Initial Characterisation of Homozygous animals

After germline transfer had been confirmed the next step was to ensure that mice homozygous for the targeted alpha-synuclein locus could be produced. Following further breeding after the first chimeric animals were obtained mice were crossed and produced a litter of four pups two of which proved to be homozygous for the targeted locus (figure 5.9, A)

Upon obtaining homozygous animals it was possible to investigate the level of α-synuclein in the presence of the neomycin cassette (figure 5.9,B). When compared to animals that were heterozygous for the targeted allele the homozygous mice displayed much lower expression of α-synuclein throughout the cortex, cerebellum and spinal cord. This reinforces the need for these animals to be crossed with the ACTFLPe line in order to remove the neomycin cassette.

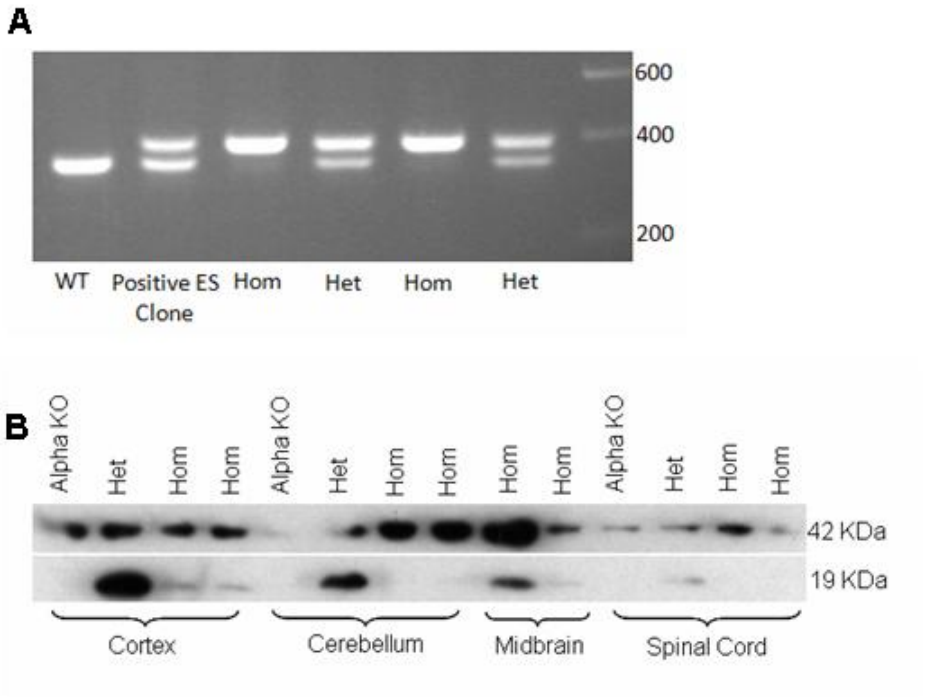


Figure 5.24 **PCR and Western Analysis of Homozygous Conditional KO Mice**
(A) 2% agarose gel showing the genotyping of a litter of mice from alpha-synuclein conditional knockout heterozygous parents. Primers "alphaint for" and "alphaint rev" were used and a 406bp band alone demonstrates animals homozygous for the targeted alpha synuclein locus. Whereas animals that are heterozygous also display the wild type (WT) alpha-synuclein alelle354bp band. DNA from a positive ES clone was used as a positive control. (B) Western blot comparing various brain regions from animals homozygous (Hom) for the targeted allele against a heterozygous (Het) animal and a conventional alpha synuclein knockout mouse

(AlphaKO). The 19KDa band represents α-synuclein and the 42KDa band demonstrates β-actin expression.

Testing FLP recombination ability in positive ES clones

As with all projects of this nature it takes time to produce chimeric animals and gain germline transfer. In addition to this, resulting animals require crosses with FLP and Cre recombinase mouse lines before the knockout can be assessed and shown to work. However it is possible to test the potential of the conditional knockout in the positive ES cell clones.

In order to establish if the neomycin cassette could be effectively removed in the ES clones, cells were grown until 70% confluent and transfected with a plasmid encoding the flippase enzyme. Lipofectamine was used for transient transfection of a positive ES cell clone 79 with the pCAGGS- Flpe vector (figure 5.10, D for map). This vector contains flippase under the chicken β-actin promoter as well as also containing a puromycin resistance cassette which could be exploited to identify recombined clones. Cells were allowed a 24 hour post transfection period before being exposed to 7 days of 3µg/ml puromycin selection. Colonies remaining after this selection period were picked and expanded across a number of plates. In order to test for Flp recombination cells on one set of plates were exposed to 105µg/ml G418 for 5-7 days. As the neomycin cassette had been flanked with *FRT* sites it was expected that if the clone that had correctly undergone recombination it would have become sensitive to G418 and died. DNA was purified from replicate plates of the sensitive clones for analysis by PCR and Southern hybrydisation.

PCR was carried out using the combination of “reverse floxed” and “exonIIrev new” primers. This combination of primers allowed a 304bp fragment for a flipped allele (i.e. with no neomycin cassette) and an internal control WT allele fragment that gave 633bp product. Clones were also analysed by Southern blot using DNA digested with BamHI. The DNA was probed using the probe outside the long arm of hybridisation to result in a WT band of 32600bp and either a complete positive targeted allele band of 18153bp or a flipped targeted allele band of 16298bp. These results are demonstrated in figure.5.10.

