Alpha-synuclein in the neurodegenerative mechanisms of Parkinson's disease and dementia with Lewy bodies

Ruth Elizabeth Joy Musgrove

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RM (candidate) (60%), AK (15%), TD (25%)

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We the undersigned agree with the stated, "proportion of work undertaken" for each of the above, published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed: _____ _____

Assoc/Prof. Tracey Dickson
Menzies Research Institute Tasmania
University of Tasmania

Prof. Alison Venn
Acting Director
Menzies Research Institute Tasmania

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Abstract

Parkinson's disease (PD) and Dementia with Lewy bodies (DLB) are characterised by Lewy body pathology and the degeneration of midbrain-dopaminergic and cortical neurons, respectively. The mechanisms underlying the selective degeneration of these neuronal populations are not known. Gene mutations associated with PD and DLB have highlighted the role of oxidative stress and mitochondrial dysfunction in the pathogenesis of these conditions. Substitution mutations in the *SNCA* gene encoding alpha-synuclein are the most common cause of familial disease and alpha-synuclein also comprises the principal component of Lewy bodies. However, the contribution of this protein to neurodegeneration is uncertain. The intent of this thesis was to further clarify the role of wild type and mutant alpha-synuclein in the pathogenic mechanisms of PD and DLB.

In vitro models of oxidative stress and mitochondrial dysfunction were applied to primary neuronal cultures derived from wild type and *SNCA* null rodent models. Alpha-synuclein expression was correlated to neuronal health, free radical production, mitochondrial function and metabolism. Results within this thesis demonstrate that wild-type alpha-synuclein protects both cortical and dopaminergic neurons from oxidative stress. This response is linked to an increase in its cytoplasmic expression within subgroups of these neuronal populations. Alpha-synuclein expression did not affect free radical production but conferred neuroprotection against caspase-dependent apoptosis. This effect was mediated through the mitogen activated protein kinase (MAPK) signalling pathway. Data within this thesis also supports a role for alpha-synuclein in facilitating neuronal energy production through oxidative phosphorylation.

In contrast to wild-type protein, expression of mutant (A35T) alpha-synuclein increased neuronal susceptibility to oxidative toxicity. However, this mutation was not associated with induction of a specific apoptotic pathway. Rather mutant (A53T) alpha-synuclein was linked to sensitisation of neurons through a toxic gain of function which was independent of mitochondrial free-radical production or calcium buffering.

In summary, the studies within this thesis have clarified the contribution of alpha-synuclein to normal neuronal function and the mechanisms of PD and DLB. The results have highlighted complexities surrounding the contentious role of alpha-synuclein in both neuroprotection and toxicity. Based on these findings, a sound hypothesis for the role of this protein in the pathogenesis of PD and DLB has been proposed. Significantly, this two-hit hypothesis validates past studies, which have detailed a role of alpha-synuclein in both neuroprotection through chaperone activity, and in neurotoxicity through a toxic gain of function mechanism. This thesis will provide a basis and direction for further investigations into the relationship between intracellular alpha-synuclein levels, and the selective nature of neurodegeneration in PD and DLB.

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Abbreviations

AD	Autosomal dominate
Akt	Protein kinase B
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid
APAF1	Apoptotic protease activating factor 1
AR	Autosomal recessive
ASK1	Apoptosis signal regulating kinase 1
ATP	Adenosine triphosphare
Bad	B-cell lymphoma (BCL) associated death promoter
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotropic factor
BH3	Bcl-2 homology domain 3
BSA	Bovine serum albumin
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
CO_2	Carbon dioxide
CSP-alpha	Cysteine-string protein alpha
DAT	Dopamine transporter
DHR	Dihydrorhodamine-1,2,3
DIC	Differential interference contrast
DIV	Days *in vitro*
DJ-1	Daisuke-Junko-1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DLB	Dementia with Lewy Bodies
Elk-1	E twenty-six (ETS)-like transcription factor 1
ETC	Electron transport chain
ER	Endoplasmic reticulum
ERK	Extracellular-signal regulated kinase
GABA	Gamma aminobutyric acid
GBA	β-Glucocerebrosidase
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibillary acidic protein
GPe	Globus pallidus pars externa
GSH	Glutathione
GSSG	Glutathione disulfide
GTP	Guanosine-5'-triphosphate
GT1-7	Gonadotropin releasing hormone (GT1-7) neuronal cell line
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	Hydrogen peroxide
H_2O	Water
HO^-	Hydroxide ion
$HO^\cdot$	Hydroxyl radical

HRP	Horseradish peroxidase
IM	Isolation media
IgG	Immunoglobulin G
IL-2	Interleukin 2
JNK	cJun N-terminal kinase
KRP	Kreb's ringer phosphate
LRRK2	Leucine-rich repeat kinase 2
LSM	Laser scanning microscopy
MAPT	Microtubule associated protein tau
MAP2	Microtubule associated protein 2
MAPK	Mitogen-activated protein kinase
Mm	Mouse monoclonal
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP+	1-methyl-4-phenylpyridinium
mtDNA	Mitochondrial DNA
NAC (domain)	Non-amyloid component
nACh	Nicotinic acetylcholine receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NDP	Nigrostriatal dopaminergic pathway
NF-M	Neurofilament medium
NMR	Nuclear magnetic resonance
$O_2 \cdot^-$	Superoxide anion
O_2	Oxygen
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PD	Parkinson's disease
PFA	Paraformaldehyde
Pfkfb3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PI	Propidium iodide
PINK1	Phosphatase and tensin (PTEN)-induced putative kinase 1
PI3K	Phosphatidylinositol 3-kinases
PLS-DA	Partial least squares - discriminant analysis
Ppm	parts per million
PRKN	Parkin gene
PSD	Paired stimulus depression
P-value	Probability value
p38	p38 Mitogen activated protein kinases
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
qPCR	Quantitative polymerase chain reaction
Rab	Ras analogue in brain
RFU	Relative fluorescent units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rp	Rabbit polyclonal
mRNA	Messenger ribonucleic acid

SEM	Standard error of the mean
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
SNCA	Alpha-synuclein gene
SOD	Superoxide dismutase
Sp	Sheep polyclonal
STN	Subthalamic nuclei
TCA	Tricarboxylic acid
TH	Tyrosine hydroxylase
VGAT	Vesicular GABA transporter
VGLUT	Vesicular glutamate transporter
WT	Wild-type
^{1}H NMR	Proton nuclear magnetic resonance
6-OHDA	6-hydroxydopamine
$\Delta\Psi_m$	Mitochondrial transmembrane potential

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1 Introduction

1.1 Parkinson's disease and dementia with Lewy bodies

Parkinson's disease (PD) is a progressive neurodegenerative disease first characterised by James Parkinson in 1817. It is clinically defined as a movement disorder, but is often accompanied by cognitive decline (Wüllner et al., 2007; Park and Stacy, 2009; Goldstein et al., 2010). Motor dysfunction in PD is caused by the selective degeneration of dopaminergic neurons in the substantia nigra (SN). Clinical characteristics include resting tremor, slowness of movement (bradykinesia). Loss of movement (akinesia) and postural instability may also develop as pathology progresses (Reichmann, 2010). A loss of olfaction (hyposmia) often precedes motor dysfunction (Wattendorf et al., 2009; Politis et al., 2010) and is attributed to defects in cholinergic circuits (Bohnen et al., 2010). Other non-motor symptoms can develop in late stage disease and include dementia, hallucinations, sleep disturbances, autonomic dysfunction and depression (Wüllner et al., 2007; Park and Stacy, 2009; Politis et al., 2010). These symptoms associate with proteinaceous intraneural inclusions called Lewy bodies (Gómez-Tortosa et al., 2000; Valls-Solé and Valldeoriola, 2002).

The extent of Lewy body pathology correlates with PD development, initially affecting brainstem and subcortical nuclei and then progressing into the neocortex (Braak et al., 2006). The primary site of pathology differentiates PD from Dementia with Lewy bodies (DLB), as the later is associated with early development of Lewy bodies in the cortex (Iseki, 2004; Neef and Walling, 2006). The involvement of these lesions in

neurodegeneration remains unclear, despite strong correlations between their presence and clinical disease progression (Lees, 2009).

Due to a lack of specific and accessible biomarkers, PD cannot be definitively diagnosed until Lewy bodies are identified in the SN at autopsy. However, a significant proportion of the aged population have asymptomatic Lewy body pathology upon post-mortem examination (Lippa et al., 2007). Clinical and post-mortem diagnosis are further complicated by concomitant neurodegenerative conditions (Lippa et al., 2007; Morgan et al., 2010), and ill-defined boundaries between PD and DLB pathology (Linazasoro, 2007). Furthermore, several clinicians have proposed that these conditions exist within a single disease entity and stem from a common pathogenic mechanism (Richard et al., 2002; Aarsland et al., 2004; Emre et al., 2007; Linazasoro, 2007; Goldmann Gross et al., 2008; Aarsland et al., 2009; Lees, 2009). This view remains contentious however, and the defined clinical outcome of each disease reflects differences in the affected neuronal populations (Richard et al., 2002). Despite continuing debate, PD and DLB are now recognised as multi-system disorders, characterised by sequential and progressive neurodegeneration of cortical and mesencephalic neurons (Linazasoro, 2007).

1.1.1 The Nigrostriatal Dopaminergic Pathway

The motor symptoms associated with PD are a direct consequence of decreased dopamine secretion in the Nigrostriatal Dopaminergic Pathway (NDP), caused by selective degeneration of dopaminergic neurons in the SN. The SN innervates the NDP, which is composed of a circuit of connections through the basal ganglia and thalamus, and include

the corpus striatum, the nucleus accumbens, the ventral striatum and the ventral pallidum. The corpus striatum is further divided into the neostriatum (consisting of the caudate nucleus and the putamen) and the globus pallidus. (Albin, 2006; Lees, 2009; Lees et al., 2009). A diagrammatic representation of the basal ganglia is given in Figure 1.1. The putamen has the most significant role in mediating motor function and is the area most affected by dopamine loss in PD (Crossman, 2000). The striatum is innervated by efferent signals from the cortex and dopaminergic projections from the substantia nigra pars compacta (SNpc), the ventral tegmental area and the retrorubal area (Smith, 2000). In turn, efferent circuits from the striatum innervate the thalamus through direct and indirect striatopallidal pathways, which are diagrammatically represented in Figure 1.2.

Disease-related neurodegeneration decreases dopaminergic input into the dorsolateral putamen and basal ganglia causing an imbalance in the relative activities of the direct and indirect striatopallidal pathways. As a result, output from the putamen is reduced and the excitatory activity of the direct pathway is consequently decreased (Marsden and Obeso, 1994; Crossman, 2000; Herrero et al., 2002). The resulting lack of activity of the ventral anterior and ventral lateral thalamic nuclei leads to hypoactivity of the motor cortex and corticospinal tract and consequent inhibition of cortically initiated motor function. These deficits are the direct cause of the motor symptoms of PD, namely akinesia and bradykinesia (Marsden and Obeso, 1994; Crossman, 2000; Galvan and Wichmann, 2008; Reichmann, 2010). The lack of dopamine release from the nigrostriatal pathway prevents activation of neurons within the globus pallidus pars externa (GPe), resulting in dis-

inhibition of the STN and hypoactivity of the indirect striatopallidal pathway (Crossman, 2000; Galvan and Wichmann, 2008; Reichmann, 2010).

1.1.2 Neurobiological basis of non-motor symptoms of PD and DLB

The pathology of non-motor symptoms in both PD and DLB correlates with the anatomical localisation of Lewy bodies. Specifically, sympathetic denervation of the heart and gastrointestional tract are associated with Lewy body pathology in sympathetic ganglia and the dorsal nucleus of the vagus nerve, respectively (Del Tredici et al., 2002; Orimo et al., 2005; Fujishiro et al., 2008). Psychological symptoms are attributed to deficiencies in noradrenaline and serotonin, which correlate with Lewy body pathology in the locus ceruleus and raphe (Cheng et al., 1991; Richard et al., 2005). Cholinergic deficits are well documented in PD and DLB and contribute to dementia (Dickson et al., 2009). However, Lewy body pathology remains the most significant correlate of cognitive decline (Maidment et al., 2006; Dickson et al., 2009).

Lewy bodies are eosinophilic intraneural lesions comprised of multiple proteins and lipids. They were identified by Frederic Lewy in 1912 (Shults, 2006) and their presence within neurons is a pathological characteristic of PD, DLB and neurodegeneration with brain iron accumulation (Hallervorden-Spatz syndrome) (Arawaka et al., 1998; Spillantini et al., 1998; Hardy et al., 2006). These inclusions are usually found within soma but can be localised to the neurites (Lewy neurites) (Shults, 2006).

Lewy bodies are classified into two major morphological subtypes, namely 'classical' and 'atypical cortical' Lewy bodies Figure 1.3. Classical Lewy bodies are localised to the SN and locus coerelus and comprise a dense granular core surrounded by a fibrillar corona (Figure 1.3A) (Goedert, 1999; Shults, 2006). Atypical cortical Lewy bodies are localised to the cortical brain regions (Kosaka et al., 1984), they appear to be entirely composed of fibrillar proteins and are of a uniform density (Figure 1.3B) (Spillantini et al., 1997; Spillantini et al., 1998; Wakabayashi et al., 2007). Lewy bodies are composed of a variety of proteins and lipids, although alpha-synuclein comprises the main constituant of protein fibrils (Figure 1.3C) (Spillantini et al., 1998).

The second major protein component of Lewy bodies is ubiquitin, which associates with proteins within the fibrilar region and the granular core (Shults, 2006). Some differences in the protein composition of classical and atypical cortical Lewy bodies have been described (Wakabayashi et al., 2007). Lewy bodies are present prior to overt cell death (Braak et al., 2006), although their causal role in neurodegeneration is undefined.

1.1.3 Neuropathological staging of PD and DLB

Braak and colleagues (2006) defined six stages of PD progression according to the extent of Lewy body pathology and dopaminergic degeneration. The first two stages of disease are associated with Lewy body development within the vagal nerve of the medulla oblongata, the caudate nucleus, the intermediate reticular zone, the gigantocellular reticular nucleus and the locus coeruleus. These stages do not correlate with symptomatic manifestations of PD (Braak et al., 2006). The SNpc is not effected until stage-three of the

disease (Shults, 2006). Stage-four is marked by the formation of Lewy bodies in the cortex (confined to the temporal mesocortex) and the basal forebrain. There notable loss of melanin-containing dopaminergic neurons of the SN, although clinical symptoms do not manifest until stage-five of the disease when Lewy body pathology advances through the mesocortex. Pathology then begins to affect the neocortex and primary sensory and motor fields of the cerebral cortex (stage-six) (Braak et al., 2006). The SN and the locus coeruleus have the highest concentration of Lewy bodies in post mortem tissue (Mezey et al., 1998). Despite strong acceptance of Braak staging, several recent post-mortem studies have shown differing patterns of Lewy body progression (Burke et al., 2008; Jellinger, 2008).

The staging of pathology in DLB is less understood. There are three pathological subtypes of the disease, which are determined by the localisation of Lewy body pathology (McKeith et al., 1996). Type-1 DLB primarily affects the subcortical and brainstem regions. Type-2 DLB describes concurrent brainstem and cortical pathology. Type-3 pathology is specifically localised to the cortex, initially in the amygdala then progressing through the limbic regions and the neocortex (McKeith et al., 1996; Yamamoto et al., 2005).

Although Lewy body pathology correlates with clinical signs of disease there is little evidence to suggest that it is the primary cause of cell loss. Post-mortem studies have identified Lewy body pathology in the surviving neurons of the SNpc, indicating that these inclusion bodies do not cause acute cell death (Samii et al., 2004). There is some evidence that Lewy body pathology and neurodegeneration are caused by distinct pathogenic

mechanisms. Specifically, mutations to the *PRKN* gene cause a Parkinsonism syndrome, which pathologically resembles PD without inclusion bodies, suggesting a lack of association between these processes. Tompkins and Hill (1997), directly compared dopaminergic neurons with and without Lewy bodies and indicate inverse correlation between these inclusions and propensity to undergo apoptosis (Tompkins and Hill, 1997). Contrary to these findings, a gene expression profile study conducted by Lu and colleagues (2005) indicates that genes associated with cell survival were expressed at a higher level in neurons lacking Lewy bodies (Lu et al., 2005).