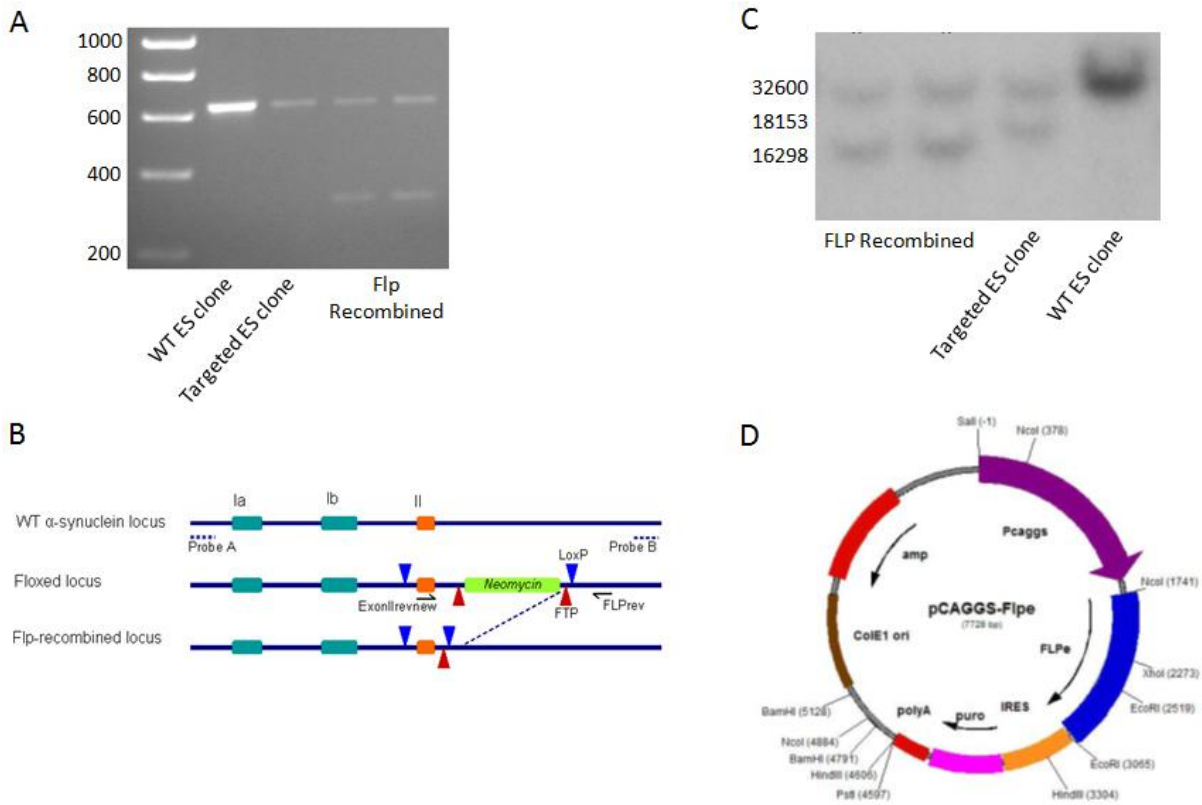


Figure 5.25 **Assessment of FLP Recombinase Activity in Targeted ES Clones**

(A) 2% agarose gel showing products of PCR amplification of 2 DNA clones positive for Flp recombination and two negative controls. The normal 633bp fragment present in the mouse genome is clearly only visible in the negative controls. The second 304bp fragment is only in clones that have undergone Flp recombination in the targeted allele. (B) Representative schematic of alpha synuclein alleles, both wild type (WT) and targeted showing the position of the primers used for screening the success of Flp recombination. (C) Southern blot confirming the PCR result showing that selected clones had undergone Flp recombination. DNA was digested with BamHI and hybridised with the probe lying outside the long arm of homology. (D) A plasmid map of pCAGGS-Flpe used to transiently transfect ES cells positive for homologous recombination with the target construct.

Testing Cre recombination ability in positive ES clones

After clones had been obtained from the Flp recombination experiment and had been confirmed both by PCR and Southern blot one clone was selected to test Cre recombination. Cells were co-transfected with the pPNT and pMC-Cre vectors using lipofectamine (figure 5.11, D for a vector map). Unfortunately the pMC-Cre vector has no desirable selectable features

within it and therefore pPNT was used to allow G418 resistance in transfected cells. After 7 days of G418 selection colonies were picked and expanded. Using the "alphaintfor" and the "alphaintrev" primers, a 354bp fragment amplified from the WT allele and 406bp fragment amplified from the targeted allele were produced when parental ES clone DNA was used as a template. Cre recombination was successful in three selected clones shown in figure 5.11 (A). Only the 354bp fragment was amplified from the WT allele of these clones because of the loss of an annealing site for "alphaintrev" primer in the Cre-recombined allele. Following PCR assessment these clones were also screened using Southern hybridisation. BamHI was again used with the probe lying outside of the long arm of homology to produce a 32600bp WT allele band and of 15450bp if correctly Cre recombined. Representative results are shown in figure 5.11 C.

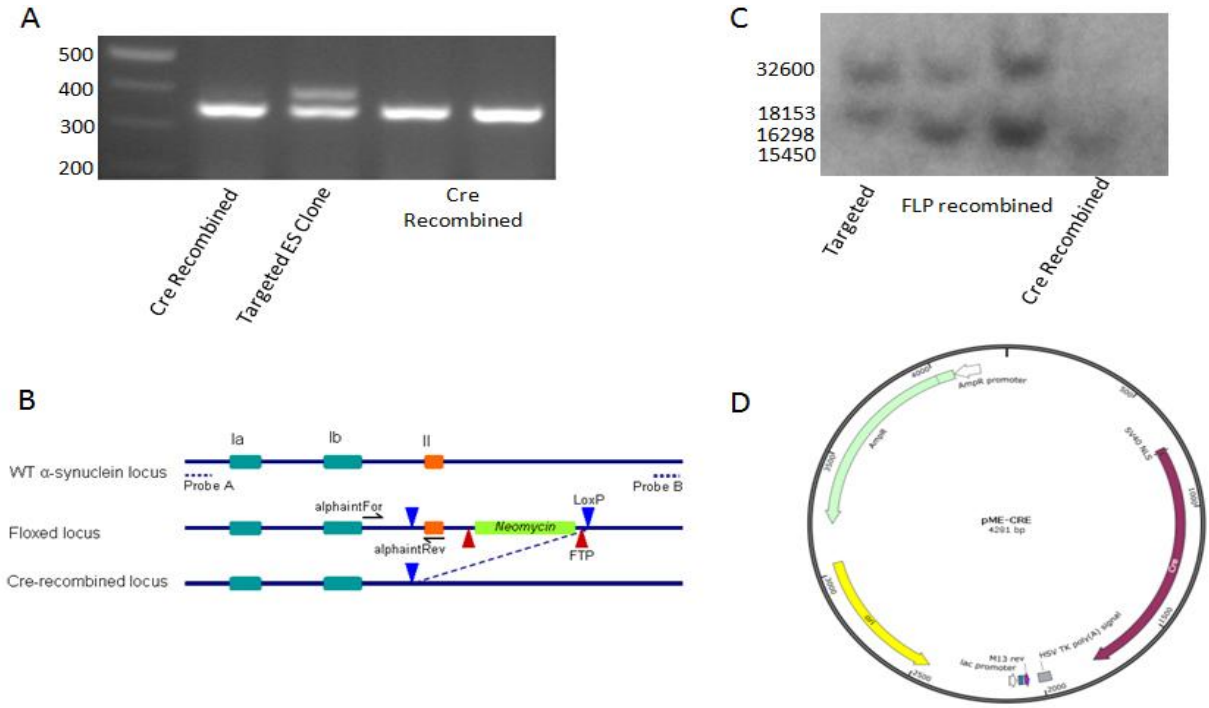


Figure 5.26 **Assessment of Cre Recombinase Activity in Targeted ES Clones**

(A) 1% agarose gel showing examples of Cre recombined ES clones and a control targeted but non recombined clone displaying both the WT 354bp band and the targeted 416bp band which is lost in Cre recombined clones. (B) Schematic of the floxed alpha synuclein locus undergoing Cre recombination. WT and targeted loci show the position of the primers used for PCR screening of successful Cre recombination. (C) Confirmation of postive Cre recombination by Southern hybridisation. DNA was digested with BamHI and hybridised with the probe adjacent to the long arm of homology. The WT allele is represented by the 32600bp band and the

targeted allele by the 18153bp band. When Flp recombination occurs the targeted allele become 16298bp and when positive for Cre recombination this decreases further in size to 15450bp. (D) Plasmid map of pMC-Cre used to transfect Flp positive clones. Note the lack of any usable resistance cassettes meaning co-transfection was required to allow easy identification of transfected cells.

Discussion

As has been shown above a targeting construct that allows the conditional knockout of α-syunuclein has been produced and successfully used to achieve homologous recombination in ES cells. These cells have been used to successfully create chimeric animals capable of germ line transfer. F3 animals have already been established and continued breeding of these animals is required to ensure a large cohort for further experiments. A breeding strategy is in place and we already possess the required lines for crossing. As has been established through studying mice homozygous for the targeted alpha-synuclein allele α-synuclein expression is greatly reduced in the presence of the neomycin cassette. This reaffirms that these animals will need to be crossed with the ACTFLPe expressing line in order to remove the cassette. The ACTFLPe line was first described in 2000 and has since become a common line for allowing FLP recombination (Rodriguez *et al.*, 2000). This line expresses FLPe under the human β-actin (ACTB) promoter. FLPe is a naturally occurring variant of FLP which contains 4 amino acid substitutions which collectively make the recombinase more thermostable and thus increase recombination activity by 4 fold at 37^{o}C and by more than 10 fold at 40^{o}C (Buchholz *et al.*, 1998). This line has been assessed and shown a wide array of FLPe expression across many tissues including the nervous system, the cardiovascular system and, most importantly the reproductive system. The recombinase activity is known to occur as early as embryonic day 10.5 and can therefore allow fast removal of the neomycin cassette in the conditional alpha-synuclein animals, which will then be transmitted to F2 progeny.

Having successfully removed the neomycin cassette, thus ensuring the normal expression of α-synuclein prior to knockout, the animals will then be crossed with the NSE-CreER line of mice in order for the conditional α-synuclein mice to possess tamoxifen inducible Cre recombinase activity (Frugier *et al.*, 2000). This line expresses Cre under the rat neuron-specific enolase gene (NSE), ensuring that the Cre recombination activity is specific to neuronal populations. The NSE promoter is not active in the embryonic nervous system before E18, when the level of ER-Cre is still very low. In postnatal development of transgenic mice the pattern of ER-Cre expression is similar to that of endogenous NSE (Forss-Petter *et al.*, 1990). As mentioned

above the Cre protein is fused to the ligand binding domain of the estrogen receptor (ER) meaning that the recombinase activity is inducible with the use of 4-hydroxytamoxifen. This will therefore allow the conditional knockout of α-synulcien with injections of 4-hydroxytamoxifen. The line of NSE-CreER animals that are possessed in the lab also express the ROSA26Sor sequence (Soriano, 1999). This entails a stop codon flanked by LoxP sites upstream of the LacZ gene meaning that when Cre recombinase is initiated the stop sequence is excised and β-galactosidase is expressed. Staining the resulting tissue with X-gal allows the areas in which efficient Cre recombinase activity has occurred. This will therefore enable the Cre recombination activity to be assessed in the brain regions of interest.

Alpha-synuclein conditional knockout animals that have been crossed with ACTFLPe and NSE-CreER lines will be bred to produce a number of cohorts for aging. It will of course be important to first assess the knockout, which will be achieved by assessing protein and mRNA levels of α-synuclein by Western blotting and qRT-PCR respectivey. X-gal staining will also be used to assess the degree of Cre recombination in the midbrain dopaminergic system. As well as these methods immunohistochemistry will also be employed against α-synuclein to ensure that the knockout is successful. It is expected that the knockout should be efficient as shown by the ability to induce both FLP and Cre recombination in targeted ES clones.

After initial analysis of the capabilities of the conditional knockout cohorts of animals will be aged and knockout induced at 3-, 9-, 12- and 24-months of age. Throughout the period of aging and following the induction of α-synuclein knockout the animals will be subjected to a variety of behavioural tests. These tests will include rotarod, inverted grid and horizontal beam tests, all as a measure of the function of the nigrostriatal system. It is anticipated that following the knockout the animals will perform less well at these tests and particularly those aged furthest before the knockout as brain plasticity decreases with age. These animals will also be challenged with psychostimulants such as amphetamine and cocaine to assess their locomotor response. These drugs work by different mechanism as described in chapter 3 and will allow an insight into the dopamine handling capacity at the striatal synapses. Further to this, tissues will be harvested at 3 and 6 months following the induction of the conditional knockout. These tissues will be assessed in a number of ways to explore any changes in the nigrostriatal system. HPLC will be used to measure any alterations in striatal dopamine levels, as well as that of its metabolites, and Western blotting will be employed to ascertain any changes to synaptic markers in the striatum. Immunohistochemistry coupled with stereological counting will be used to determine the number of dopaminergic neurons of the SNpc and VTA.

It is hoped that electron microscopy can be used to assess changes to the synapses in the striatum. This would include the evaluation of synaptic vesicle density and arrangement into their various pools, the size of synaptic boutons and length of the active zone as well as the number of mitochondria. These measurements will be carried out on projections to the striatum as well as those to the VTA. Following on from this fast-scan cyclic voltametry will also be used to explore the release and reuptake dynamics of dopamine at the synapse in the striatum. These techniques have all be previously employed to look at conventional knockout models and have been useful in helping to unravel some intriguing data. It is hoped that these techniques will also allow a clear insight into the changes that occur in the absence of α-synuclein at the dopaminergic synapse.

In order to explore any compensation mechanism that may occur in the conventional knockout animals during critical development periods as previously discussed, animals could be crossed with a reporter strain of GFP mice that show expression in the midbrain structures. This could be done with both conventional and conditional knockout α-synuclein animals as well and WT controls and the GFP positive SNpc and VTA neurons laser dissected. These neurons could then be used to extract mRNA that could be used for comparative whole genome expression profiles of these dopaminergic neurons. Any significant changes can then be confirmed using other methods such as qRT-PCR. Through this process it would be hoped that pathways which are altered during changes in normal α-synuclein expression, and thus in the neurons response to diminishing α-synuclein levels seen in disease, could be identified for further study.

This novel conditional α-synuclein model could also be used to further investigate the role of α-synuclein in MPTP toxicity. Animals could be aged to 6- months prior to induction of the knockout and then after a month be exposed to MPTP. This may provide further knowledge with regards to the scenario of conventional knockout of α-synuclein possibly causing a more robust population of midbrain neurons and thus being described in a number of studies as resistant to the effects of MPTP (Drolet *et al.*, 2004, Robertson *et al.*, 2004).

It is hoped that the development of the novel model will give a new insight into the role of α-synuclein loss of function in the disease process. This is an important facet of knowledge that as yet has not been properly explored. Previous α-synuclein knockout animals have failed to reveal any overt phenotypes, as discussed in the introduction in detail, which may be due to functional compensation of the other synuclein family members or that of a prenatally induced mechanism not related to β- or γ-synuclein which allows for the functional compensation of α-synuclein. Identification of mechanisms which dopaminergic neurons use to cope with

depletion of α-synuclein may also represent important new pathways that could be targeted therapeutically to limit the loss of these neurons.

Chapter 6

Final Discussion

This thesis has studied the role of the synuclein family within the midbrain dopaminergic system in detail and has revealed evidence to further bolster support for the involvement of these proteins in normal dopamine neurotransmission. This is in agreement with a previous body of work which suggests this population of neurons is sensitive to small changes in the levels of the synucleins. Further to this it is hoped that the creation of the conditional α-synuclein knockout model will allow for a more specific role for the protein to be determined and produce insight into its role in the disease process.

The role of the synucleins in dopamine neurotransmission

As yet there has been no single clearly defined role identified for any of the synucleins. Previous work has concentrated mainly on the role of α-synuclein due to its clear function in disease and has linked it to a role in neurotransmission, including dopamine neurotransmission. Although less work has focussed on the other two synucleins, tentative hints have also pointed to a similar role for them in normal neurotransmission (Chandra et al., 2005, Senior et al., 2008). This is not surprising due to their highly similar amino acid sequences and overlapping expression patterns with α-synuclein. To gain a better general insight into the role of the synuclein family, triple synuclein null mice have proved insightful. Work presented in this thesis with the triple synuclein null mice supports this sentiment of a role in dopaminergic neurotransmission, and in particular suggests that these proteins are involved in dopamine neurotransmission at the vesicular level.