1.1.4 Current treatment

Current therapies can aid in controlling the motor symptoms of PD but do not reverse cell loss or prevent further degeneration. Dopamine replacement therapy (levodopa) is the first line of drug treatment for PD patients (Antonini, 2010; Poewe et al., 2010). However, the symptomatic benefits of levodopa decrease over time and fluctuations in motor response occur in the majority of patients after three to five years of treatment. The severity of these fluctuations correlates with the duration of therapy (Abbruzzese, 2008). Levodopa is often used concomitantly with second line therapies such as monoamine oxidase-B inhibitors, dopamine receptor agonists and catechol-*o*-methyl transferase inhibitors. These agents act to augment the effects of levodopa (Singh et al., 2007).

Non-pharmacological therapies are recognised as potential methods in PD treatment. Deep brain stimulation of the subthalamic nucleus is a well established technique of improving motor function (Kopell et al., 2006), although, its use is appropriate for just 5-10% of

people with PD due to the strict patient criteria (Pereira and Aziz, 2006). Foetal mesencephalic graft transplantation is an alternative therapy, which offers potential restoration of the NDP and reversal of motor symptoms. Recent clinical trials indicate that these grafts can improve motor function (Olanow et al., 2009), although the success of these grafts is limited and post-mortem analysis indicates Lewy body pathology in dopaminergic neurons of the graft tissue (Snyder and Olanow, 2005; Li et al., 2010). Further development of this technique is hindered by current legal and ethical limitations (Singh et al., 2007). Gene therapy is also undergoing clinical trial in PD patients and some vectors show early indications of success (Feng and Maguire-Zeiss, 2010; Marks et al., 2010).

1.1.5 Aetiology

Age is the greatest risk factor associated with PD (Albin, 2006; Fahn, 2010). PD affects approximately 2% of the male population and 1.3% of females over 60 years of age. The incidence increases exponentially past this age to affect up to 5% of the total population over 85 years (de Rijk et al., 1995; Rao et al., 2006). Females are less susceptible owing to the neuroprotective properties of oestrogen. This female sex hormone stimulates the production of brain-derived neurotrophic factor (BDNF), which facilitates differentiation and survival of dopaminergic neurons (Nishio et al., 1998; Cyr et al., 2002; Sohrabji and Lewis, 2006). BDNF levels are reportedly decreased within the SN in PD patients (Nishio et al., 1998; Mogi et al., 1999; Parain et al., 1999; Howells et al., 2000).

The underlying cause of PD and DLB is often attributed to an 'interplay between genetic and environmental factors' (Horowitz and Greenamyre, 2010), although 90% of cases have no known genetic cause (Vance et al., 2010). It is thought that behavioural factors, specifically high consumption of iron, manganese, lutein, cholesterol and saturated fats, as well as incidences of head trauma, contribute to idiopathic disease pathogenesis (Johnson et al., 1999; Bower et al., 2003; Goldman et al., 2006; Powers et al., 2009). However, these risk factors do not consistently correlate with disease across independent studies (Powers et al., 2009; Spangenberg et al., 2009) and in most incidences the evidence for environmental contributors is weak (Hardy, 2006). Discrepancies within the segregation of PD among identical twins suggest that environmental factors do contribute to disease pathogenesis (Tanner et al., 1999). However, these findings are equally representative of a self-propagating disease such as a prion disease (Hardy, 2006). A 'prion-like phenomenon' is currently proposed as an alternative hypothesis for the pathogenesis of neurodegenerative conditions associated with protein aggregation, including PD and DLB (Desplats et al., 2009; Olanow and Prusiner, 2009; Angot et al., 2010; Goedert et al., 2010).

There is a strong inverse relationship between cigarette smoking and PD, with regular smokers having up to a 50% decreased risk of developing the disease (Tanner et al., 2002). Nicotine acts directly on nicotinic acetylcholine (nACh) receptors on dopaminergic neurons of the nigrostriatal pathway. This binding interaction stimulates the release of dopamine, increasing dopaminergic innervation of striatal neurons and relief of PD symptoms (Khwaja et al., 2007; Quik et al., 2007; Quik et al., 2009).

Clinical manifestations of PD have been linked to exposure to the common pesticides rotenone and paraquat (Langston and Ballard, 1983; Giasson and Lee, 2000; Mccormack, 2002). These agents cause toxicity through induction of mitochondrial and cytosolic free-radical production, respectively (Richardson et al., 2005; Ramachandiran et al., 2007). Administration of either paraquat or rotenone directly into the NDP of rodents causes selective degeneration of nigrostriatal dopaminergic neurons (Somayajulu-Nițu et al., 2009; Pan-Montojo et al., 2010). Rotenone toxicity is also linked to the formation of intracellular inclusions which biochemically resemble Lewy bodies (Betarbet et al., 2000).

There is a strong correlation between familial PD and symptomatic onset prior to the age of fifty years (Fahn, 2010). Multiple mutations in two distinct genes (*SNCA* and *LRRK2*; Table 1.1) cause dominant Mendelian inheritance of PD. These familial cases are characterised by both neurodegeneration and Lewy body pathology (Hardy et al., 2006; Shulman et al., 2011). Mutations to three genes (*PRKN*; *PINK1* and *DJ-1*; Table 1.1) are associated with recessive inheritance of PD or Parkinsonism, although the occurrence of Lewy body pathology in these conditions is variable (Shulman et al., 2011). There is a disproportionately high incidence of glucocerebrosidase gene (*GBA*) mutations in sporadic PD patients (Neumann et al., 2009; Sidransky et al., 2009). It remains undetermined whether these incidences represent a strong susceptibility locus or a Mendelian inheritance pattern with incomplete penetration (Shulman et al., 2011). Other susceptibility loci are found within the *MAPT* gene, encoding the microtubule associated tau protein (Pankratz et al., 2009).

Substitution mutations within the *SNCA* gene encoding alpha-synuclein are the most common cause of familial PD and DLB. Four mutations (Ala53Thr (A53T); Ala30Pro (A30P); Glu46Lys (E46K) and Gly209Ala (G204A)), in addition to genomic multiplications of the gene, are associated with dominant disease inheritance (Polymeropoulos et al., 1997; Teive et al., 2001; Seidel et al., 2010). PD caused by mutations to alpha-synuclein has an aggressive phenotype characterised by extensive Lewy body pathology and early age of onset (Polymeropoulos et al., 1997; Spira et al., 2001). The Glu46Lys mutation segregates with a PD phenotype particularly associated with dementia (Zarranz et al., 2004).

1.2 Synucleins

1.2.1 Protein properties

Alpha-synuclein belongs to a family of synuclein proteins (alpha-, beta- and gamma-synuclein) (Maroteaux and Scheller, 1991). Synucleins are biochemically characterised by a homologous N-terminal amphipathic domain containing a consensus motif of KTKEGV, which facilitates their lipid biding properties (Jensen et al., 1995; Lavedan, 1998). There is significant overlap in the cellular and regional localisation of synuclein family mambers. They are all enriched in neural tissue and alpha- and beta- synuclein colocalise at some pre-synaptic nerve terminals (Iwai et al., 1995; Quilty et al., 2003) and there is some evidence of functional redundancy in the family (Chandra et al., 2004).

Single gene knockout mice for alpha-, beta- and gamma-synuclein are viable, although some physiological abnormalities have been described (Abeliovich et al., 2000; Chandra et

al., 2004). *SNCA* null mice have no significant compensatory upregulation of gamma- or beta-synuclein or synaptic proteins (Abeliovich et al., 2000; Chandra et al., 2004). However, alpha- and beta-synuclein double knockout mice show a 50% increase in the level of gamma-synuclein, and 30% increases in both 14-3-3 family proteins and complexin. The significance of this compensatory upregulation remains unclear due to the low basal level of gamma-synuclein expression (Kuhn et al., 2007). There is also evidence that alpha- and beta-synuclein have corresponding roles in regulating dopamine uptake (Chandra et al., 2004).

1.3 Alpha-synuclein

1.3.1 Biochemical characteristics of alpha-synuclein

Alpha-synuclein comprises 0.5-1% of cytosolic protein in human brain homogenate (Iwai et al., 1995). It is an acidic, soluble and heat stable protein, with an observed molecular weight of approximately 19kDa (Maroteaux et al., 1988; Jakes et al., 1994). It is comprised of three distinct domains; an amphipathic N-terminal domain, a hydrophobic non-amyloid component (NAC) domain and an acidic C-terminal domain (Lücking and Brice, 2000). The N-terminal domain has seven imperfect repeats of 11 amino acids, predicted to form five amphipathic helices (Jensen et al., 1995). The NAC is the second major component of amyloid plaques in the brains of Alzheimer's disease patients (Irizarry et al., 1996). A specific stretch of twelve amino acids within this domain enables the protein to form a beta-pleated sheet structure (Giasson et al., 2001).

The biochemical properties of alpha-synuclein predict functions in both membrane and protein binding (Davidson et al., 1998; Uversky and Eliezer, 2009). The protein was thought to lack secondary structure and exist as an unfolded random coil in aqueous solution (Weinreb et al., 1996). This property would facilitate rapid recognition of binding partners and reduces the level of sequence specificity required between binding sites (Bisaglia et al., 2009). However, recently it was shown that physiological alpha-synuclein exists as an alpha-helix, which has high affinity for membrane binding (Bartels et al., 2011) Structural motifs within the C-terminal region resemble binding domains of tubulin/actin binding proteins, calcium/calmodulin interacting proteins and phosphate/kinase inhibitors (Lücking and Brice, 2000). Alpha-synuclein also shares specific regions of structural homology with functional binding domains of the 14-3-3 family of molecular chaperones (Ostrerova et al., 1999).

Alpha-synuclein is highly conserved across species, indicating that its structural characteristics are functionally significant (Clayton and George, 1999). Post-translational modifications may however, influence properties such as protein and membrane binding (Oueslati et al., 2010). Pathological conditions such as oxidative stress increase the phosphorylation (Anderson et al., 2006), tyrosine(tyr)-nitration (Souza et al., 2000a; Takahashi et al., 2007), methionine(met)-oxidation (Uversky et al., 2002), and ubiquitination (Hasegawa et al., 2002; Nonaka et al., 2005) of alpha-synuclein. The functional significance of these post-translational modifications in normal health is unknown, although tyr-nitration hinders its membrane binding properties (Hodara et al., 2004).

Alpha-synuclein has a number of splice variants (Lavedan, 1998). The complete protein transcript is the major isoform and is 140 amino acids in length. Alpha-synuclein isoforms SNCA126 and SNCA112 result from in-frame deletions of exons 3 and 5, respectively. Differential roles of the three isoforms are not well understood and functional studies have focussed on the role of the full length protein (Beyer, 2006). Because of the small variation in size, distinguishing protein levels of each isoform is difficult, although, quantitative polymerase chain reaction (qPCR) studies have shown differential changes in the expression on isoforms SNCA126, SNCA140, SNCA112 and SNCA98 in PD, DLB and Alzheimer's disease patients (Beyer et al., 2008).

1.3.2 Alpha-synuclein localisation and function

Alpha-synuclein is most abundant in regions of the CNS associated with synaptic plasticity; predominantly within the hippocampus, amygdala, cerebral cortex and olfactory bulb. It is also expressed in the thalamus, striatum and brainstem (Iwai et al., 1995; Abeliovich et al., 2000). The regional localisation of alpha-synuclein may reflect its involvement in synaptic function and plasticity. The association between proposed functions of alpha-synuclein and its involvement in neurodegeneration remain unclear.

1.3.2.1 Alpha-synuclein in development

The expression and cellular localisation of alpha-synuclein is dynamically regulated throughout brain development. These alterations correlate with significant points of neuronal migration, maturation and synaptogenesis (Hsu et al., 1998; Murphy et al., 2000;

Quilty et al., 2003; Zhong et al., 2010). In rodent models, alpha-synuclein expression is low in early embryogenesis (prior to E15), but increases in later stages of neuronal development (E18) extending into the postnatal period, (P7) (Hsu et al., 1998). A relatively high level of expression is maintained throughout adulthood, indicative of a function in homeostasis (Hsu et al., 1998). Post-mortem studies in humans have however, shown altered levels of alpha-synuclein expression during aging (Chu and Kordower, 2007).

1.3.2.2 Alpha-synuclein at the synapse

The pre-synaptic localisation of alpha-synuclein suggests an involvement in synaptic function (Maroteaux et al., 1988; George et al., 1995). Early studies identified correlations between increased synaptic expression and critical stages of development, such as periods of learning and synaptic plasticity (Maroteaux et al., 1988; George et al., 1995; Hsu et al., 1998; Murphy et al., 2000). Alpha-synuclein exists in equilibrium between membrane bound and free forms, approximating 15:85% respectively (Lee et al., 2002). The protein preferentially binds with acidic D2 class phospholipid membranes as a class 3 amphipathic alpha-helix (Payton et al., 2004). There is general agreement that the membrane affinity of alpha-synuclein is tightly linked to its function.

Abeliovich et al, (2000), Murphy et al, (2000) and Cabin et al, (2002), were the first to investigate neuronal development and synaptic function in the absence of alpha-synuclein. *SNCA* null mice are viable and have no gross morphological or functional defects during development. Original characterisation of these mice focused on physiological alterations in dopaminergic cells of the nigrostriatal pathways. Abeliovich and colleagues (2000)

report no significant deficits in the morphology, development, or synaptic density within dopaminergic nuclei, or alterations in the release and reuptake of dopamine. However, these authors did report reduced paired stimulus depression of striatal dopaminergic neurons and decreased total dopamine content (Abeliovich et al., 2000).

Subsequent studies of hippocampal neurons from *SNCA* null mice also signify decrease in the number of distal synaptic vesicles docked in the pre-synaptic terminal, both in culture and *in vivo*, (Abeliovich et al., 2000; Murphy et al., 2000; Cabin et al., 2002). These deficits correlated with reductions in synaptic vesicle recycling after repetitive stimulation (Cabin et al., 2002). In contrast, Nemani and colleagues (2010) identified an inverse correlation between increased cellular concentration of alpha-synuclein and synaptic transmission of both glutamate and dopamine in hippocampal and mesencephalic neurons, respectively. However, there are no apparent reductions in the number of synaptic vesicles or the efficiency of neurotransmitter re-uptake through endocytosis, rather the authors suggest that defects in the ability to cluster synaptic vesicles at the synaptic release sites underlie these deficits (Nemani et al., 2010; Surmeier, 2010). Collectively, these data indicate a role for alpha-synuclein in maintaining and clustering the distal pool of synaptic vesicles, although the mechanisms for this association remain unclear.

1.3.2.3 Lipid binding properties of alpha-synuclein

Impaired lipid metabolism is a feature of PD and there is evidence that alpha-synuclein can regulate lipid synthesis and membrane composition. Alpha-synuclein interacts with phospholipid membranes as well as free fatty acids and recent studies have described a

regulatory role for this protein in fatty acid metabolism and synthesis. Golovko and colleagues (2007) report an increase in the docosahexaenoic acid concentration and turnover in brain phospholipids of mice lacking alpha-synuclein (Golovko et al., 2007). Independent studies indicate that *SNCA* null mice have reduced levels of arachidonic acids within their cell membranes, accompanied by increases in the concentrations of non-polar lipids in the brain (Barceló-Coblijn et al., 2007). Specific changes in mitochondrial lipid composition, including reduced levels of phosphatidylglycerol are also reported (Ellis et al., 2005). Mutations to alpha-synuclein alter its phospholipid binding affinities. Specifically the A53T mutation decreases membrane association, whilst the E45K has an increased binding affinity for phospholipids (Bodner et al., 2010).

In addition to its direct effects on lipid metabolism, alpha-synuclein regulates both lipid and protein trafficking to the membrane through interactions with the Ras analogue in brain (Rab) family of GTPases (Cooper et al., 2006). Using a yeast model, Cooper and co-workers (2006) demonstrated that overexpression of alpha-synuclein inhibited trafficking between the endoplasmic reticulum and Golgi. Further investigation by these authors revealed that alpha-synuclein specifically acts to inhibit vesicle docking at the golgi. These results are consistent with independent studies correlating vesicle clustering with localised alpha-synuclein accumulation near the plasma membrane (Gitler et al., 2008).