A previous study on an independently produced line of triple synuclein null mice demonstrated no neuronal loss in the CA1 and CA3 subfields of the hippocampus (Greten-Harrison et al., 2010). To expand on this dopaminergic midbrain populations of the SNpc and VTA were investigated in the triple knockout mice obtained in our laboratory. This confirmed that there were no significant alterations in the number of TH positive cells in these regions therefore supporting previously obtained evidence that these proteins are not essential for the survival or development of the dopaminergic midbrain neurons. Work carried out on γ-, α- and αγ-synuclein null mice has previously shown that there is a small but significant decrease in the number of tyrosine hydroxalse positive neurons in the SNpc (Robertson *et al.*, 2004). However another study investigating the loss of α-synuclein on the survival of midbrain dopaminergic neurons failed to describe any cell loss (Abeliovich *et al.*, 2000). It is likely that the disparities in the two described studies arose from strain background variations as the mice used in the Robertson *et al* study were on a pure C57Bl6 background whereas those in the Abeliovich *et al* study were intercrosses between C57Bl6 and 129SV/j mice. A possible explanation for the

differences between the triple synuclein null mice and other combinations is that in the case where all synucleins are absent a compensatory mechanism may be established that can fully compensate for the loss of the synucleins, but where at least one of the synucleins is remaining it is probable that this mechanism cannot be fully established and therefore leads to the loss of a subset of dopaminergic neurons that are particularly sensitive to changes in synuclein levels.

No changes were noted in levels or distribution of synaptic markers in the triple synuclein null animals, but a significant reduction of 40% in striatal dopamine concentrations was apparent. Despite this the animals proved to have a hyperdopaminergic phenotype which can be explained by increased releasability of dopamine that was demonstrated in these animals using FCV studies (Anwar *et al.*, 2011). Interestingly the impact that the loss of all three synucleins has on dopamine releasability was specific to the dorsal striatum with no significant changes being noted in the ventral striatum, therefore displaying clear differences in the sensitivities of these different subsets of dopaminergic populations to the loss of the synucleins (Anwar *et al.*, 2011). The differences in susceptibility to PD pathology is well described in these two sets of dopaminergic neurons and it is generally considered that SNpc dopaminergic neurons that innervate the dorsal striatum differ in many ways from those that arise in the VTA and innervate the ventral striatum. These differences include heterogeneity of ion channels and protein expression which includes specific markers of dopaminergic neurons such as VMAT2 and DAT (Lissa and Roeperb, 2008, Bjorklund and Dunnett, 2007, Korotkova *et al.*, 2004). This disparity is considered to explain the apparent increased sensitivity of the dorsal striatal neurons to the neurodegenerative process. Due to previously well described differences in these two neuronal populations it is not surprising that they display varying sensitivities to the loss of synuclein expression. This work has uncovered clear differences in these two regions with regards to the role of the synuclein family and may suggest that different areas of the brain are more dependent on these proteins than others. This highlights the importance of exploring various subpopulations of neurons in such studies to unveil any disparities in sensitivity.

Earlier work has shown α-synuclein can regulate the dynamics of dopamine release at the presynaptic membrane. Previous α-synuclein as well as double synuclein knockout animal models identified increased releasability (Abeliovich *et al.*, 2000, Senior *et al.*, 2008, Yamada *et al.*, 2004). In contrast to this, studies that have used mice overexpressing α-synuclein have demonstrated an inhibition of dopamine release, possibly through alterations in normal

synaptic vesicle pool dynamics (Nemani *et al.*, 2010, Larsen *et al.*, 2006). This evidence suggests that α-synuclein levels are important in the normal release dynamics of dopamine and that the protein may act as a negative regulator for dopamine release under normal circumstances. However the method by which this is instated remains unclear. One possibility is through the regulation of the synaptic vesicle pools.

Much of the work relating to the investigation of the affects of the synucleins on vesicle pool dynamics has been obtained through studies on hippocampal neurons. These neurons are distinctly different from those of the striatum as the midbrain dopaminergic neurons lack any clearly defined reserve and recycling pools of vesicles. Although these studies are very useful in terms of studying basic aspects of neural transmission this use of different neuronal populations for studies of this nature cannot be directly compared and for this reason it is likely the cause of differing results between studies. Early work on cultured hippocampal neurons that were treated with antisense oligonucleotides in order to knockdown α-synuclein expression showed a decreased availability of a reserve pool of synaptic vesicles (Murphy *et al.*, 2000). Consistent with this Cabin *et al* demonstrated that α-synuclein knockout animals have a significant impairment to prolonged repeated stimulation which depleted the docked vesicle population and due to the reserve pool being diminished, replenishment of the readily releasable pool was slower (Cabin *et al.*, 2002). Recently a study in which α-synuclein was over-expressed at levels predicted to mirror those seen in patients with multiplication mutations, but which were not great enough to display any marked toxicity, also displayed a specific reduction in the size of the recycling pool of synaptic vesicles and therefore resulted in inhibition of normal neurotransmitter release which was evident prior to any detectable pathology (Nemani *et al.*, 2010). As well as this when either of the mutant forms of α-synuclein, A53T or E46K, were overexpressed in cultured hippocampal neurons, there was a noted inhibition of exocytosis of synaptic vesicles. Taken together these studies demonstrate an important role for α-synuclein and highlight the problems that even small alterations in the expression level of this protein can cause.

Indeed, in an independent study using triple synuclein null mice there were measurable alterations in hippocampal neurotransmission, but this was not attributed to changes in neurotransmitter releasability dynamics (Greten-Harrison *et al.*, 2010). This study failed to reveal any changes in the number of synaptic vesicles in hippocamal neurons. In agreement with this, work done by our collaborators on the triple synuclein null mice produced in our lab showed no significant alterations in the number of synaptic vesicles, however these results

were gained from neurons of the dorsal striatum (Anwar *et al.*, 2011). These results from two independent cohorts of triple synulcien null animals and in two different populations of neurons are not in agreement with some previous studies that would suggest that a decrease in the number of the synaptic vesicles would be expected. One possibility is that in the triple synuclein null mice a more effective compensation mechanism is activated whereas in animals only lacking a single synuclein this is not the case. Although the results of these two studies are not completely in agreement with each other on certain phenotypes seen in these animals, they do both consistently display alterations of normal basic neurotransmission suggesting an important role for the synucleins.

In our studies regarding synaptic vesicle dynamics in the triple synuclein null mice it was revealed that although normal levels of synaptic vesicles were present they contained less dopamine due to a reduced capacity to compartmentalise it. It was demonstrated through studies investigating ^{3}H-dopamine uptake in synaptic vesicles purified from triple synuclein null mice, as described in chapter 4, that β-synuclein appears to have an important role in allowing efficient dopamine transport into the these vesicles and it is likely that the other two members of the synuclein family also posses this capability, but to different degrees. Further investigation is required in order to explore this hypothesis. Previous work carried out by Schulter *et al* supports this work by demonstrating that synaptic vesicles purified from α-synuclein null animals show no alteration in their ability to sequester dopamine in comparison to those purified from WT animals. In this case it could be proposed that due to β-synuclein still being present it could function to help in vesicle dopamine storage (Schluter *et al.*, 2003). This data is consistent with the body of work showing the synucleins to have a role in synaptic vesicle management.