1.4 Alpha-synuclein and mechanisms of neurodegeneration

Although the role of alpha-synuclein in neurodegeneration remains unknown, the molecular interactions of this protein may mediate the mechanisms of dopaminergic cell

loss. The properties of dopaminergic neurons render them specifically vulnerable to pathogenic mechanisms of PD. These include the metabolic handling of dopamine, the contribution of cell-type specific receptors and the environmental attributes of the SN. Much less is known about the vulnerability of cortical neurons, which degenerate in PD dementia and DLB. Cortical neurons may be equally susceptible to broad pathogenic mechanisms such as mitochondrial dysfunction, oxidative stress, excitotoxicity and protein mishandling. Alpha-synuclein is implicated in dopamine-mediated neurotoxicity as well as mitochondrial and cytosolic causes of neurodegeneration. However, the direct effect of this protein on cell function and health is unclear. The following sections discuss the role of alpha-synuclein in putative mechanisms of neurodegeneration in PD and DLB. Specific emphasis is placed on its role in oxidative stress, proteolysis and mitochondrial function.

1.4.1 Oxidative stress

Oxidative damage is a common consequence of aerobic metabolism. It is mediated by free radicals, which accumulate as by-products of cellular redox reactions. These reactions are essential for energy production, some enzymatic activities and signalling pathways (Patten et al., 2010). The mammalian brain is specifically vulnerable to oxidative stress due to its high-energy requirements and relative lack of antioxidant defence mechanisms (Butterfield, 2006; Halliwell, 2006). Neurons demand vast amounts of ATP to maintain ion status and electrochemical potential despite continual membrane depolarisation, which occurs during action potential propagation and neurosecretion. The respiratory quotient for the brain is effectively one, indicating that most neuronal ATP is synthesised through the oxidative metabolism of glucose. Oxidative phosphorylation is the most efficient pathway

of ATP production and occurs on the electron transport chain (ETC) on the inner mitochondrial membrane. The ETC is dependant on O_2 as the final electron acceptor. To accommodate for its O_2 demand, the brain (constituting 2% of body weight) utilizes 20% of total body O_2 consumption (equivalent to 1.68 μM/g·min) (Siesjo, 1984; Zilberter, 2011).

The chemical properties of O_2 facilitate the formation of highly reactive species. Because the unpaired electrons of O_2 exist in parallel spin states, it is predisposed to univalent reduction and necessitates the production of radical intermediates.

The mitochondrial ETC is the major generator of free radicals in the CNS (Kudin et al., 2005; Halliwell, 2006). The electrochemical potential of the inter-mitochondrial environment facilitates the production of superoxide anion ($O_2{\cdot}^-$) through premature 'leaking' of electrons directly onto O_2. In the CNS, complex I (NADH dehydrogenase) contributes to the majority of mitochondrial $O_2{\cdot}^-$ (Herrero and Barja, 2000). Significantly, complex I impairment has been reported in PD and DLB (Navarro and Boveris, 2009).

Cytosolic production of $O_2{\cdot}^-$ and other reactive oxygen species (ROS) occurs through metal ion catalysed reactions, advanced glycosylation end products and other redox-dependent enzymatic reactions. In catecholaminergic neurons, monoamine neurotransmitters auto-oxidize on exposure to O_2, generating $O_2{\cdot}^-$. As this reaction is catalysed by Fenton chemistry, its rate is dependent on the both the partial pressure of O_2 and the concentration of transition metals.

Cells defend themselves from oxidative damage through enzyme-catalysed conversion of reactive species into inert compounds. The superoxide dismutase (SOD) family of enzymes eliminate $O_2^{\cdot -}$ through alternate oxidation and reduction reactions, yielding H_2O_2 and O_2.

The elimination of H_2O_2 produced by SOD's and other oxidases is largely dependant on the free radical scavenger glutathione (GSH). The family of glutathione peroxidise enzymes require GSH as a cofactor for the enzyme coupled redox reaction of H_2O_2 to water (H_2O). GSH is an abundant tripeptide (Glu-Cys-Gly) localised to the cytoplasm and mitochondria. Its potent antioxidant capacity is attributed to the thiol group of its cysteine residue (Rabinovic and Hastings, 1998). Two GSH molecules are able to reduce H_2O_2 by forming a disulfide bridge between them. This reaction yields oxidised glutathione (GSSH), which is rapidly recycled to GSH by glutathione reductases. Post-mortem tissue form PD patients have reduced levels of total GSH in the SN, accompanied by a reduction in the GSH:GSSG ratio (Fitzmaurice et al., 2003; Zeevalk et al., 2007).

Saturation of antioxidant defences results in the accumulation of reactive species. Cells typically maintain a balance between antioxidants and free radicals in order to limit oxidative damage. However, synthesis of antioxidants is energetically expensive and this equilibrium is rarely perfect. Most aerobic cells therefore experience continual, yet limited, oxidative damage (Halliwell, 2007). Oxidative stress describes an augmented imbalance in the production of oxidative species and their reduction by cellular defence mechanisms.

1.4.1.1 The contribution of dopamine to oxidative stress in PD

There is widespread evidence of increased oxidative damage in the SN of PD patients upon post mortem examination (Wersinger and Sidhu, 2006). Several unique environmental characteristics of the SN further contribute to decreased redox potential. These include neuromelanin, increased iron concentration and the oxidative by-products of dopamine metabolism. The SN is therefore more susceptible to age-related and pathological causes of oxidative stress, including genetic and environmental factors, and has a high basal level of ROS production and oxidative toxicity.

The characteristics of nigral dopaminergic neurons have been extensively studied due to their selective vulnerability in PD. Of specific focus is the potential of dopamine to yield oxidative metabolites. This process occurs through both enzymatic breakdown and auto-oxidation which yield H_2O_2, $O_2^{\cdot -}$ and reactive quinone species. Although H_2O_2 reacts poorly with most biological targets, it is converted into highly reactive hydroxyl radical $HO^{\cdot}$ in the presence of transition metals. This is specifically relevant to pathology within the SN due to its high iron content (Friedman et al., 2009).

Dopaminergic neurons defend themselves from the oxidation of cytosolic dopamine by actively sequestering the neurotransmitter into synaptic vesicles (Staal et al., 2000; Staal et al., 2004; Caudle et al., 2007; Guillot and Miller, 2009). This is mediated by vesicular monoamine transporter 2 (VMAT2), the inhibition of which causes a sustained increase in cytoplasmic dopamine adducts (Fornstedt and Carlsson, 1989). Dopamine is taken up at the synapse by the dopamine transporter (DAT). A9 dopaminergic neurons have a higher

DAT:VMAT2 ratio than ventral tegmental area neurons, which may contribute to their selective vulnerability through cytosolic dopamine-oxidation (Uhl, 1998). Deficiencies in VMAT2 function are associated with PD (Harrington et al., 1996; Sala et al., 2010).

Alpha-synuclein controls cytoplasmic dopamine concentration through regulation of its synthesis, uptake and storage. Overexpression of alpha-synuclein decreases the rate of synaptic dopamine release both *in vitro* and *in vivo* (Yavich et al., 2004; Senior et al., 2008). This effect is likely due to reduced vesicle recycling following endocytosis (Nemani et al., 2010) and impairment of vesicle docking and fusion (Cooper et al., 2006; Gitler et al., 2008). Alpha-synuclein also regulates membrane localisation and function of DAT *in vitro*, although its affect on dopamine uptake is controversial (Gosavi et al., 2002; Wersinger and Sidhu, 2003; Fountaine et al., 2008). Overexpression of monomeric alpha-synuclein can inhibit activity of the DAT (Wersinger et al., 2003; Wersinger and Sidhu, 2003), however, it may also augment dopamine uptake (Lee et al., 2001a). The differential response varies according to cell type, the cytoplasmic concentrations of both dopamine and the DAT, and the membrane localisation of DAT expression (Venda et al.; Lehmensiek et al., 2006; Fountaine and Wade-Martins, 2007). Direct assays of DAT function in *SNCA* null mice show no deficiencies in activity (Wersinger and Sidhu, 2003; Yavich et al., 2004). However, alpha-, beta-synuclein and alpha-, gamma-synuclein double knockout mice show small, yet significant reductions in dopamine uptake (Chandra et al., 2004; Senior et al., 2008). Despite this, decreases in DAT function cannot fully account for the deficiencies in synaptic dopamine uptake.

Alpha-synuclein can modify cytoplasmic dopamine content through mechanisms independent of its direct affects on DAT function. Specifically, this protein inhibits dopamine synthesis by binding to inactive tyrosine hydroxylase (TH) and preventing its phosphorylation into the active form (Perez et al., 2002; Peng et al., 2005). Alpha-synuclein aggregation prevents its inhibition of TH and the DAT and disrupts the membrane integrity of dopamine storage vesicles, allowing the release of dopamine into the cytoplasm (Volles et al., 2001; Volles and Lansbury, 2002; Lashuel and Hirling, 2006). The consequent increase in the cytoplasmic dopamine concentration can lead to accumulation of its oxidative metabolites. However, alpha-synuclein aggregates may also decrease dopamine uptake by reducing the cell surface expression of DAT, secondary to golgi fragmentation (Gosavi et al., 2002).

The dopamine oxidation hypothesis cannot fully account for the staging and extent of neurodegeneration in PD, despite clear of evidence of oxidative stress within nigrostriatal dopaminergic neurons (Ahlskog, 2005). Other inconsistencies for this hypothesis are outlined below: 1) Neurodegeneration begins outside the NDP and is not limited to nuclei containing dopaminergic neurons (Braak et al., 2006; Linazasoro, 2007). 2) Cellular dopamine content does not dictate the pattern of cell death in disease as outlined by Braak and colleagues (2006). 3) Neurons which synthesis other monoamine neurotransmitters, with similar metabolic properties of dopamine, are relatively resistant to PD pathology. 4) Many clinical symptoms, including early deficiencies in olfaction, do not reflect loss of dopaminergic neurons (Dickson et al., 2009). 5) Dopamine replacement therapy does not alleviate all symptoms of PD and there is little evidence to suggest it augments oxidative

stress (Schapira, 2008). Based on these facts it is evident that dopamine is not the sole source of neurotoxicity in PD, although its metabolism may promote disease pathology and contribute to dopaminergic degeneration.

1.4.1.2 Alpha-synuclein and oxidative stress

There are two opposing views on the relationship between oxidative stress and alpha-synuclein. The common view is that oxidative stress is an environmental trigger of the 'toxic' accumulation of the protein. This relationship is well founded as there is strong evidence that chronic exposure to free radicals induces alpha-synuclein aggregation (Hashimoto et al., 1999b; Hashimoto et al., 1999a; Manning-Bog et al., 2002; Betarbet et al., 2005; Fornai et al., 2005). It remains difficult however, to ascertain the role of oxidative stress in the complex biochemistry of alpha-synuclein aggregation. Alpha-synuclein is able to form both oligomeric and fibrillar species, although oligomeric species are more highly associated with cellular toxicity (Conway et al., 2000a; Conway et al., 2000b; Rochet et al., 2000; Li et al., 2001). The production of both oligomers and fibrils is regulated by oxidative modifications to the protein. Specifically, alpha-synuclein can be oxidised by the conversion of four methionine (met) residues into sulfoxides (Uversky et al., 2002). It can also undergo nitration (secondary to oxidative stress) at tyrosine (tyr) residues (Souza et al., 2000b; Takahashi et al., 2002). Both met-oxidation and tyr-nitration of alpha-synuclein inhibit its fibril formation (Uversky et al., 2002; Yamin et al., 2003). However, tyr-nitration induces partial folding of alpha-synuclein and the formation of stable oligomers (Souza et al., 2000b; Yamin et al., 2003). Significantly, these oligomeric

species disrupt membrane integrity and compromises homeostatic regulation of ions (Danzer et al., 2007; van Rooijen et al., 2010b, a)

The alternative view regards the role of monomeric alpha-synuclein in the neuronal response to oxidative stress. Several studies have correlated the expression of alpha-synuclein to oxidative cytotoxicity. Alpha-synuclein augments toxicity induced by rotenone (Orth et al., 2003; Ved et al., 2005), hydrogen peroxide and nitrosative stress (Prasad et al., 2004), and can directly contribute to oxidative stress (Hsu et al., 2000). Specifically, alpha-synuclein is able to generate hydroxyl radicals in metal catalysed reactions (Turnbull et al., 2001; Wang et al., 2010) and can stimulate nitric oxide synthase, thereby promoting free radical production (Adamczyk and Kazmierczak, 2009).

Alpha-synuclein gene silencing attenuates the neurotoxicity of complex I and II respiratory chain inhibitors (MPTP, malonate and 3-nitropropionic acid) in primary dopaminergic neurons (Klivenyi et al., 2006; Fountaine et al., 2008). However, lack of alpha-synuclein does not affect the rate of superoxide production in MPTP treated dopaminergic neurons or neuronal viability following rotenone treatment (Fountaine and Wade-Martins, 2007; Fountaine et al., 2008). These data suggest alpha-synuclein contributes to the neurotoxicity of MPTP via a mechanism independent of complex I function. Its differential association with MPTP, but not rotenone toxicity, further indicates a dopamine-dependant mechanism of alpha-synuclein toxicity in this model.

In contrast to the above data, alpha-synuclein is able to mediate neuroprotection against oxidative mechanisms of neurotoxicity (Hashimoto et al., 2002; Seo et al., 2002; Manning-Bog et al., 2003; Quilty et al., 2006). Endogenous expression of alpha-synuclein is upregulated in primary neurons exposed to chronic oxidative stress, both *in vivo* and *in vitro* (Vila et al., 2000; Manning-Bog et al., 2002; Quilty et al., 2006). These endogenous levels of protein expression mediate neuroprotection against hydrogen peroxide and 6-hydroxydopamine (6-OHDA) (Quilty et al., 2006; Monti et al., 2007). Given that protein expression is both energetically expensive and tightly regulated, these data provide compelling evidence for the involvement of alpha-synuclein in the homeostatic response of neurons to oxidative stress.

Multiple factors are likely to dictate the ability of alpha-synuclein to mediate either toxicity or protection in conditions of oxidative stress. One key determinant is the intracellular concentration of the protein. Multiplication of the *SNCA* gene is linked to familial PD. In these rare cases the symptomatic age of onset correlates with gene dosage and alpha-synuclein load (Singleton et al., 2003; Chartier-Harlin et al., 2004; Ibanez et al., 2004; Ross et al., 2008). A dose dependant induction of protein aggregation is thought to contribute to its toxicity (Cookson, 2006; Kim and Lee, 2008).

1.4.1.3 Concentration dependent protein aggregation

The cytosolic concentration of alpha-synuclein is synergistically controlled by its transcription, metabolic breakdown and compartmental localisation. In the mature brain transcription of alpha-synuclein is independent of a TATA promoter (Liang and Carr,

2006). Rather, its expression is induced through mitogen-activated protein kinase (MAPK) pathways (Clough and Stefanis, 2007), indicating a regulation by stress stimuli, growth factors, or inflammatory cytokines. It has a long half-life, suggesting a homeostatic function dependent on steady-state expression (Kim and Lee, 2008). *In vivo* upregulation of alpha-synuclein is reported in response to various neuronal stress models, including oxidative stress (Manning-Bog et al., 2002), mitochondrial inhibition (George et al.; Vila et al., 2000), chronic cocaine abuse (Mash et al., 2003; Qin et al., 2005), alcoholism (Bonsch et al., 2005) and age (Chu and Kordower, 2007). These data support a role for stress stimuli in the induction of alpha-synuclein upregulation.

Alpha-synuclein aggregates are derived from its partially folded intermediates (Figure 1.4) (Uversky et al., 2001). Although unstable, increasing concentration of these intermediates allows self-association into a stable dimeric conformation, which mediate oligomeric fibril formation (Uversky et al., 2001; Uversky, 2007; Kim and Lee, 2008). The concentration of partially folded intermediates is therefore critical in alpha-synuclein aggregation (Kim and Lee, 2008) and central to the formation of Lewy bodies (Conway et al., 2000a). Alpha-synuclein exists in steady-state equilibrium between native protein, partially folded intermediates and unfolded polypeptides (Figure 1.4) (Uversky et al., 2001; Uversky, 2007).