Continuing on the theme of dopamine neurotransmission, α-synuclein has also been implicated in a role allowing it to interfere with normal dopamine synthesis. It is apparent, at least in cell culture systems, that the overexpression of α-synuclein is able to reduce the activity of tyrosine hydroxylase, the rate limiting enzyme in the dopamine synthetic pathway, whereas conversely the knockdown of α-synuclein leads to increased activity of this enzyme (Liu *et al.*, 2008, Yu *et al.*, 2004, Gao *et al.*, 2007). In the triple synuclein null mice used in this study it is apparent that the activity of tyrosine hydroxylase is normal and so the translation between *in vitro* and *in vivo* studies may not hold true.

Although the work above along with numerous other studies clearly links the synucleins to dopamine neurotransmission their specific role remains poorly defined. This will require

further studies that do not solely concentrate on the dopaminergic nigrostriatal or hippocampal circuits. It will be important that other dopaminergic systems are researched as well as non-dopaminergic systems. It is apparent from the work carried out on the triple synuclein null animals that the loss of the synucleins affects different closely related subpopulations of dopaminergic neurons, suggesting that different neuronal subtypes have different sensitivities to changes in the levels of these proteins. This may help explain why the dopaminergic neurons of the SNpc are particularly sensitive to pathology in PD. Helping to reveal why this may be so will also be important in order to better understand the disease process. As yet most research has concentrated on the dopaminergic system due to its clear link to the motor symptoms of PD but little is known about the impact of changes in synuclein levels on the non dopaminergic systems of the brain, despite the fact many symptoms seen in patients with PD arise from non dopaminergic circuits, and so studying these may prove insightful. The triple synuclein null model should prove a valuable tool in the hunt for the affects of altered synuclein dynamics on neurotransmission in other neuronal populations and may help explain some of the more varied symptoms of the disease. This is a very important area that needs to be researched as these symptoms are often debilitating to the patients, but also because it may allow new insight into the role of the synucleins that could be translated back to the dopaminergic system.

The role of familial redundancy within the synuclein family

In the lack of a member of the synuclein family it has always been considered that the remaining protein(s) can effectively allow functional compensation. Although the work presented in this thesis demonstrates this to an extent it is unlikely to be the most important source of compensation in these models. Even in triple synuclein null animals there were no overt phenotypes and those that were observed were not drastically altered from those previously observed in other knockout models. The loss of dopamine, despite normal TH activity, in the nigrostriatal system was greater than has been demonstrated in other synuclein knockout combinations and this severity of loss was observed in 4 month old animals (Abeliovich *et al.*, 2000, Chandra *et al.*, 2004). The only comparable loss of dopamine was reported in α-synuclein knockout animals that were aged to 24 months (Al-Wandi *et al.*, 2010). It therefore appears that there is a degree of familial compensation amongst the synuclein family that can work at least temporarily but it is more likely that a developmental compensatory mechanism is responsible for the diminished phenotypes in these animals that lack three abundant synaptic proteins.

Further evidence that supports this hypothesis comes from small changes in behavioural tests in an age dependent manner in the triple synuclein null animals. These knockout animals illustrate a significantly diminished ability to perform the accelerating rotorod test by 4 months, whereas they display a progressive inability to carry out the inverted grid test. This may infer that the compensation mechanism that these triple synuclein null animals have is less effective, particularly with age when other stresses on the nervous system are apparent. Put together it is likely that another compensatory mechanism is instated during embryonic development of these animals in the loss of the synucleins which are abundant synaptic proteins and it is this reason that a clear role for the synucleins remains masked.

The importance of the novel α-synuclein conditional knockout model

As has been detailed in this thesis a novel α-synuclein conditional knockout model has been successfully produced. Using targeted ES clones both FLP and Cre-recombinase activity has been confirmed to induce knockout of the neomycin cassette and α-synuclein respectively. However due to the nature of this project as yet it remains to be confirmed in the mice themselves. Currently, germline transfer has been established and these animals are being crossed with ACTFLPe mice, following which they will be bred initially with NSECreER but also at a later date with DAT/ER-Cre mice to produce working cohorts. These mice will be kept on a pure C57Bl/6 background in line with the rest of the synuclein knockout animals we obtain. The issue of background is important as variation in strain does appear to cause varied results as discussed above and the chosen background is one of the most commonly used for PD modelling. It is also the strain of choice for the EUCOMM project, which aims to produce a conditional knockout model of disease related genes, highlighting the importance of having animal models on comparable backgrounds.

This model has been produced in order to give further and clearer insight into the role of α-synuclein with particular interest in the degree to which a loss of function of the protein contributes to the disease process in PD. The fact that the model is conditional allows the aging of the animals prior to the knockout of the gene occurring; meaning the role of α-synuclein can be fully assessed in the aged nervous system, an important consideration in the study of diseases of aging. As well as this it is hoped that any compensatory mechanism, which seems apparent in previously produced synuclein knockout animals, will be overcome as the brain will be less plastic and therefore unable to adapt. This is something that as yet no other knockout model of α-synuclein has been able to achieve.

This model will first be used to assess the midbrain dopaminergic system in these animals as has been described in detail in the case of the triple synuclein null animals. The numbers of dopaminergic neurons will be assessed in the SNpc and VTA structures along with their gross morphology. Importantly, ultrastructure analysis using electron microscopy will be employed to assess the number of synaptic vesicles, synaptic bouton size, distance of vesicles to the synapse and other synaptic parameters. This model will also be carefully assessed using FCV and dopaminergic neurotransmission-altering drugs in order to gain insight into neurotransmitter release dynamics, which as discussed above appears highly important. Behavioural assessment will also occur to consider how the aged loss of α-synuclein impacts on normal motor ability. However, the fact that the inducible NSECre-recombinase is not specific to any one subtype of neuron will allow the effects of the loss of α-synuclein to be assessed in any population of neurons throughout the brain. As has been demonstrated from the studies of the triple synuclein null animals, it is apparent that different subpopulations of neurons show varying sensitivities to the loss of the synucleins, meaning it should be expected that the loss of α-synuclein will have similar outcomes. For this reason categorical assessment of other neurotransmitter systems should be carried out, for example the hippocampus could be explored as it was in the Greten-Harrison *et a*l study which proved fruitful and will also allow for more direct comparisons between previous studies (Greten-Harrison et al., 2010). As well as crossing these animals with NSECreER mice to induce the knockout of α-synuclein, a separate line will be created in which CreER recombinase expression is under the control of the DAT promoter meaning that the knockout is specific to dopaminergic neurons only. This will allow for the detailed investigation of the dopaminergic system with no possible interference from other neuronal subpopulations that may be affected by the inactivation of α-synuclein. Taken together this should allow for an unprecedented insight into the loss of α-synuclein on synaptic function both in specific populations of neurons as well as enabling a wider outlook with the use of a more broadly expressed Cre recombinase line. This may ultimately lead to new therapeutic strategies.

Final Conclusions

This thesis has contributed further evidence of the role of the synucleins in normal dopaminergic synaptic function. Work with triple synuclein null mice has revealed that these animals have a normal complement of SNpc and VTA neurons and normal gross brain architecture with no overt phenotypes. However, significant reductions in dopamine concentrations were observed and despite this a clear hyperdopaminergic phenotype was revealed. This body of work has also disproved the hypothesis that the synucleins are required

for MPTP toxicity and that triple knockout animals are in fact more sensitive to MPTP than WT animals. In addition to this it was revealed that synaptic vesicles of triple synuclein null animals are not able to efficiently store dopamine and that β-synuclein expression can restore this deficit. However, as with many previous studies this project has concentrated on the midbrain dopaminergic systems due to their clear association with PD. It is clear that although the main movement related symptoms of PD are caused by the loss of the SNpc dopaminergic neurons many of the other symptoms are not. This is a complex disorder that should not be simplified to one system and for this reason it will be fruitful to carry out similar studies in other affected regions. This will not only impact on our understanding on the disease process but is also likely to allow a broader understanding to the role of the synucleins at a basic level and how this may differ in different neuronal populations.

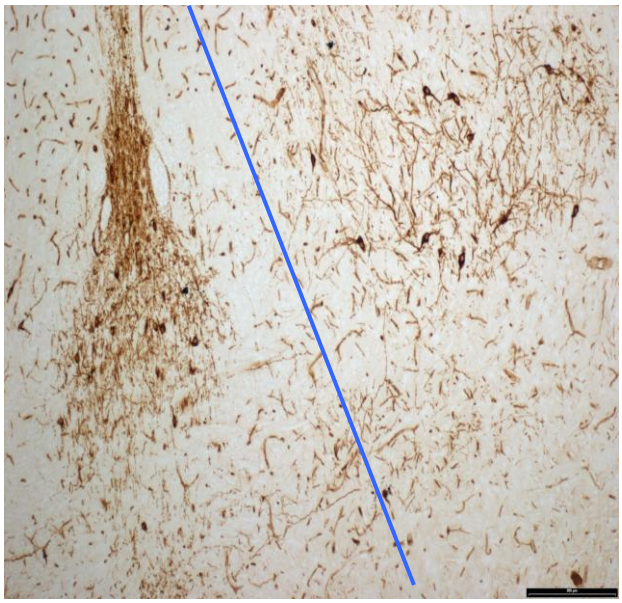
The work completed for this thesis has also involved the development of a completely novel conditional α-synuclein knockout model, which is important to allow the study of the effect of a loss of normal function of α-synuclein at the synapse. Although this is a very plausible idea it has until recently gained little attention. The main school of thought regarding α-synucleins role in PD pathogenesis is through a simple gain of function mechanism. However, as is becoming ever clearer, this seems to be a somewhat naïve view of events and it is likely that there is an interplay between gain and loss of function of α-synuclein that leads to a complex pathway of events which begins with subtle alterations in the normal dynamics of the affected neurons but which eventually culminate in cell death. It is hoped that the characterisation of this new model will allow for the role of α-synuclein at the synapse to be made clear and that this model will therefore impact on the way that PD pathogenesis is viewed.

Chapter 7

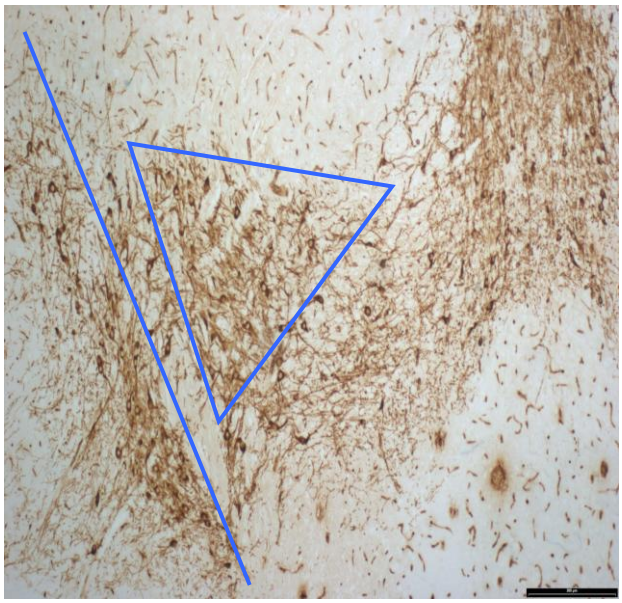
Appendix

Appendix 1: the separation of VTA and SNpc and cell body diameter measurements

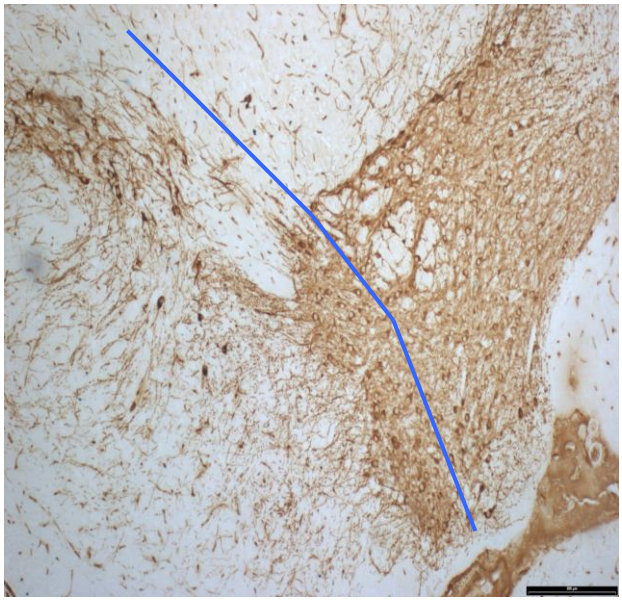
The below diagram shows how the VTA, area eight and SNpc were defined for counting of TH positive cells. This definition was kept constant throughout all counting. Images were taken at 10x and scale bar represents 200µm.



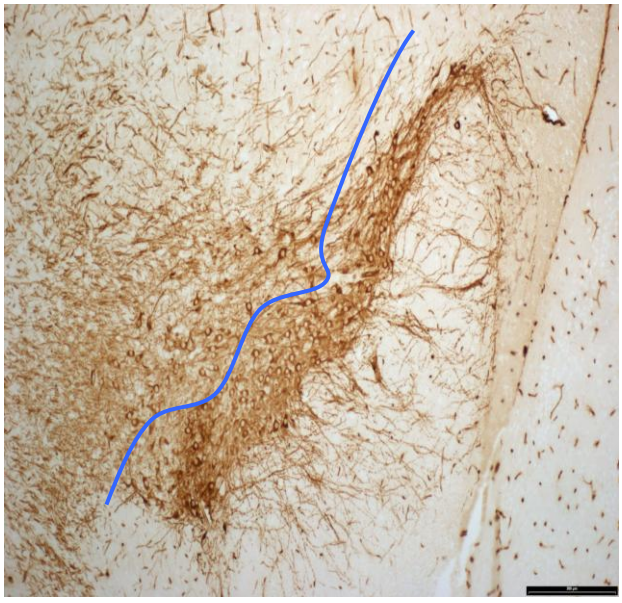
1) VTA is shown to the left side of the blue line. On the right side of the line area 8 is present which was not counted.