Post-mortem studies of human tissue report decreased *SNCA* mRNA in degenerating neuron populations (Kingsbury et al., 2004). These authors identify a 50% reduction in alpha-synuclein mRNA at the cellular level. Comparatively spared brain regions, including

the occipital cortex and granule layer of the cerebellum, had relatively high levels of *SNCA* mRNA. However, conflicting evidence indicates that elevated alpha-synuclein expression is specifically localised to brain regions affected by PD pathology (Chiba-Falek et al., 2006). Although high levels of expression are specific to surviving neurons of the SN (Grundemann et al., 2008), indicating its expression is associated with cell health. The major limitation of these studies is the selective use of tissue from symptomatic PD patients, often in final stages of the disease. For this reason, changes in the expression of alpha-synuclein in early or pre-symptomatic stages of disease remain undetermined.

1.4.2 Dysfunction of protein degradation

Equally important to the concept of concentration dependent toxicity, is the role of protein degradation. Alpha-synuclein catabolism is mediated by both the ubiquitin proteasome system (UPS) and autophagy (Bennett et al., 1999; Webb et al., 2003; Cuervo et al., 2004). Dysfunction of these systems is implicated in the pathogenesis of neurodegenerative diseases including PD and DLB, among others (McNaught and Jenner, 2001; McNaught et al., 2002b; McNaught et al., 2003; Ravikumar and Rubinsztein, 2004, 2006).

The UPS maintains cellular homeostasis through the degradation of short-lived cytoplasmic proteins (Rock et al., 1994; Sherman and Goldberg, 2001). It has a major function in acutely controlling levels of regulatory proteins and is the primary means for eliminating mutant, misfolded and oxidatively damaged proteins (Sherman and Goldberg, 2001). Protein accumulation following saturation or impairment of the UPS may therefore

catalyse concentration-dependant aggregation of damaged or misfolded proteins. Significantly, pharmacological inhibition of the UPS causes an accumulation of alpha-synuclein aggregates in primary mesencephalic neurons (McNaught et al., 2002; Rideout et al., 2005) and PC12 cells (Rideout et al., 2001). Post-mortem tissue of PD patients provides direct evidence of UPS dysfunction in the SN. McNaught and colleagues (2001) specifically report decreases in the expression of proteasomal subunits in nigral neurons, but not cortical or striatal tissue (McNaught and Jenner, 2001). These data may indicate a role for UPS impairment in the selective vulnerability of nigral neurons (Betarbet et al., 2005). However, they may equally signify that proteasome dysfunction is secondary to PD pathology.

Lewy bodies provide direct evidence that mechanisms of protein catabolism are impaired in PD and DLB. The presence of proteasomal subunits within Lewy bodies suggests an involvement of alpha-synuclein in UPS dysfunction (Ii et al., 1997; Wakabayashi et al., 2007). This is supported by studies linking alpha-synuclein overexpression with impaired proteasome activity, an effect that is potentiated by mutant or aggregated alpha-synuclein (Stefanis et al., 2001; Snyder et al., 2003). The antagonistic affect of alpha-synuclein on proteasome function is likely to be conferred through an interaction with the S6′ subunit of the 19S proteasome cap (Snyder et al., 2003). In addition to proposed involvement of UPS dysfunction in Lewy body formation, inhibition of this system is directly associated with neurodegeneration (McNaught et al., 2004). Significantly, cell death following systemic exposure to proteasome inhibitors preferentially affects neurons of the SNpc locus coeruleus, dorsal motor nucleus of the vagus and the nucleus basalis of Meynert,

suggesting involvement in PD. Proteasome inhibition is additionally associated with the formation of alpha-synuclein and ubiquitin positive eosinophilic inclusions (Rideout and Stefanis, 2002; Rideout et al., 2005).

1.4.2.1 Autophagy

In contrast to the UPS, autophagy is the major mechanism for the degradation of long-lived cytoplasmic proteins and is the only mechanism of organelle recycling. It is specifically involved in the catabolism of large membrane proteins and protein complexes, which are physically unable to be degraded within the narrow barrel of the proteosome (Pan et al., 2008). For this reason, autophagy may be critical in the degradation of aberrant proteins aggregates, which characterise multiple neurodegenerative diseases. In the CNS, autophagy impairment causes neurodegeneration with intraneural accumulation of polyubiquitnated proteins (Hara et al., 2006; Komatsu et al., 2006; Massey et al., 2006). Alpha-synuclein is catabolized, at least in part, by chaperone-mediated autophagy (CMA) (Webb et al., 2003; Cuervo et al., 2004). Suppression of autophagy may therefore be a mechanism of both neurodegeneration and Lewy body formation in PD and DLB (Cuervo et al., 2004). There is some evidence that mutant alpha-synuclein inhibits CMA as it is poorly translocated to the lysosomal lumen and accumulates on the membrane surface. Consequently, the clearance of other CMA substrates is suppressed through steric hindrance of their translocation (Cuervo et al., 2004).

1.4.2.2 The unfolded protein response

The unfolded protein response (UPR) is a universal cellular mechanism that is activated by the accumulation of unfolded proteins within the lumen of the Endoplasmic Reticulum (ER). The major function of the UPR is to restore homeostasis, by temporarily halting protein translation and increasing the concentration of molecular chaperones in order to facilitate protein folding. When these functions do not successfully restore correct protein folding the UPR initiates host defence mechanisms and in long term ER stress can activate apoptotic pathways. The UPR is shown to be active in both PD and DLB and may be initiated by pathogenic mechanisms of these diseases, including oxidative stress (Holtz et al 2006). The phosphorylation of alpha-synuclein at Ser-129, as well as its accumulation in the ER causes activation of the UPR (Sugeno et al., 2008). Alternatively, the stress-induced upregulation of alpha-synuclein may indicate a homeostatic function in the UPR, consistent with its putative role as a molecular chaperone (Souza et al., 2000a).

1.4.3 Mitochondrial dysfunction

Neurons have limited glycolytic capacity and depend on mitochondria for ATP synthesis through oxidative phosphorylation. This series of redox reactions occurs on the inner mitochondrial membrane and involves the transfer of electrons down the ETC. Oxidative phosphorylation is driven by an electrochemical gradient across the inner-mitochondrial membrane ($\Delta\Psi_m$), which is maintained by the transport of protons out of the mitochondrial matrix by complex' I, III and IV of the ETC. Mitochondria also regulate cellular Ca^{2+}

homeostasis and are the major endogenous generators of ROS (Kudin et al., 2005; Chan et al., 2009). Excitable tissues, such as neurons and muscle, are particularly susceptibility to mitochondrial dysfunction due to their high energy demand (Morais and De Strooper, 2010). Impaired mitochondrial function disrupts homeostatic mechanisms causing energy crisis, oxidative stress and calcium influx.

Impaired ETC activity is implicated in PD and DLB, specifically there are reports of decreased complex I activity in both the SN and frontal cortex in sporadic PD and DLB (Parker et al., 1989; Parker et al., 2008; Navarro and Boveris, 2009). Keeney and colleagues (2006) have further identified oxidative damage of complex I in PD, which may contribute to its disassembly and functional impairment (Keeney *et al.*, 2006). Several studies also show decreased function of complex' I, II, III and IV in muscle, platelets and lymphocytes (Bindoff et al., 1991; Mann et al., 1992; Cardellach et al., 1993; Blin et al., 1994), although these findings remain contentious (Martin et al., 1996).

Selective inhibition of complex I was first associated with Parkinsonism in pethidine (Demerol) and heroin addicts, through contamination of synthetic heroin with MPTP (Langston and Ballard, 1983). MPTP is oxidised to its active metabolite MPP+ by monoamine oxidase B and selectively taken into cholinergic neurons by the dopamine transporter (DAT) (Chiba et al., 1984; Javitch et al., 1985). Its toxic effect is primarily mediated through inhibition of the electron and proton transfer functions of complex I. Deficiencies in these functions cause both free-radical production and energy depletion. However, MPP+ treatment also results in massive release of cytoplasmic dopamine, which

contributes to oxidative toxicity (Rollema et al., 1986). Neuronal vulnerability to MPP+ correlates with DAT density and mimics the progression of dopaminergic neuronal loss in early PD (Sanghera et al., 1997; Manning-Bog et al., 2007).

Recent studies have confirmed the involvement of mitochondria in PD. Rotenone-induced complex I inhibition reproduces the anatomical stages of cell loss during the disease (Pan-Montojo et al., 2010). Furthermore, cybrid cell lines containing cytoplasm from PD patients have altered mitochondrial morphology and impaired complex I activity (Trimmer et al., 2000; Veech et al., 2000). These results indicate that mitochondrial dysfunction is independent of the nuclear genome and may suggest a role for age-dependant mitochondrial DNA mutations in disease pathogenesis. The involvement of mitochondria may, however, be specific to neurodegeneration and independent of Lewy body formation. Specifically, proteins involved in mitochondrial function are genetically linked to Parkinsonism independent of Lewy body formation (Table 1.1).

1.4.3.1 Genetics and mitochondrial dysfunction in PD

Several genes linked to recessive Parkinsonism encode proteins, which function in mitochondrial activity. Of these, *PTEN-induced kinase 1*, (PINK1) and *PRKN* are known to have direct associations with the mitochondria. Loss of function of either protein is associated with mitochondrial dysfunction (Greene et al., 2003; Clark et al., 2006; Yang et al., 2006). Single and double gene knockout studies in drosophila provide compelling evidence that PINK1 and parkin are involved in the same biochemical pathways (Clark et al., 2006; Park et al., 2006). This is supported by the indistinguishable phenotype of PD

patients carrying either mutation (Ibanez et al., 2006). Gene silencing or mutation of either *PINK1* or *PRKN* sensitises neurons to complex I inhibition and oxidative stress (Casarejos et al., 2006; Wood-Kaczmar et al., 2008; Grunewald et al., 2010). Furthermore, deficiencies in ETC activity are reported in human PD patients with mutations to either protein (Muftuoglu et al., 2004). It is therefore likely that these proteins are involved in energy production through oxidative phosphorylation. This effect may be mediated by the ability of parkin to regulate the transcription of respiratory chain complexes transcribed from mtDNA through association with mitochondrial transcription factor A (Kuroda et al., 2006).

Daisuke-Junko-1 (DJ-1) is a redox sensitive molecular chaperone that is involved in the neuronal response to oxidative stress (Shendelman et al., 2004; Meulener et al., 2006). Mutations to *DJ-1* are a cause of early onset PD (Bonifati et al., 2003). Loss of DJ-1 function sensitises neurons to cytosolic and mitochondrial ROS production (Meulener et al., 2005; Meulener et al., 2006), as well as MPP+ toxicity in dopaminergic neurons (Kim et al., 2005). These studies indicate that the activity of DJ-1 is not specific to differential cellular compartments. Hao and colleagues have recently identified a direct role of DJ-1 in mitochondrial function and ATP production. Overexpression of this protein overwrites the phenotypic toxicity of PINK1-deletion in drosophila (Hao et al., 2010). These results indicate that DJ-1 plays an imperative role in mediating the response to cytosolic ROS production, as well as mitochondrial function.

1.4.3.2 Alpha-synuclein in mitochondrial function and dysfunction

Alpha-synuclein may regulate mitochondrial function through both direct and indirect interactions with these organelles. Mutant alpha-synuclein expression is a direct cause of mitochondrial dysfunction. Specifically, transgenic expression of the A53T mutation causes mitochondrial defects including morphological abnormalities and mtDNA mutations (Martin et al., 2006). Mutant (A53T) alpha-synuclein further disrupts multiple protein interactions involved in mitochondrial metabolic pathways (Xun et al., 2008). Mice expressing the A30P alpha-synuclein mutation accumulate oxidative damage to specific proteins involved in mitochondrial energy production (Poon et al., 2005). Both the A53T and A30P mutations increase oxidative stress (Giasson et al., 2000) and may therefore impair mitochondrial function through mtDNA damage and protein modification.

Overexpression of wild-type alpha-synuclein induces both mitochondrial damage and oxidative stress through unknown mechanisms (Hsu et al., 2000). These data may provide mechanistic insight into the neuroprotective effect of alpha-synuclein depletion against MPTP, malonate and 3-nitropropionic acid (Klivenyi et al., 2006). In addition to the involvement of mitochondrial activity in neurodegeneration, inhibition of complex' I and IV induce alpha-synuclein expression and aggregation (Betarbet et al., 2000; Lee et al., 2001b), suggesting a role in Lewy body formation. Direct administration of rotenone, which acts through inhibition of NADH oxidoreductase activity, into the NDP of rodents reproduces both neurodegeneration and inclusion body formation associated with PD. However, chronic MPTP administration does not induce Lewy body pathology in non-human primate models (Halliday et al., 2009). Further investigation is therefore required to

understand the relationship between mitochondrial function and alpha-synuclein pathology in disease pathogenesis.

Direct interactions between wild-type alpha-synuclein and mitochondria have been described (Devi et al., 2008; Zhang et al., 2008; Liu et al., 2009). The N-terminal region of alpha-synuclein contains an unidentified mitochondrial targeting sequence (Devi et al., 2008; Liu et al., 2009). It localises primarily to the inner and outer mitochondrial membrane *in vitro* and *in vivo* (Li et al., 2007; Devi et al., 2008). However, this association is specific to select brain regions, which include the striatum, thalamus, olfactory bulb and hippocampus (Liu *et al.*, 2009). There is no evidence of mitochondrial localisation of alpha-synuclein in the cortex, despite its high level of cytosolic expression (Liu *et al.*, 2009). The import of alpha-synuclein into the mitochondria is associated with a dose-dependant loss of complex I activity, presumably mediated by a direct interaction with halo complex I (Devi et al., 2008; Liu et al., 2009). Alpha-synuclein may be translocated to the mitochondria in response to oxidative and metabolic stress, as well as acidic intracellular environment (Cole et al., 2008). This could result from N-terminal post-translational modifications, which increase its mitochondrial targeting. Mutant (A53T) alpha-synuclein has a faster rate of mitochondrial import than wild-type protein (Devi *et al.*, 2008), further correlating this process with pathogenesis.

Contrary to reports of its involvement in mitochondrial toxicity, alpha-synuclein may maintain homeostatic functions of ETC complexes involved in oxidative phosphorylation. Alpha-synuclein null mice have a decreased linked-activity of complex I/III (Ellis et al.,

2005) and NADH cytochrome c reductase (Devi *et al.*, 2008). Ellis and colleagues (2005) further report a decrease in the biosynthetic precursor of cardiolipin in *SNCA* null mice. Cardiolipin is an inner-membrane protein that has multiple roles in maintaining the physical association between complexes of the ETC, as well as the electrochemical gradient across the membrane (Chicco and Sparagna, 2007). Alpha-synuclein is thought to regulate the production or chaperone mediated transport of cardiolipin precursors in the cytoplasm (Ellis et al., 2005). However, its effect on cardiolipin activity may be secondary to oxidative stress. Cardiolipin is susceptible to loss of function by peroxidation due to the abundance of double bonds in its tertiary structure and its physical proximity to the ETC (Chicco and Sparagna, 2007). Therefore, a reduction in ROS production by alpha-synuclein, may indirectly promote ETC function through cardiolipin.

1.5 Summary and aims

The mechanisms underlying selective neurodegeneration in PD and DLB remain unclear. Genetic mutations associated with these conditions highlight the role of oxidative stress and mitochondrial dysfunction in disease pathogenesis. Mutations to alpha-synuclein account for the majority of familial cases, although the contribution of this protein to cell loss is undefined. This thesis aimed to investigate the role of alpha-synuclein in mitochondrial function, free radical production and the neuronal response to oxidative stress.

These studies follow previous reports that alpha-synuclein mediates neuroprotection against oxidative stress (Quilty et al., 2006). It was therefore hypothesised that the

differential regulation of alpha-synuclein expression among neuronal subpopulations indirectly contributes to selective vulnerability to oxidative stress. This hypothesis was addressed in the following aims:

Aim 1 – Compare the effect of alpha-synuclein expression on the vulnerability of primary dopaminergic and cortical neurons exposed to an *in vitro* model of chronic oxidative stress.