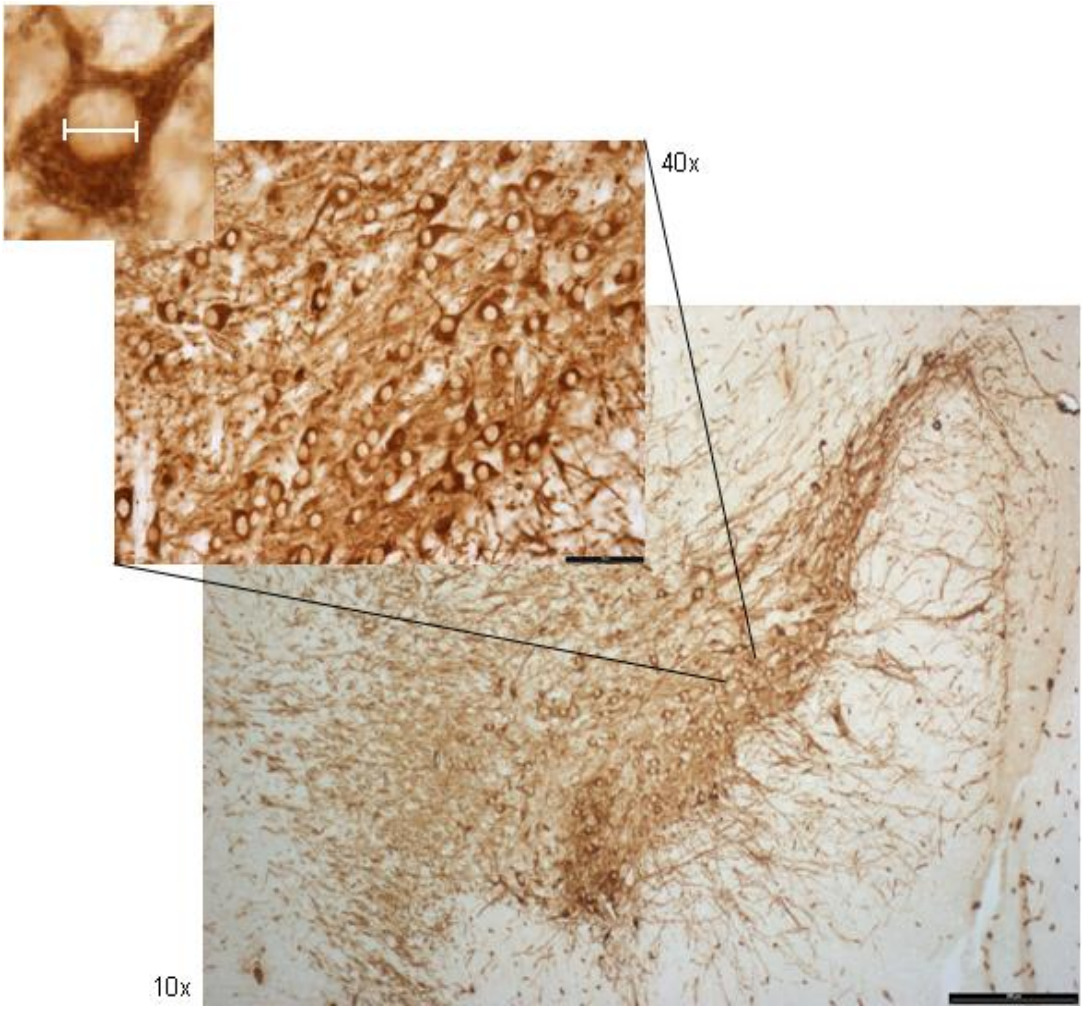
2) The VTA is shown to the right of the picture and the remainder of area 8 is highlighted in the triangle. To the left of the blue line the beginning of the SNpc



3) Here the VTA and SNpc become hard to separate. To the right of the blue line was counted as VTA and to the left of the blue line was counted as the SNpc.

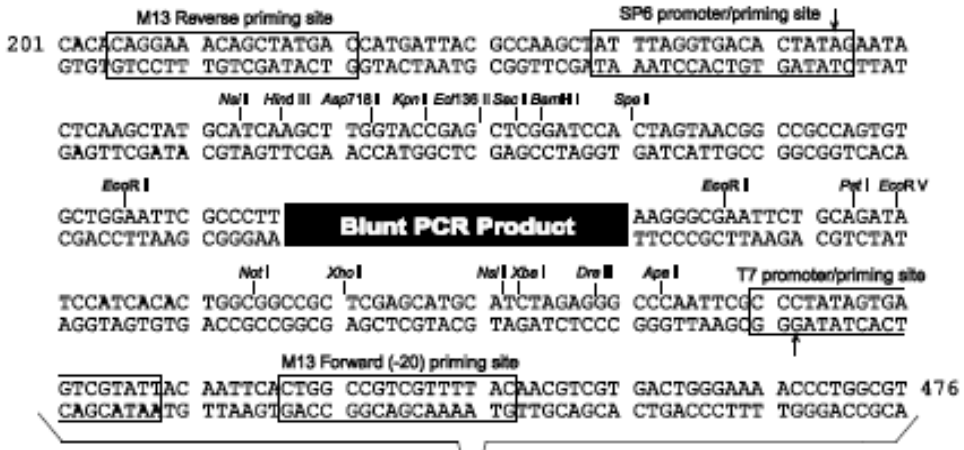


4) The VTA is coming to its end on the left of the line and to the right of the line the SNpc is now very clear.



TH immunostained sections of the SNpc at 10x and 40x magnification. In the third inlay, in which only one cell body is present, the method of measuring cell body diameter is demonstrated. This was done across the middle horizontal plain of a random selection of cell bodies from each slide in order to calculate the Abercrobie correction factor. The cell body must have a clear centre in order to be counted as demonstrated in the inlay showing the single cell body. 10x scale bar represents 200µm and 40x scale bar represents 50µm.

Appendix 2: pCR-Blunt II-TOPO (Invitrogen) plasmid map



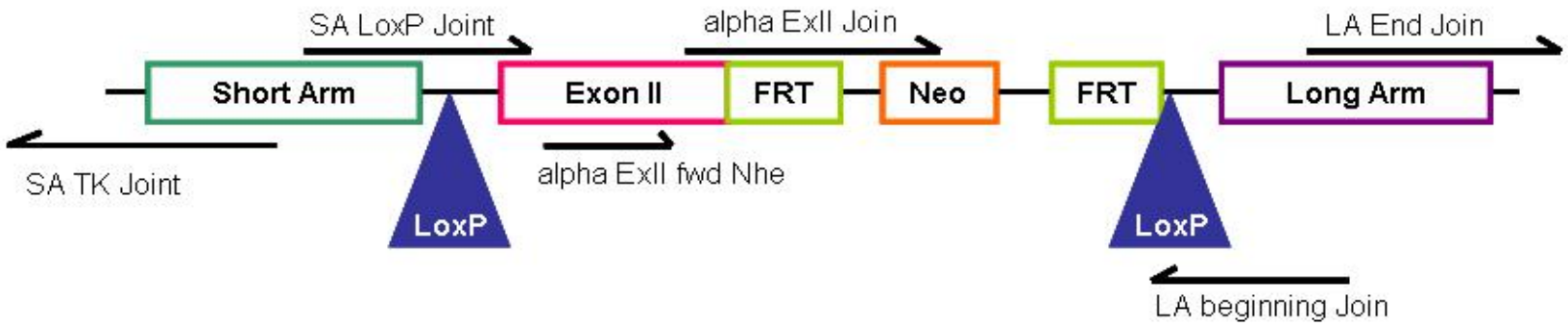
Comments for pCR™-Blunt II-TOPO®
3519 nucleotides

lac promoter/operator region: bases 95-216
M13 Reverse priming site: bases 205-221
LacZ-alpha ORF: bases 217-576
SP6 promoter priming site: bases 239-256
Multiple Cloning Site: bases 269-399
TOPO®-Cloning site: bases 336-337
T7 promoter priming site: bases 406-425
M13 (-20) Forward priming site: bases 433-448
Fusion joint: bases 577-585
ccdB lethal gene ORF: bases 586-888
kan gene: bases 1099-2031
kan promoter: bases 1099-1236
Kanamycin resistance gene ORF: bases 1237-2031
Zeocin resistance ORF: bases 2238-2612
pUC origin: bases 2724-3397