PD is characterised by the degeneration of dopaminergic neurons in the SN (Lees et al., 2009). The selective vulnerability of these neurons is attributed to the metabolism of dopamine, which can increase cytosolic free radical production (Jenner, 2003). Alpha-synuclein increases cytoplasmic dopamine in neuronal-derived cell lines, thereby subjecting them to additional oxidative toxicity (Guo et al., 2008; Bisaglia et al., 2010). In contrast, alpha-synuclein has been shown to protect primary cortical neurons from oxidative stress (Quilty et al., 2006). This thesis therefore aimed to compare the effect of endogenous alpha-synuclein expression in dopaminergic and cortical neurons exposed to an *in vitro* model of chronic oxidative stress.

Aim 2 - Establish the mechanism by which endogenous alpha-synuclein modifies neuronal vulnerability to oxidative stress and mitochondrial dysfunction.

Oxidative stress and mitochondrial dysfunction are genetically implicated in the pathogenesis of PD and DLB, and this association is supported by experimental evidence. Overexpression of alpha-synuclein compromises mitochondrial function through direct

inhibitory binding to complex I (Devi et al., 2008), and is associated with an increase in free radical production. However, the role of endogenous levels of alpha-synuclein in these processes remains undefined. This thesis examined the affect of endogenous alpha-synuclein expression on free radical production, antioxidant capacity and apoptosis. The aim of these investigations was to determine the mechanism by which endogenous alpha-synuclein confers neuroprotection.

Aim 3 - Determine the effect of endogenous alpha-synuclein on neuronal metabolism.

Biochemical, behavioural and morphological analysis of alpha-synuclein knockout models has identified a number of functions for this protein. However, these studies have failed to provide significant insight into the contribution of this protein to disease-related neurodegeneration. The current thesis employed a metabolite screening approach to further characterise this unknown role. Metabolic flux profiles were compared between alpha-synuclein knockout and wild-type (C57BL6) mice using primary neuronal cultures. This investigation was not hypothesis-driven; rather the aim was to employ specific techniques to identify novel functions of this protein.

Aim 4 - Define the molecular mechanism by which mutant (A53T) alpha-synuclein modifies neuronal vulnerability to oxidative stress.

The A53T alpha-synuclein is linked to familial PD, and current studies link this mutation to a toxic gain of function (Cuervo et al., 2004). To determine the mechanism by which

mutant alpha-synuclein confers neuroprotection this thesis examined its effect on free radical production, and mitochondrial function.

2 Materials and methods

2.1 Solutions

Chemicals used for the following solutions were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.1.1 Genotyping solutions

0.5 M EDTA **1 L**

- EDTA 146.12 g
- 4 N NaOH
- EDTA was dissolved in Milli-Q with stirring. NaOH was used to adjust pH to 8.0, to aid solubility.

50 X TAE Buffer (Tris-Acetate-EDTA) **1 L**

- Tris base 242 g
- Acetic Acid (Spectrum, Gardena, CA, USA) 57.10 mL
- 0.5 M EDTA 100 mL
- Solution was made to 1 L with Milli-Q, pH was adjusted to 8.5 with 4 N NaOH.

1 X TAE Buffer

1:50 dilution of 50 X TAE buffer in Milli-Q.

Agarose gel 2% (w/v) **100 mL**

- Agarose (Bioline Alexandria NSW, AUS) 2 g
- 1X TAE buffer 100 mL

2.1.2 Cell culture solutions

0.01 M Phosphate buffered saline (PBS)	**1 L**
• 9% (w/v) NaCl	100 mL
• 200 mM Na_2HPO_4 (28.3 g/L)	40 mL
• 226 mM $NaH_2PO_42H_2O$ (27.1 g/L)	10 mL
• Milli-Q	850 mL

Poly-$_L$-lysine solution

- poly-$_L$-lysine solution (0.01% v/w in H_2O; catalogue number P8920) was diluted 1 in 10 in sterile 0.01M PBS at pH 7.0.

4% Paraformaldehyde, 4% sucrose	**1 L**
• paraformaldehyde (PFA)	40 g
• sucrose	40 g
• 200 mM Na_2HPO_4 (28.3 g/L)	400 mL
• 120 mM NaH_2PO_42 (31.2 g/L)	100 mL
• Milli-Q	500 mL

- Milli-Q was heated to 80^{o}C in a microwave and transferred to fume hood where the PFA and sucrose were added with stirring. pH was adjusted to 8.0 with 4 N sodium hydroxide (NaOH) to aid solubility. Once dissolved, the solution was filtered through Whattman filter paper into sodium phosphate buffer (100 mL $NaH_2PO_42H_2O$; 400 mL 200 mM Na_2HPO_4). pH was adjusted to 7.4. Solution was stored at 4ºC for up to one week, or frozen at -20ºC and thawed directly before use.

2.1.3 Mitochondrial solutions

Mitochondrial Isolation Medium (IM)	**1 L**
• 255 mM Sucrose	38.25 g
• 75 mM Mannitol	23.96 g
• 5 mM HEPES	1.19 g
• 1 mM EGTA	0.38 g

• Buffer components were dissolved in Milli-Q and pH was adjusted to 7.2 with 4 N NaOH.

Krebs Ringer Phosphate (KRP)	**100 mL**
• 145 mM NaCl	847.40 mg
• 5.7 mM Na_2PO_4	93.37 mg
• 4.86 mM KCl	36.23 mg
• 0.54 mM $CaCl_2$	5.99 mg
• 1.22 mM $MgSO_4$	14.68 mg
• 5.5 mM Glucose	99.08 mg
• 50 μM HEPES	1.19 g

• Buffer components were dissolved in Milli-Q to a total volume of 100 mL, with stirring. pH was adjusted to 7.4.

2.2 Animals

Hooded-Wistar rats were obtained from the Central Animal House of University of Tasmania. Rats were housed at 20ºC, on a 12 hour light/dark cycle with free access to food and water.

Alpha-synuclein knockout (*Snca* -/-) mice were obtained from Jackson Laboratory (Sacramento, California, USA), and maintained as a homozygous colony. C57Bl/6 mice were purchased from the STEPS Animal facility at the University of Tasmania. Transgenic mice overexpressing mutant (A53T) alpha-synuclein were purchased from Jackson Laboratory. Positive males (A53T alpha-synuclein expressing) were bred with negative females to obtain both hemizygous-positive and transgene (A53T alpha-synuclein) negative embryos, which were used for cell culture. Experiments on adult mice were performed on hemizygous A53T alpha-synuclein positive males, and results were compared with A53T alpha-synuclein negative littermates. Mice were housed in microisolator cages on a 12 hour light/dark cycle with free access to food and water. All animal experiments were approved by the Ethics Committee (Animal Experimentation) of the University of Tasmania (approval number A11952, for the principal investigator Associate Professor Tracey Dickson).

2.3 Genotyping

2.3.1 DNA extraction

Mice were genotyped at the time of weaning (28 days post-natal) from a small end-portion of the tail, which was removed and stored at -20^{o}C until DNA extraction. Cultured embryos were genotyped from liver tissue, which was removed immediately after decapitation and stored at -20^{o}C until DNA extraction. A REDExtract-n-amp tissue kit (Sigma-Aldrich) was used to extract DNA from liver and tail samples according to the manufacturers recommendations.

2.3.2 Polymerase chain reaction (PCR)

Snca -/- mice were genotyped during initial breeding and maintained as a homozygous colony. Primers to the wild-type *SNCA* gene (SNCA tm1Rosl), and region flanking the deleted gene region (SNCA tm1Rosl neo generic) were used as detailed below:

SNCA tm1Rosl neo generic (oIMR0013) (5'- CTTgggTggAgAggCTATTC -3')

SNCA tm1Rosl neo generic (oIMR0014) (5'- AggTgAgATgACAggAgATC -3')

SNCA tm1Rosl (oIMR1286) (5' - ggCgACgTgAAggAgCCAgggA -3')

SNCA tm1Rosl (oIMR1287) (5'- CAgCgAAAggAAAgCCgAgTgATgTACT -3')

The PCR products for the SNCA tm1Rosl neo generic was 280 base-pairs (bp), and for the wild-type gene SNCA tm1Rosl was 320 bp. A single band at 280 bp therefore signified a homozygous knockout.

All mutant (A53T) alpha-synuclein transgenic mice were genotyped for the *SNCA* A53T transgene with a PCR product of 248 bp. *IL-2* was used as an internal control and had a PCR product of 324 bp.

The primer sequences were as follows:

IL-2 (oIMR0042) (5' - CTAggCCACAgAATTgAAAgATCT-3')

IL-2 (oIMR0043) (5' - gTAggTggAAATTCTAgCATCATCC-3')

SNCA (oIMR1772) (5' - TgTAggCTCCAAAACCAAgg -3')

SNCA (oIMR3560) (5' - TgTCAggATCCACAggCATA -3').

DNA was amplified by polymerase chain reaction (PCR) in an Eppendorf Mastercycler gradient (North Ryde, NSW, AUS), according to reaction conditions outlined below:

Initialisation 94°C 3 mins

Denaturation	94°C 30 secs	35 cycles
Annealing	63°C 30 secs	
Elongation	72°C 45 secs	
Final elongation	72°C 2 mins	
Hold	11°C <24 hours	

2.3.3 Gel electrophoresis

Gel electrophoresis (2% agarose; 110 mV, 25 mins) was used to separate PCR product. DNA bands were stained with Sybr safe (Invitrogen Life Technologies; Carlsbad, CA, USA), and detected on a ChemiDOC XRS (BioRad; Gladesville, NSW, AUS). The size of PCR products was estimated in comparison to a 100 bp ladder (NewEngland Biolabs, Ipswich, MA, USA).

2.4 Mouse cortical neuron culture

2.4.1 Substrate preparation

Dissociated neurons were plated onto #1 (130 µm thick) 12 mm glass coverslips (Marienfeld, Lauda-Königshofen, GER). Prior to use, coverslips were etched in 70% Nitric acid (BDH Chemicals, Poole, Dorset, UK) for four hours, rinsed three times in Milli-Q, heat sterilized and placed in 24-well tissue-culture-treated trays (Iwaki, Crown Scientific, Minto, NSW, AUS). Glass coverslips were coated with 0.001% poly-$_L$-lysine (Sigma-Aldrich) for 24 hours unless otherwise stated. Prior to culture, substrate was removed and coverslips were dried in a biosafety cabinet. Initial plating media (0.5 mL), consisting of Neurobasal TM (Appendix A), with 2% B-27 supplement (Appendix B), 10% (v/v) fetal calf serum (heat inactivated for one hour at 60^{o}C), 0.5 mM $_L$-glutamine, 25 µM glutamate

and 1% antibiotic–antimycotic (Gibco/Invitrogen Life Technologies, Carlsbad, CA, USA) was added to each well. Culture trays were incubated at 37°C; 5%CO_2 for a minimum of one hour prior to the addition of cells.

2.4.2 Tissue dissection

Primary dissociated cortical cell cultures were prepared from embryonic (E14) C57Bl/6, mutant (A53T) alpha-synuclein transgenic and *Snca-/-* mice. The mother was sacrificed by CO_2 affixation (Flow rate 3 L/min) the placentas were immediately removed and placed on ice. The embryos were decapitated in a biosafety cabinet. Cortical tissue was isolated with the aid of a light microscope and placed in Hanks-Balanced-Salt-Solution (HBSS; Gibco). Tissue was digested with 0.025% trypsin (Gibco) in 0.01 M sterile PBS for three mins. Cortical neurons were plated at 2.5×10^4 cells per well in 24-well tissue culture trays (Iwaki) in initial plating media. At 24 hours, the media was replaced with 1 mL of growth media containing Neurobasal TM, 2% B-27 supplement, 0.5 mM glutamine and 1% antibiotic–antimycotic. Cultures were incubated 37°C, 5% CO_2 for up to 15 days *in vitro* (DIV).

2.5 Culture stress models

Oxidative stress and excitotoxicity were induced in mouse cortical neurons grown on a substrate of poly-$_L$-lysine at 15 DIV. Mild oxidative stress was modelled by the application of antioxidant-free growth media consisting of Neurobasal TM with 2% antioxidant-free B-27 (A0-B27) supplement (Appendix C), 25 µM glutamate and 1% streptomycin/penicillin (Gibco). Mitochondrial-free-radical production was induced by inhibition of the rotenone sensitive NADH-reductase activity of complex I of the

mitochondrial electron transport chain. Rotenone was added to growth media to final concentrations of 5, 10, 15 and 20 nM. Cell cultures were imaged or fixed at three and six hours. Excitotoxicity was by the addition of 25 μM glutamate diluted in fresh growth media for six hours.

2.6 Immunocytochemistry

2.6.1 Cell fixation

Cultures were fixed in 4% PFA/ 4% sucrose for 25 mins on an orbital mixer at room temperature. After fixation, coverslips were washed three times in 0.01 M PBS for 10 mins. Fixed cultures were stored in 0.01 M PBS at 4^{o}C until required, or in 0.01 M PBS with 0.02% (w/v) sodium azide (NaN3; Sigma-Aldrich) if stored longer than three days.

2.6.2 Immunocytochemistry

After fixation cell membranes were permeabilised in 0.3% (v/v) triton-X-100 (Fluka/ Sigma-Aldrich) in 0.01 M PBS. Primary antibodies were diluted in the triton-X-100/ PBS solution and incubated at room temperature for two hours on an orbital mixer, then at 4^{o}C overnight. Cultures were washed three times in 0.01 M PBS (10 mins, room temperature) to remove non-bound antibody. The specifications and concentrations of commonly used antibodies are given in Table 2.1. Concentrations of other antibodies are given in respective chapters. All antibodies were IgG.

Primary antibodies were fluorescently tagged with species and isotype specific Alexafluor fluorescent secondary antibodies (Moleculer probes/ Invitrogen Life Technologies,

Carlsbad, CA, USA) as listed in Table 2.2. Secondary antibodies were applied in 0.01 M PBS to a final concentration of 1:1000 and incubated in the dark for two hours on an orbital mixer (room temperature). Nuclear yellow (Hoechst S769121, 0.0001% w/v in 0.01 M PBS) was added for the final 15 mins of the secondary antibody incubation. Cells were washed three times in 0.01 M PBS, for 10 mins, and once in Milli-Q (room-temperature and in the dark). Coverslips were mounted onto glass microscope slides using fluorescent mounting medium (Dako, Glostrup, DNK).

2.7 Cell viability assays

Changes in cell viability were determined by the percentage of propidium iodide labeled neurons and compared to non-treated controls from the same culture. Propidium iodide (Sigma-Aldrich) was reconstituted in growth media and used at a final concentration of 1 μg/mL (1.4 μM). Dihydrorhodamine (50 μM; Moleculer probes) was used as a marker of viable neurons in live cultures (Figure 2.1). A minimum of 100 neurons were counted per treatment.

2.8 Microscopy and image analysis

2.8.1 Microscopy

Live cell counts were performed on a Leica DMLB2 fluorescent microscope with a X40 water immersion lens objective (Leica Biosystems, Melbourne, VIC, AUS), and fixed fluorescence was viewed with X20, X40 and X100 objectives. Images were captured with an Optronics Magnifire CCD camera (Meyer Instruments, Houston, TX, USA). Laser Scanning Microscopy (LSM) was performed on a Zeiss LSM 510 confocal microscope

(X63) with ZEN imaging software (Carl Zeiss Inc, Thornwood, NY, USA).

2.8.2 Image analysis

Exposure time and aperture were kept constant for images where comparisons in fluorescent intensity were made. Optical density thresholds were set using ImageJ software (National Institute of Health; Bethesda, Meryland, USA), and were kept constant within culture sets. Two distinct thresholds were set for alpha-synuclein immunofluorescence as previously described (Quilty et al., 2006). These thresholds distinguished subpopulations of neurons expressing 'low' and 'high' levels of alpha-synuclein, based on the intensity of fluorescent immunolabelling. The 'low' threshold was set at 50% of the intensity of the 'high' intensity threshold, based on an 8-bit image (Figure 2.2).