Appendix 3: table of primers

Fragment Synthesis Primers	Sequence 5'-3'	Internal Resriction site
Alpha ex2 fwd NheI	AGCTAGCCCTTCTGCAACTCTTCTTCTG	NheI
Alpha ex2 rev' FRT NheI	CGCTAGCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCCAGGCTTACTGCCATACATG	NheI
Alpha ex2 nest fwd	TGGGGTAGAGGATGCCCACCT	
Alpha ex2 nest Rev1	CAACCTCTTACTGTTCAGCATG	
Alpha ex2 nest Rev2	CAGGCTTACTGCCATACATG	
Alpha SA fwd EcoRI	CGAATTCCCCAACACAAGTTCAGAATAC	EcoRI
Alpha SA rev BamHI	AGGATCCGGAGGTGGGCATCCTCTAC	BamHI
Sbf FRT fwd	ACCTGCAGGGAAGTTCCTATTCTC	SbfI
NotSal loxP rev	GCGGCCGCTTTTGTCGACATAACTTCGTATAATG	NotI SalI
Alpha LA fwd SalI	AGTCGACGTGAGCCACAAGTACTCATG	SalI
Alpha LA rev NotI	AGCGGCCGCCATTTCTCTATAGTCTCTGAGTC	NotI
Nested LA Fwd	CACATAGTTCAGGCTGGCAT	
Nested LA Rev	TACCTCCTGCAGATGCTAAA	
SAprobeUP	GACTGGTATAAACAAAGGCC	
SAprobeDOWN	CCAGATGAGAGGACACAAAC	
LAprobeUP	CATAACCAAGCTGCCAAAGC	
LAprobeDOWN	CAGGATGTCTACTTCAGAAC	
Template DNA		
Sbf FRT loxP Sal	AGGGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCCCAAATAACTTCGTATAGCATACATTATACGAAGTTATGTC	
Seqeuncing Primers		
Alpha exII fwd join	GGCACAGTGTTGATTGATTG	
Alpha exII join	ACCTGCATATTAGCTCTCAG	
Short arm internal for sequencing forward	ATGGTGATAGTGGAGTGGGA	
SA Internal fwd 2	TTATTCCCTTGCTCCCACAG	
SA TK joint	ACCTCTTACCTTTCCGACTTCTGGC	
SA LoxP Joint	AGCGCCTGCTCTGGGCAGATA	
LA beginning join	TGAGGGTCAAAGGAGTTGTA	
LA finish Join	GCTGTACTGTTGGCTAT	
LA End joint	TTTTGTTGGTCTCTGTGAGGCCATT	
LA internal Fwd	CTTCCACAACTACAGGCTTG	
SP6	CATTTAGGTGACACTATAG	
T7	TAATACGACTCACTATAGGG	
Primers for Positive Clone Screening		
AlphaLAfor	CATGAGTACTTGTGGCTCAC	
Alphaint1for	TGCTGGGCACAGTGTTGATTG	
Alphaint1rev	AAAGGCTGGGCTTCAAGCAG	
Exon1revnew	GACATGTATGGCAGTAAGCC	
NeoB	GAAGAACGAGATCAGCAGCC	
NeoC	CTACCGGTGGATGTGGAATG	
Primers for Flippase Genotyping		
Reverse Floxed primer	CCACTGACAAGTGATCCCTA	
Primers for Cre Recombined Genotyping		
Cre For	AGACTGACTCCTCTCTGGGT	
Cre Rev	CATGAGTACTTGTGGCTCAC	

Appendix 4: Sequencing of final construct



Presented above is the completed targeting construct with primers used for sequencing highlighted.

Appendix 5: Sequence of targeted α-synuclein locus also showing Southern hybridisation probes

TGATTTATGCAACAGCATAGGTGAACTAACACATCATGCTGACTGGTATAAACAAAGGCCATATACTCCATGGATATGTACAGAATCAAATAGAATTATAAACATAGTTCAAAGGGATGA
AACATTTCCTTTTATCTTTTGAGATTTCACTCAGGTCAGATAACTGGCCAGACTGTGTGACTGAAGATAATAGAAACCAGACAGTGCTGATGTTAGGAGCAACACCCTGACCAGTACCGCT
TAGTTTTGCATGCAATGAGTGTTCTAGATATTGAAATAGTCTCTCTTTAAAATGGTATGCTATCACTTGGACTTTTTCAAAATCTGCAGACACAAAATCAGAGCAGTTCACTCTATAAACTA
TAATTCAATGTAGAATATCATTTGATGCCATCCTGGGTATTTCAGTCATTCTCACATTTATTAATGTGTGCTAGAATGTTCCCAGATGGAAAAACATGAAAAGCTTAAATCTCTAGAAGGA
GAGAAGTCGATAGTGACAGAGTAGCCATGCTGAAGGCACAGAATGATGCTTGTGGAAGCTGGTGATATTTATGTAGGAATCTTAGTCTCACAACTGTAAATATGTTTAAATGTTTTACAT
TCTAAAATTTTAGAGGAGAGGTGTCATCTCAATTCACTTTCTCTTCTATAATAGAAAAAAAAAAAACCTGGCTAAATAGAACATAACTTGGTAAAGTTCTGAGAGGCAGAAAACCAACGC
CCAGACGCAACCAAAACAGGCCTGGCAAAACATTATCCCGAGGAAACGTTTGTGTCCTCTCATCTGGCTTTAGACTATTGACAAATAGACCCCAAGAAATTGGAAGTCCTCCAGGAATTT
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GTACTTTTATACTTTTTTTGAGAGATTCCAGCTTCCCCCCAAAAAAGCCCATCTCAGTTTCTCTTGCTCTGGGTCTTTGTTAAATGACATCTTCCTTGCAATGCCTAATTTATTTAAAGTTGGA
ACCATTCTCACCCATGAAAACCATAACCTTTCTATTCTAATTTCTTCTTGTTTGATAAAGTGTCATTGCATTTAAAATAAATTAAATAATCTACTTGTTTTGAGTATGTTATTTTTCTTTGTCTA
TGTAGGCACTATCATAATGTAAATATTTATTTTGCTTGTTGATACTTCATGTGTCTAGGCAAGTTCCTAACTACAAATTCAGTAATGAATAAGAGCTTATTAAGGATCGAAAGAATGGATA
AATGACAATTTTCTAAGGATTAATAATCATATACATGGTGTAAAACCTTTGGCTATTGACTGATCCAAAAGTTGTAATCAAATGGGTTCTGAAGTAGACATCCTGAAACACAAAAGAAAG
ATACTTTCACCTGTGGGCAGACT

Short Arm Southern Hybridisation Probe

Short Arm

LoxP

Exon II

FRT

Neomycin Cassette

Long Arm

Long Arm Southern Hybridisation Probe

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