2.9 Mitochondrial isolation

Mitochondria were isolated from adult mice according to the methods of (Kristian, 2010) with some modifications. Mice were sacrificed with pentibarbitone (180 mg/kg, Illium Troy Laboratories, Glendenning, NSW, AUS) and perfused transcardially with 25 mL Isolation Medium (IM, section 2.1.3). Brains were placed in ice-cold IM and the cerebellum was removed. The remaining brain was homogenized in ice cold IM (10% w/v), with eight strokes of a glass dunce homogenizer. Homogenate was centrifuged at 1300 *g* for three mins at 4^{o}C. The supernatant was removed and kept on ice. The pellet was resuspended in 5 mL IM and centrifuged at 1300 *g* for three mins at 4^{o}C. The supernatant was combined with that obtained from the previous step, and centrifuged at 21000 *g* for 10 mins at 4^{o}C. The pellet was resuspended in 15% percoll (GE healthcare, Björkgatan,

Uppsala, SWE) in IM (v/v). This suspension was layered onto a percoll density gradient of 1.5 mL 40%, and 3.7 mL 24% percoll in IM (v/v), and centrifuged at 37500 *g* for eight mins at 4°C, with slow acceleration and deceleration. The synaptosomal fraction was isolated from the interface between the 15% percoll and the 24% percoll layers. Somatic mitochondria were isolated from the interface between the 24% percoll and the 40% percoll layers. Synaptosomes were incubated on ice with with 0.1% digitonin (Biosynth AG, Gstaad, CHE) in IM to disrupt membranes. Both the somatic and synaptic mitochondrial preparations were centrifuged in IM at 16700 *g* for 10 mins at 4°C. Supernatant was carefully removed and the pellet resuspended in 1 mg/mL BSA in IM. Mitochondria were centrifuged at 6900 *g* for 10 mins at 4°C. The pellet was resuspended in 100-200 µL of IM. Isolated mitochondria were centrifuged briefly to pellet, and suspended in 100-200 µL of Krebs-ringer phosphate (KRP) unless otherwise stated.

Mitochondrial protein concentration was determined using a Bradford Assay (Bio-Rad reagent) with concentration measured against a BSA standard constituted in KRP buffer (or alternative buffer corresponding to that used for mitochondrial suspension), according to the manufacturers instructions.

2.10 Estimation of mitochondrial H_2O_2

Mitochondrial free-radical production was determined according to the methods described by (Whiteman et al., 2009). Mitochondrial H_2O_2 production was measured in isolated mitochondria using horseradish peroxidase (HRP) and Amplex ultraRED reagent (both from Molecular Probes). The reaction was initiated by the addition of 50 µg mitochondria

to a solution of 0.1 units/mL HRP and 50 μM Aplex ultraRED, to a total volume of 200 μL in KRP. Results were compared with a standard curve constructed from known H_2O_2 concentrations and with a linearity of less than 2 μM. To control for background fluorescence, KRP was added in place of mitochondria. Fluorescence was read on a dual monochromatic plate reader at 15 sec intervals for 30 mins (excitation 568/ emission 581 nm). The linear phase of increase in absorbance was used to calculate the rate of H_2O_2 production per mg of mitochondrial protein and calculated as relative fluorescent units (RFU). Mitochondrial sample H_2O_2 production was calculated as total RFU minus background RFU, and expressed as pmol H_2O_2/mg protein/30min. All measures were taken in triplicate and statistical differences between groups were determined using the student's t-test.

2.11 Statistical analysis

Microsoft Excel (Mac Os X) was used for statistical analysis and to generate graphical representations of data, with the exception of statistical tests relating to data in chapter five. Statistical analyses of data obtained from cultures were from at least four separate culture repeats. Averages and means are reported as ± standard error of the mean (SEM). Comparison of statistical significance between groups and treatment conditions was determined using the student's t-test, unless otherwise stated. Probability values (P-values) less than 0.05 were considered statistically significant.

3 Neuroprotective upregulation of endogenous alpha-Synuclein precedes ubiquitination in cultured dopaminergic neurons

3.1 Introduction

The metabolic properties of dopaminergic neurons render them specifically vulnerable to oxidative stress and may contribute to their selective degeneration in PD (Chapter 1.4.1.1; Przedborski, 2005) Recent studies have indicated that alpha-synuclein can potentiate dopamine oxidation (Bisaglia et al., 2010) through modulation of dopamine uptake (Lee et al., 2001a; Wersinger and Sidhu, 2003). However, relatively little is known about factors contributing to the degeneration of cortical neurons in DLB. The current chapter aimed to compare the role of endogenous alpha-synuclein in the degeneration of dopaminergic and cortical neurons exposed to an *in vitro* model of chronic oxidative stress.

Quilty and colleagues (2006) correlated oxidative stress-induced upregulation of alpha-synuclein with neuroprotection (Quilty et al., 2006). However, conflicting genetic and experimental data indicate a dose-related toxicity of wild-type protein, which is attributed to the tendency for alpha-synuclein to self-aggregate at high concentrations (Outeiro and Lindquist, 2003; Singleton et al., 2003; Chartier-Harlin et al., 2004; Kim and Lee, 2008). From these data it was proposed that endogenous upregulation of alpha-synuclein is an innate neuroprotective response. However, additional increases in the intracellular concentration may drive the neurotoxic aggregation of this protein and contribute to cell death. It was further hypothesised that interactions between dopamine and alpha-synuclein potentiate oxidative dopamine metabolism and sensitise dopaminergic neurons to oxidative toxicity.

3.2 Experimental procedures

3.2.1 Primary rat cortical culture

Primary dissociated cortical cell cultures were prepared from embryonic (E18) Hooded Wistar rats as described by (Dickson et al., 2000) following similar protocol as mouse neurons (Chapter 2.4). Four culture repeats were used for each experiment. Cortical neurons were plated at a density of 4.5×10^4 cells per 12 mm round coverslip in plating media as described for mouse cortical cultures in chapter 2.4.2. The plating media was replaced with 1 mL of growth media at one *day in vitro* (DIV). Cultures were grown to 21 DIV at which time they are considered relatively mature due to the absence of developmentally specific proteins and the expression of markers associated with maturity, such as synaptic markers and the neurofilament triplet proteins (Dickson et al., 2000; Haas et al., 2004; King et al., 2006). Growth media was replenished every 3–4 days, up to 21 DIV.

3.2.2 Primary rat mesencephalic culture

Primary mesencephalic cultures were prepared from embryonic (E15) Hooded Wistar rats using modifications to published methods (Engele, 1998; Feng et al., 1999). Briefly, the ventral mesencephalon was dissected, and washed in HEPES buffer. Tissue was mechanically dissociated, without trypsin. Neurons were plated at a density of 2.0×10^5 cells per 12 mm round coverslip. Neurotrophic factors, brain derived neurotrophic factor (BDNF; 2.5 ng/ml) and glial derived neurotrophic factor (GDNF; 1 ng/ml; both from Chemicon, USA) were added to media at 24 hours *in vitro*, as previously described (Engele, 1998). Half the growth media was replaced every 3–4 days, without subsequent

addition of neurotrophic factors.

3.2.3 Oxidative stress and proteasome inhibition

Cortical and mesencephalic cultures were subjected to an *in vitro* model of chronic oxidative stress as previously described (Quilty et al., 2006). Briefly, at 11 DIV media was replaced with 1 mL growth media containing antioxidant-free B-27 supplement. Non-treated cultures were also subjected to a complete media change. Cultures were maintained in antioxidant-free media to 21 DIV, with half the media replaced every 3-4 days. Acute oxidative insult was modeled by the addition of 20 μM hydrogen peroxide (H_2O_2; Sigma-Aldrich) to the growth media six hours prior to fixation. All cultures were fixed (Chapter 2.6.1) at 21 DIV. To determine the ability of neurons to recover from chronic oxidative stress, a subset of cortical cultures were treated with antioxidant free media at 7 DIV. This media was replaced with standard growth media at 17 DIV and cultures were maintained to 21 DIV. The 20S proteasomal subunit was inhibited with lactacystin (Clabiochem, Damstatd, GER), which was added to culture media at a concentration of 10 μM for 48 hours prior to fixation.

3.2.4 Immunocytochemistry, microscopy and data analysis

Immunocytochemistry was performed as described in chapter 2.6 using previously characterised antibodies (Table 2.1). For quantification of alpha-synuclein expression, a minimum of one hundred TH (mesencephalic cultures) or MAP2 (cortical cultures) immunopositive neurons were imaged from at least five randomly selected fields of view per culture, for each time point and antibody combination. Images for quantification were

acquired with a Leica (DMLB2) fluorescent microscope and analysed as described in section 2.8. Statistical significance was determined with the student's *t*-test.

3.3 Results

3.3.1 Alpha-synuclein is upregulated in dopaminergic neurons in response to chronic oxidative stress

The effect of alpha-synuclein expression was determined in primary dopaminergic neurons exposed to an *in vitro* model of chronic oxidative stress. Dopaminergic neurons were identified with immunolabelling to TH (Engele, 1998) (NOTE: these neurons will be termed 'dopaminergic' from this point onwards). At 21 DIV (no treatment), 7.2% (± 4) of the mesencephalic-derived neurons were dopaminergic; alpha-synuclein immunoreactivity was detected in a subset (39.9% ± 4) of these neurons. There was variation in the level of alpha-synuclein expression between individual cells, and neurons were subclassed as expressing either high or low levels of the protein, based on the fluorescence intensity (Chapter 2.8; Quilty et al., 2006). A high level of alpha-synuclein immunolabelling was detected in 2.8% (± 3) of dopaminergic neurons and this subclass was distinguished by a 53.6% (± 10) increase in the mean fluorescent intensity of alpha-synuclein labelling ($F_0/\mu m^{-1}$) (Figure 3.1A, 3.2A).

Acute and chronic models of oxidative stress were used to determine the effect of oxidative toxicity on the expression of alpha-synuclein. Acute exposure to hydrogen peroxide did not significantly ($P>0.05$) alter the proportion of dopaminergic neurons that expressed high levels of alpha-synuclein, 3.1% (± 3) (Figure 3.2A). However, ten days chronic exposure to antioxidant free media was associated with an ($P<0.05$) upregulation of alpha-synuclein expression (Figures 3.1A,B, 3.2A). There was a 3-4 fold increase (8.9% ± 3) in the proportion of dopaminergic neurons expressing high levels of alpha-synuclein following

10 days exposure to antioxidant-free media (Figures 3.1, 3.2A). Chronic oxidative stress did not alter ($P>0.05$) the proportion of alpha-synuclein positive neurons at 21 DIV (6.1% ± 5), compared with age-matched controls (7.2% ± 4).

3.3.2 High alpha-synuclein expression in dopaminergic neurons is associated with neuroprotection

The relationship between alpha-synuclein expression and cell health was determined with an additional hydrogen peroxide challenge. Nuclear morphology and chromatin fragmentation was used as an indicator of early apoptosis (Wyllie et al., 1980; Roy and Sapolsky, 2003; Quilty et al., 2006). Chronic exposure to antioxidant free media did not ($P>0.05$) affect the viability of dopaminergic neurons. However, additional acute hydrogen peroxide treatment caused a 73.2% (± 4), increase in the proportion of dopaminergic neurons with apoptotic nuclear morphology. Dopaminergic neurons expressing high levels of alpha-synuclein were 18.1% (± 6) less likely ($P<0.05$) to have undergone apoptosis after exposure to chronic oxidative stress and subsequent hydrogen peroxide treatment (Figures 3.2B; 3.3). Those expressing low levels of the protein also showed an 8.2% (± 3) decrease in apoptotic phenotype following additional oxidative toxicity, when compared to alpha-synuclein negative neurons.

Acute hydrogen peroxide treatment alone was associated with a 15.5% (± 3) decrease ($P<0.05$) in the viability of dopaminergic neurons. However, apoptotic nuclear morphology did not significantly correlate to the differential levels of alpha-synuclein expression following acute oxidative stress. Furthermore, this treatment did not ($P>0.05$) alter the

proportion of dopaminergic neurons expressing high levels of alpha-synuclein, 3.1% (± 3) (Figure 3.2A).

3.3.3 Dopaminergic and cortical neurons do not significantly differ in their response to chronic oxidative stress

There is increasing interest in the contribution of dopamine to alpha-synuclein toxicity and its involvement in the pathogenesis of PD (Xu et al., 2002; Zhou et al., 2006; Bisaglia et al., 2010). The metabolic attributes of dopaminergic neurons render them selectively susceptible to oxidative stress and energy impairment. However, there has been relatively little discussion regarding the comparative vulnerability of cortical neurons. In the current study we compared the response of dopaminergic and cortical neurons to chronic oxidative stress. Alpha-synuclein expression is specific to 68.5% (± 3) of MAP2 positive cortical neurons, with high-level immunoreactivity in 9.7% (± 3) of these. The response of cortical neurons to chronic oxidative stress was comparable ($P>0.05$) with that of dopaminergic neurons. Treatment caused a three-fold increase in the proportion expressing high-level alpha-synuclein 24% (± 4) (Figure 3.2A). This high-expressing subpopulation was relatively resistant to subsequent acute oxidative toxicity. Specifically there was a 25.2% (± 4) reduction in relative proportion of these neurons with condensed nuclei, than those with low or no immunoreactivity to the protein (Figure 3.2B).

To determine whether the initial increase in alpha-synuclein expression was dependant on continuous exposure to oxidative stress, or a longer-term alteration, normal growth media containing antioxidants was reintroduced to a subset of cortical cultures for three days

following the chronic exposure (10 DIV) to antioxidant free media. Under these conditions, the relative number of high alpha-synuclein neurons remained significantly greater (23.9% ± 2) compared with non-treated, age-matched controls (9.8% ± 1).

3.3.4 Chronic oxidative stress, followed by acute toxicity results in punctate nuclear accumulation of alpha-synuclein

Alpha-synuclein immunolabelling in non-treated cultures was diffuse throughout cell soma (Figures 3.4A; 3.5A,B), with punctate immunoreactivity within the neuritis (Figure 3.4B). This pattern of alpha-synuclein immunoreactivity has been shown to colocalize with synaptic markers in the current *in vitro* model (Quilty et al., 2006), and reflects its pre-synaptic expression as previously reported (George et al., 1995; Hsu et al., 1998; Murphy et al., 2000). Alpha-synuclein expression was upregulated in subpopulations of both dopaminergic and cortical neurons subjected to chronic oxidative stress. There was a specific increase in the fluorescent intensity of synaptic labelling within the neurites, whilst the labelling throughout the soma remained diffuse (Figures 3.4C,D; 3.5E,F). Further acute oxidative toxicity resulted in punctate accumulations of alpha-synuclein within the soma (Figure 3.5G,H). There was an additional increase in synaptic alpha-synuclein labelling (Figure 3.4E,F).

The effect of UPS inhibition on the structural properties of alpha-synuclein and neuronal viability has previously been described in both dopaminergic and cortical neurons (McNaught et al., 2002a; Rideout and Stefanis, 2002). In the current study we report that proteasome inhibition was associated with altered cellular localisation of alpha-synuclein,

which was distinct from that induced by oxidative toxicity. Proteasome inhibition caused small punctate accumulations of alpha-synuclein immunoreactivity within the cytoplasm, which were of an extranuclear localisation (Figure 3.4G). These accumulations were clearly distinguished from those associated with acute oxidative toxicity by their size and location. There were no further changes in alpha-synuclein immunoreactivity within the neurites (Figure 3.4H). Proteasome inhibition did not appear associated with changes in the proportion of cortical neurons with apoptotic morphology, irrespective of the level, or pattern of alpha-synuclein immunoreactivity.

3.3.5 Both nuclear and cytoplasmic alpha-synuclein accumulations colocalise with ubiquitin

In non-treated cultures immunolabelling for ubiquitin was diffuse throughout the cytoplasm and punctate within the neurites (Figures 3.6C; 3.7C). Confocal microscopy revealed that there was limited colocalisation of alpha synuclein and ubiquitin (Figures 3.6A-D; 3.7A-D). Treatment with chronic oxidative stress resulted in the formation of punctate ubiquitin immunopositive accumulations. These were localised to the soma and neurites of a subset of cortical and dopaminergic neurons (Figures 3.6I-L; 3.7I-L) but did not colocalise with areas of increased alpha-synuclein immunolabelling.

Ubiquitin immunolabelling did not colocalise with the punctate accumulations of alpha-synuclein induced by acute oxidative toxicity (Figures 3.6E-H; 3.7E-H). However, a combination of chronic and additional acute oxidative toxicity caused nuclear localisation of distinct punctate accumulations of alpha-synuclein. Significantly, these puncta

colocalised with ubiquitin (Figures 3.6M-P; 3.7M-P). Ubiquitin was not associated with synaptic alpha-synuclein labelling.

UPS inhibition after chronic oxidative stress also resulted in the formation of punctate alpha-synuclein accumulations that colocalised with ubiquitin. However, the cellular distribution of these puncta was distinct from that induced by oxidative toxicity and was associated with an extranuclear concentration of these accumulations within the soma (Figure 3.6Q-T). Table 3.1 provides a summary of the localisation of alpha-synuclein and ubiquitin, across each treatment group.

3.4 Discussion

The current study aimed to clarify the role of alpha-synuclein and dopamine in the neuronal response to oxidative toxicity. The effect of alpha-synuclein expression was compared between dopaminergic and cortical neurons exposed to an *in vitro* model of chronic oxidative stress. The results indicate that alpha-synuclein upregulation is an innate and neuroprotective response conserved between these neuronal subpopulations. However, subsequent acute oxidative toxicity caused the formation of ubiquitinated alpha-synuclein accumulations within the soma. These results fit a two-hit hypothesis for the role of alpha-synuclein in neurodegeneration, characterised by an initial protective upregulation preceding its toxic aggregation. Significantly, the role of alpha-synuclein in oxidative stress did not differ between dopaminergic and cortical neurons.

Alpha-synuclein expression has been linked with neuroprotection in *in vitro* cell-line models (da Costa et al., 2000; Hashimoto et al., 2002), primary cortical neurons (Quilty et al., 2006), as well as in cerebellar granules cells *in vivo* (Monti et al., 2007). Although these data indicate that the protective effect of alpha-synuclein is conserved among multiple cell populations, conflicting evidence is given to the effect of this protein in dopaminergic neurons (Bisaglia et al., 2010). Lee and colleagues propose that alpha-synuclein drives the toxicity of dopaminergic neurons through enhanced DA function and increased dopamine uptake (Lee et al., 2001a). The current study directly compared the differential effect of endogenous alpha-synuclein expression in dopaminergic and cortical neurons. Significantly, the response of these neuronal subtypes did not differ in the current

model, rather alpha-synuclein upregulation was a conserved and neuroprotective response to oxidative stress. These data are consistent with a role for alpha-synuclein in preconditioning primary neurons from oxidative stress, and are in accordance with its proposed role as a molecular chaperone (Souza et al., 2000a).

This study aimed to further determine if endogenous increases in alpha-synuclein predisposed dopaminergic neurons to degeneration following additional toxic insult. Cappai and colleagues (2005) reported that dopamine drives the toxic aggregation of alpha-synuclein (Cappai et al., 2005) and may neutralise the neuroprotective properties of this protein (Alves Da Costa et al., 2002). Alpha-synuclein aggregation is also driven by increases in its concentration (Tofaris et al., 2001; Outeiro and Lindquist, 2003; Kim and Lee, 2008), and is further augmented by oxidative stress (Hashimoto et al., 1999b). It was therefore proposed that oxidative stress-induced upregulation of alpha-synuclein would precede its toxic aggregation by dopamine. However, no association between alpha-synuclein upregulation and selective toxicity of these neurons was found, following chronic oxidative stress treatment.

The current study indicates that high intracellular levels of alpha-synuclein are maintained following the withdrawal of oxidative stress conditions. Failure of immediate clearance may contribute to a concentration dependent aggregation of alpha-synuclein as previously reported in yeast cells (Outeiro and Lindquist, 2003). Elevated levels of intracellular alpha-synuclein are also associated with saturation and impairment of the UPS (Bennett et al., 1999; Betarbet et al., 2005; McGeer and McGeer, 2008). Although the current study found

no direct link between endogenous alpha-synuclein upregulation and altered ubiquitin immunolabelling following chronic oxidative stress, additional acute oxidative toxicity caused the formation of ubiquitinated accumulations of alpha-synuclein in both cortical and dopaminergic neurons. These inclusions were morphologically distinct from those induced by proteasomal inhibition, indicating that UPS impairment was not the cause of aggregate formation in the current model. Based on current findings we propose that alpha-synuclein aggregation may be a consequence of its neuroprotective upregulation and that this process is facilitated by additional insult, however, aggregation of this protein is not a specific consequence of dopamine.

The current findings do not suggest that dopaminergic neurons are selectively sensitive to oxidative stress. Rather they show that alpha-synuclein expression influences neuronal vulnerability. These results further support the increasingly accepted view that PD, DLB, and related Lewy body diseases share common pathogenic mechanisms affecting two differential, although equally susceptible cell populations (Goldmann Gross et al., 2008; Jellinger, 2008). These data fit an emerging 'two-hit' hypothesis for the role of alpha-synuclein in neurodegeneration. Specifically, chronic low dose toxicity activates a protective cellular stress response involving alpha-synuclein upregulation; however increasing intracellular levels of this protein may drive its aggregation upon exposure to additional insult.

4 Alpha-synuclein protects neurons from oxidative stress downstream from free radical production through modulation of the MAPK signaling pathway

4.1 Introduction

A direct correlation between the upregulation of alpha-synuclein and resistance to apoptosis has previously been reported in primary neurons subjected to an *in vitro* model of oxidative stress (Chapter 3; Quilty et al., 2006). The current chapter aimed to determine the mechanisms by which alpha-synuclein expression mediates this neuroprotective effect.

Several studies have investigated the molecular pathways by which alpha-synuclein may confer neuroprotection. This protein can covalently bind with cytochrome c in a Fenton-chemistry-dependant reaction (Hashimoto et al., 1999a; Bayir et al., 2009) and Bayir and colleagues (2009) also propose that alpha-synuclein acts to scavenge cytochrome c released from the mitochondria to avert downstream activation of effector caspases. Alpha-synuclein decreases activation of caspase-3 through reduction in p53 expression (Alves Da Costa et al., 2002) and can prevent apoptosis upstream from cytochrome c release. Specifically, induction of alpha-synuclein expression was associated with increased cell survival through direct inhibition of cJun N-terminal Kinase (JNK) phosphorylation in GT1-7 cells (Hashimoto et al., 2002). Overexpression of alpha-synuclein further protects neurons from MPTP toxicity through direct interactions with Bad and protein kinase c (PKC) delta (Kaul et al., 2005). However, this protein may also promote cell death by disrupting the relative balance of the JNK, mitogen activated kinase (MAPK)/ extracellular regulated kinase (ERK), and p38 MAPK pathways (Iwata et al., 2001b). Ostrerova and

colleagues suggest that direct interactions with Bad, ERK and PKC underlie the neurotoxicity of alpha-synuclein overexpression in HEK cells (Ostrerova et al., 1999).

In addition to its effects on apoptosis, alpha-synuclein may also modify neuronal tolerance to oxidative stress through the production and scavenging of free radicals. Recently, alpha-synuclein has been shown to bind to redox sensitive copper ions with high affinity, although it is unclear if this interaction contributes to its neuroprotective role or can result in oxidative damage during disease (Davies et al., 2010). Cytosolic overexpression of the protein causes oxidative stress and morphological alterations of mitochondria in GT1-7 cells (Hsu et al., 2000). Mitochondria have since been identified as the source of free radical production in alpha-synuclein over-expressing cell lines. This toxic process is linked with the direct association of oligomeric-alpha-synuclein with the mitochondrial membrane (Parihar et al., 2008b). Specifically, the N-terminal region of alpha-synuclein contains a mitochondrial targeting sequence, which enables its translocation to the inner membrane. Alpha-synuclein forms an inhibitory interaction with complex I of the electron transport chain (ETC) (Devi et al., 2008). However, mitochondrial dysfunction may result from an upstream effect of alpha-synuclein on nitric oxide synthesis activity, leading to increased free radical production (Adamczyk and Kazmierczak, 2009).

The intent of the current study was to clarify the contribution of wild-type protein to oxidative stress and apoptosis in primary neurons. It was hypothesised that physiological levels of alpha-synuclein do not contribute to free-radical production; rather they act to prevent cell death through inhibitory binding interactions with pro-apoptotic proteins. To

my knowledge the contribution of alpha-synuclein to these processes has primarily been studied through genetic overexpression of the protein. However, high cytosolic concentrations of alpha-synuclein can induce its self-aggregation and cellular toxicity (Kim and Lee, 2008). For this reason, the effect of endogenous alpha-synuclein expression on mitochondrial free radical production and the neuronal response to oxidative stress was evaluated in the current chapter. The specific aim of this study was to determine the mechanism by which alpha-synuclein confers neuroprotection in primary neurons. The current results do not support an association between physiological alpha-synuclein expression and the generation of reactive oxygen intermediates, but demonstrate that this protein inhibits the MAPK pathway to prevents cytochrome c release and caspase activation.

4.2 Experimental procedures

4.2.1 Primary mouse cortical culture

Primary dissociated cortical cell cultures were prepared from embryonic (E14) C57Bl/6 and *Snca -/-* mice, as described in chapter 2.4. Cortical neurons that were used for superoxide imaging were plated onto poly-$_L$-lysine-coated 35 mm imaging dishes (Iwaki). At 24 hours the plating media was replaced with clear growth media using Neurobasal TM without phenyl red (Gibco).

4.2.2 Glutathione assay

Total glutathione was measured with a glutathione detection kit (Northwest Life Science Specialties), according to the manufactures specifications. This kit had a lower level discrimination of 0.1 μM.

4.2.3 Immunocytochemistry, microscopy and data analysis

Immunocytochemistry was performed as described in section 2.6 using previously characterised antibodies as listed in Table 2.1. Cell viability was determined as described in chapter 2.7; Figure 2.1. Immunocytochemical analysis of cultures, and LSM image acquisition was performed as described in chapter 2.8. Data is represented as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Student's *t*-test. P-values <0.05 were considered significant. Immunocytochemical analysis of neurons treated with antioxidant-free growth media for three and six hours were identified with the

cytoskeletal markers MAP2 and NF-M. Caspase-depedent cell-death was defined by strong nuclear labeling of anti-active caspase-3. Cytochrome c release was defined as lack of cytochrome c immunoreactivity within the cell soma, as previously described (Neame et al., 2004).

4.2.4 Oxidative stress, mitochondrial impairment and excitotoxicity

Mild oxidative stress and excitotoxicity were induced as described in chapter 2.5. Inhibition of complex I was induced by the addition of rotenone at final concentrations of 5, 10, 15 and 20 nM in growth media.

4.2.5 Activity-dependent superoxide production

Activity-dependent superoxide production was measured as the rate increase in fluorescent intensity of DHE following membrane depolarization (50 μM KCl in growth media) at 7-8 DIV. Changes in the fluorescent intensity of DHE were measured with laser-scanning microscopy (scan area 594 μm^2; scan time 3.12 sec; continuous scanning), during and immediately following depolarisation. The rate of superoxide production was calculated as the linear increase in DHE labeling within the soma of each neuron in the captured field of view over three minutes.

4.2.6 Mitochondrial H_2O_2 production

Mitochondria were isolated from adult (four month-old) *Snca -/-* and C57Bl/6 mice, as described by Kristian (2010), and in section 2.9. Mitochondria were suspended in 400 μl of Krebs-Ringer phosphate at pH 7.35 (2.1.3), and 1X protease inhibitor (Roche). Protein concentration was determined by the BioRad dye-binding assay. Mitochondrial H_2O_2 production was measured in triplicate as described in chapter 2.10.

4.2.7 Apoptosis inhibition

The activation of pro-apoptotic pathways was assessed in primary cortical neurons at 15 DIV by the addition of the following apoptosis inhibitors; the MAPK inhibitor PD98059, the JNK inhibitor SP600125 (Santa Cruz) (Ciani and Salinas, 2007) and the caspase-8 inhibitor Z-IEDT-FMK (R&D systems), each dissolved in DMSO and used at a final concentration of 10 μM, as previously described (Rakhit et al., 2004; Ciani and Salinas, 2007; Wang et al., 2008). Inhibitors were added to culture media one hour prior to treatment. Cultures were treated with antioxidant-free growth media for six hours in the presence of apoptosis inhibitors. The fixable mitochondrial marker (Mitotracker red CMXRos) was added to media with inhibitors. Cytochrome c release from mitochondria was measured using immunocytochemical analysis of cytochrome c localisation relative to mitochondria (Mitotracker red CMXRos staining). Loss of colocalisation between these markers was considered indicative of cytochrome c release. For quantification a minimum of 12 fields of view were acquired across two coverslips for each culture and treatment. The cell soma, and a surrounding region equivalent to the radius of the soma, was removed from each image to minimize discrepancies caused by differences in cell size and somatic mitochondrial clustering. Analysis of colocalisation was performed on binary representations of colocalised pixels acquired using the RG2B colocalisation plugin in ImageJ (Mauer, 2004; thresholds set at 0). Results were represented as percent colocalisation of cytochrome c to the Mitotracker red CMXRos labeling. Changes in cytochrome c release for each treatment were compared to a DMSO treated control.

4.3 Results

4.3.1 Alpha-synuclein specifically mediates neuroprotection to oxidative stress and complex I inhibition but not excitotoxicity

To characterise the role of alpha-synuclein in neurodegeneration, cortical neuron cultures derived from WT and *Snca -/-* mice were subjected to oxidative stress, complex I inhibition and excitotoxicity. Loss of membrane integrity (Propidium Iodide; PI staining) was used as an early quantitative measure of cell death. Mild oxidative stress was modelled in cortical neurons through six hours treatment in antioxidant-free media. WT neurons were more resistant ($P<0.05$) to cell death (PI staining), following antioxidant depletion ($33\% \pm 4$) than those derived from *Snca -/-* ($45\% \pm 3$) mice (Figure 4.1). As neuronal oxidative stress is primarily driven by mitochondrial free-radical production we further characterised the neuroprotective effect of alpha-synuclein expression against rotenone-mediated inhibition of complex I. Alpha-synuclein expression was associated with neuroprotection ($P<0.01$) against low level (5 nM rotenone) complex I inhibition at six hours. Specifically WT neurons were more resistant to cell death ($16\% \pm 3$ increase from control), relative to *Snca -/-* ($31\% \pm 2$ increase from control) cultures, as determined with PI staining (Figure 4.1). The effect of alpha-synuclein on excitotoxicity (25 μM glutamate; 6 hours) was assessed in order to establish whether this neuroprotective effect was maintained across divergent mechanisms of neurotoxicity. There was no difference ($P>0.05$) in the vulnerability of WT ($30.9\% \pm 4.6$) compared with *Snca -/-* ($24.8\% \pm 4$) cortical neurons to excitotoxicity, as determined by PI staining (Figure 4.1).

4.3.2 The neuroprotective effect of alpha-synuclein is subject to the level of oxidative toxicity

To further characterise the neuroprotective effect of alpha-synuclein, cortical neurons were treated with increasing concentrations of rotenone, to model mild to moderate oxidative toxicity. Alpha-synuclein expression was associated with neuroprotection against low-level complex I inhibition (5 nM rotenone) at three hours. Specifically, WT (28.9% ± 3) cultures had a decreased (P=0.01) proportion of PI positive neurons compared to *Snca -/-* cultures (40.3% ± 7). However, this correlation was abolished by higher rotenone concentrations (10-20 nM) (Figure 4.2).

4.3.3 Alpha-synuclein does not effect the rate of mitochondrial free-radical production

This study aimed to determine if an increase in basal-level free radical production underlies the vulnerability of *Snca -/-* neurons to oxidative stress. Mitochondria are the major source of reactive oxygen intermediates, which are rapidly converted to H_2O_2 in the mitochondrial matrix. In the current study, the effect of alpha-synuclein expression on total mitochondrial free radical production was measured. There was no difference (P>0.05) in the rate of H_2O_2 production in somatic mitochondria isolated from whole brains of WT (163.4 pmol/mg/min ± 23) and *Snca -/-* mice (110.2 pmol/mg/min ± 18). As alpha-synuclein is localised to the pre-synaptic membrane, we further determined the differential rate of H_2O_2 production in permeabilised synaptosomes; this rate did not differ (P>0.05) between WT (315.1 pmol/mg/min ± 62) and *Snca -/-* (330.8 pmol/mg/min ± 54) whole brain.

4.3.4 Alpha-synuclein does not modulate the activity-dependant rate of free radical production in cultured cortical neurons

The affect of alpha-synuclein expression on the activity-dependent rate of superoxide production was determined in cortical neurons (7 DIV) following membrane depolarization. The fluorescent intensity of DHE was used to quantify the change in superoxide concentration immediately following the addition of KCl (final concentration of 50 mM) into growth media. There was no significant difference in this rate between cortical neurons derived from WT (0.4 F_0/min ± 0.2), and *Snca* -/- (0.7 F_0/min ± 0.3) mice.

4.3.5 Alpha-synuclein does not affect antioxidant-capacity in whole cortex

Antioxidant buffering of free radicals can prevent the induction of cell death by oxidative toxicity. The current study verified the role of glutathione (GSH)-mediated antioxidant-capacity in the differential vulnerability of WT and *Snca* -/- neurons was. There was no significant (P=0.42) difference in the levels of total GSH in whole, homogenised cortex of WT (67.6 μM ± 4) and *Snca* -/- (59.1 μM ± 9) mice at 12 weeks of age.

4.3.6 Alpha-synuclein expression protects neurons downstream from free radical production

As alpha-synuclein did not effect the production or scavenging of free radicals we aimed to determine its role in downstream pathogenic mechanisms leading to apoptosis. Cellular destruction during apoptosis is executed by effector caspases (caspase-3, 6 and 9), which are activated through both mitochondrial and death receptor pathways. Six-hour antioxidant depletion was associated with a 10% (± 5) increase in the number of neurons immunoreactive for active caspase-3 (Figure 4.3A). Caspase activation occurred through a

mitochondrial dependant pathway involving cytochrome c release, as indicated by a complete loss or diffuse redistribution of somatic cytochrome c labelling, in 32.6% (± 3) of WT neurons (Figure 4.3A). We compared the effect of oxidative stress on the activation of caspase-3 in WT and *Snca -/-* neurons before loss of cell viability, which we had previously determined at six hours treatment (Figure 4.1). At three hours antioxidant depletion there was no difference in immunoreactivity for active caspase-3 between WT (11.9% ± 2) and *Snca -/-* (12.3% ± 2) cultures. *Snca -/-* cultures did however, have a higher proportion ($P<0.01$) of neurons with loss of cytochrome c (20.3% ± 1) compared to WT (8.6% ± 2) cultures (Figure 4.3B). The correlation between alpha-synuclein expression and resistance to apoptosis was strengthened by subdivision of WT neurons according to their level of alpha-synuclein expression. Alpha-synuclein positive neurons comprise 40% (± 4.1) of cultured cortical neurons at 15 DIV. High-level alpha-synuclein expression was associated with a two–fold greater resistance ($P<0.05$) to caspase-3 activation (6.8% ± 3) at six hours antioxidant depletion, when compared to the subpopulation expressing low levels (12.9% ± 4) or no (16.6 ± 4) alpha-synuclein (Figure 4.3C, 4.4A-C). Endogenous alpha-synuclein expression correlated with resistance to cytochrome c release, as indicated by loss, or diffuse redistribution of cytochrome c labelling (Figure 4.3D, 4.4D-F). This was demonstrated by a four-fold increase in the proportion of alpha-synuclein negative neurons with release of cytochrome c, compared to the alpha-synuclein positive subpopulation (Figure 4.4D,F).

4.3.7 Alpha-synuclein acts through the MAPK pathway to prevent cytochrome c release

We aimed to determine the mechanism by which alpha-synuclein prevents cytochrome c release in response to oxidative stress. Pharmacological inhibitors of pro-apoptotic proteins were applied to the current model of oxidative stress to define the neuroprotective pathway of alpha-synuclein. Cytochrome c release was used as an early determinant of apoptosis and was calculated as the percent of colocalisation between Mitoctracker Red CMXRos staining and cytochrome c immunolabelling. Inhibition of the stress-activated MAPK pathway increased ($P<0.01$) the proportion of mitochondria with colocalised cytochrome c in *Snca* -/- neurons following six hours treatment in antioxidant-free growth media. Specifically, MAPK inhibition increased mitochondrial colocalisation of cytochrome c by 8.5% (± 1) in *Snca* -/- but not WT (3.6% decrease from control ± 1) neurons (Figure 4.5; 4.6). The JNK specific inhibitor SP600125 was applied to the cultures in an attempt to further define the MAPK pathway affected by alpha-synuclein expression. However, this did not prevent ($P<0.05$) cytochrome c release within WT (1.7% ± 2 decrease in colocalisation from control) or *Snca* -/- (0.07% ± 2 increase in colocalisation from control) neurons. Caspase-8 inhibition also did not alter the level of cytochrome c release following oxidative stress in WT (1.5% increase in colocalisation from control ± 2) or *Snca* -/- (1.2% increase in colocalisation from control ± 1) cortical neurons.

4.4 Discussion

Alpha-synuclein is a pre-synaptic molecular chaperone involved in synaptic plasticity, clustering and fusion of vesicles (Cooper et al., 2006; Nemani et al., 2010; Surmeier, 2010), and regulating brain lipid content (Ellis et al., 2005; Rappley et al., 2009). Despite an obvious contribution to disease, its direct involvement in neuronal health and degeneration is argued to be that of both a hero and a villain. The role of alpha-synuclein in cell health has primarily been studied using models of protein overexpression in transformed cell lines (da Costa et al., 2000; Saha et al., 2000; Hashimoto et al., 2002; Seo et al., 2002; Monti et al., 2007; Bayir et al., 2009). Therefore the aim of this chapter was to clarify the effect of endogenous alpha-synuclein in primary neurons exposed to *in vitro* models of neurotoxicity.

The previous chapter and current results (Figure 4.2) suggest that physiological concentrations of alpha-synuclein confer neuroprotection against oxidative stress and mitochondrial dysfunction. It was hypothesised that alpha-synuclein modulates the neuronal response to oxidative stress through either upstream regulation of basal-level free-radical production, or downstream inhibition of cell death. The current data suggest that the protective action of alpha-synuclein is not conferred through decreased free radical production, but by inhibition of caspase-dependent apoptosis (Figure 4.4). Specifically, alpha-synuclein regulates the pro-apoptotic MAPK pathway to prevent cytochrome c release (Figure 4.6).

Previous studies have identified a direct correlation between alpha-synuclein expression and resistance to oxidative stress in primary cortical and dopaminergic neurons (Manning-Bog et al., 2003; Quilty et al., 2006; Monti et al., 2007). This study aimed to clarify whether this response was maintained against multiple mechanisms of neurotoxicity, or was specific to the apoptotic pathways induced by oxidative stress. Induction of excitotoxicity in primary neurons is associated with both apoptotic and necrotic cell death, depending on the extracellular glutamate concentration (Leist and Nicotera, 1998; Seo et al., 2009), whereas mild oxidative toxicity primarily activates pro-apoptotic pathways (Avery, 2011). These data suggest that alpha-synuclein expression specifically confers resistance against oxidative mechanisms of cell death but not excitotoxicity, suggesting a role in modification of free radicals or specific inhibition of apoptotic pathways.

Mitochondrial dysfunction contributes to both PD and DLB pathology. Several disease-associated mutations as well as environmental causes are known to effect mitochondrial function (Onyango, 2008), and respiratory chain deficiencies have been reported in PD and DLB patient brains (Parker et al., 2008; Navarro et al., 2009). Mitochondrial dysfunction, and particularly inhibition of the ubiquinone reduction by complex I, causes significant increases in reactive oxygen intermediates (Sherer et al., 2003). Alpha-synuclein facilitates mitochondrial function through indirect modulation of the ETC by positive regulation of cardiolipin levels (Ellis et al., 2005; Devi et al., 2008). Based on these findings, it was proposed that *Snca -/-* neurons have a higher rate of total free radical production and basal increases in oxidative damage underlie their susceptibility to additional oxidative stress.

This would be of particular significance at the synapse considering the presynaptic localisation of alpha-synuclein and the relative sensitivity of synaptic mitochondria to complex I impairment (Davey et al., 1998). However, in the current study alpha-synuclein deficiency did not increase total free radical production (H_2O_2) from isolated synaptosomes or somatic mitochondria. This effect was independent of relative cellular activity, as determined by superoxide production following membrane depolarisation. These data therefore negate a role for endogenous alpha-synuclein in the suppression of mitochondrial-generated oxidative stress.

The current data appear inconsistent with the established role of alpha-synuclein in the neurotoxicity of primary dopaminergic neurons treated with the complex I respiratory chain inhibitor MPTP (Klivenyi et al., 2006; Fountaine et al., 2008). However, Choi and colleagues indicate that the toxicity of MPTP is independent of complex I inhibition (Choi et al., 2011). Furthermore, alpha-synuclein does not affect the rate of superoxide production in MPTP treated neurons, indicating that this protein mediates MPTP toxicity independent of complex I activity. In further contrast to its proposed role in augmenting complex I inhibition, Alves da Costa and colleagues identified an anti-apoptotic effect of alpha-synuclein expression in HEK cells exposed to low-dose MPP+ (1-methyl-4-pphenylpyridinium) (Alves Da Costa et al., 2006). These results are in accordance with the current findings and suggest that alpha-synuclein protects against low level complex I inhibition.

Results within this study suggest that alpha-synuclein confers neuroprotection against oxidative toxicity downstream of free-radical production and scavenging. This investigation therefore aimed to further determine the involvement of this protein in apoptosis and cell survival. Previous reports have shown alpha-synuclein to mediate cell survival through direct scavenging of cytosolic cytochrome c (Bayir et al., 2009), inhibition of caspase activation (da Costa et al., 2000) and down regulation of the caspase-dependent apoptotic kinase PKC∂ (Jin et al., 2010). However, our data indicates that alpha-synuclein acts upstream from these events, to prevent cytochrome c release (Figure 4.4).

Oxidative toxicity activates several pro-apoptotic pathways, which induce mitochondrial mediated cell death. Alpha-synuclein modifies the activity of some of these pathways, including the Bcl-2 family proteins, JNK, ERK/MAPK (Iwata et al., 2001b; Hashimoto et al., 2002) and downstream P13/Ark (Ostrerova et al., 1999; Seo et al., 2002) p53 (Alves Da Costa et al., 2002) pathways. However, these studies have relied on exogenous application, or induced expression of alpha-synuclein in neuronal derived cell lines. High intracellular concentrations of alpha-synuclein can down-regulate the pro-survival proteins Akt, Gsk3ß and p53, induce mitochondrial damage, and promote its toxic aggregation (Hsu et al., 2000; Kim and Lee, 2008; Ebrahim et al., 2010). This study therefore aimed to further ascertain the affect of endogenous alpha-synuclein levels on common pro-apoptotic pathways. The current data indicate that alpha-synuclein prevents cytochrome c release through inhibition of the MAPK signalling pathway. Binding interactions between alpha-synuclein and ERK/MAPK have been well documented (Iwata et al., 2001b; Hashimoto et al., 2003). Specifically, alpha-synuclein overexpression is associated with induction of

apoptosis through a mechanism involving MAPK inhibition (Iwata et al., 2001b). However, data from Hashimoto and colleagues suggest the inhibitory interaction between alpha-synuclein and ERK cause a downstream increase in caveolin-1 (Hashimoto et al., 2003). Significantly, caveolin is able to suppress apoptotic-signalling pathways including PKC and ERK. Current data supports these previous results, and further suggest that inhibitory interactions between alpha-synuclein and MAPK van prevent oxidative-pathways of apoptosis. From these results one can propose that endogenous alpha-synuclein regulates MAPK activity to prevent cytochrome c release, and down-stream apoptosis. Direct comparisons between *Snca -/-*, wild-type and alpha-synuclein overexpressing systems would aid understanding of the dose dependant interactions of this protein and its role in cell survival.

There are three major mammalian MAPK subfamilies; the ERK, the JNK and the p38 kinases (Subramaniam and Unsicker, 2010). JNK is a major pro-apoptotic signaling pathway implicated in PD and DLB (Ferrer et al., 2001) and is involved in the induction of apoptosis in the MPTP rodent model of PD (Wang et al., 2004; Kim and Choi, 2010; Wang et al., 2011). ERK is conversely associated with cell survival through induction of transcription and activation of the pro-survival PI3K/Akt pathway (Subramaniam and Unsicker, 2010), although chronic activation and nuclear translocation of ERK is also associated with apoptosis (Stanciu and DeFranco, 2002). Significantly, oxidative stress activates both the JNK and p38MAPK signalling pathways through phosphorylation of apoptosis signal-regulating kinase 1(ASK1) reviewed in (Kim and Choi, 2010).

This study investigated the specific effect of alpha-synuclein on JNK inhibition, in order to further characterise the MAPK pathway affected by alpha-synuclein. However, JNK inhibition did not influence cytochrome c release following oxidative stress in *Snca -/-,* relative to wild-type neurons. These results indicate that alpha-synuclein promotes cell survival through either the MAPK/ERK1 or p38 MAPK pathways. Further definition of the inhibitory interactions between alpha-synuclein and its MAPK targets are beyond the scope of this study. However, there is increasing evidence that alpha-synuclein interacts with the MAPK/ERK pathway, and may activate the pro-survival P13k/Akt pathway (Seo et al., 2002; Opazo et al., 2003; Yuan et al., 2010).

The identification of LRRK2 mutations as a genetic cause of familial PD further exemplifies the significance of MAPK regulation in neurodegeneration. LRRK2 is essential for the guanine triphosphate (GTP)-linked activity of MAPK kinase kinase and can directly activate MAPK kinases upstream of MAPK p38 and JNK (Gloeckner et al., 2006; Ito et al., 2007). Mutations in LRRK2 increase cellular susceptibility to oxidative stress through activation of these kinase-signalling pathways (West, 2007; Gloeckner et al., 2009). The pathological outcomes of these mutations closely model those of idiopathic and *Snca*-mutant causes of PD.

Findings from the current study indicate that endogenous alpha-synuclein does not contribute to free-radical production but protects primary cortical neurons from oxidative stress. These results contribute to the increasing understanding of the role of MAPK regulation in the neuronal response to oxidative stress.

5 Lack of alpha-synuclein expression drives glycolytic metabolism in cultured cortical neurons

5.1 Introduction

Gene silencing and overexpression techniques have provided a systematic platform to study the role of alpha-synuclein in neuronal function and viability. These genetic models have been extensively characterised through morphological, proteomic and biochemical analysis (Abeliovich et al., 2000; Murphy et al., 2000; Cabin et al., 2002; Chandra et al., 2005). Although these studies have provided insight into the role of this protein, they have failed to clarify the precise cellular function of alpha-synuclein and its involvement in neurodegeneration. The current study characterised the role of alpha-synuclein in the development, viability, neuronal differentiation and function of primary cortical neurons *in vitro*. The primary aim was to determine potential effects of endogenous alpha-synuclein on neuronal metabolism, using metabolic profiling of media (exometabolomics) derived from wild-type (WT) and alpha-synuclein null (*Snca -/-*) cortical neurons.

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy is widely used to define metabolic pathways affected by gene deletion (Bundy et al., 2007; Atherton et al., 2009; Guengerich et al., 2010). This method of establishing phenotypic variation does not require prior prediction of gene activity and is therefore distinguished from traditional hypothesis-based analysis (Kuchel, 2010). To my knowledge this approach has not yet been used to assess the role of alpha-synuclein in neuronal metabolism. In the current study, metabolic characterisation of *Snca -/-* neurons was therefore used to identify novel functions of

alpha-synuclein.

5.2 Experimental procedures

5.2.1 Cell culture

Primary dissociated cortical neuron cultures were prepared from embryonic (E14) C57Bl/6 and *Snca -/-* mice, as previously described (Chapter 2.4). Dissociated neurons were plated into 6-well culture trays (Iwaki Japan) treated with 0.001% poly-$_L$-lysine (Sigma-Aldrich), at a density of 1.5×10^5 cells/well. Cultures were incubated 37^{o}C, 5% CO_2 for up to 15 days *in vitro* (DIV).

5.2.2 Immunocytochemistry, microscopy and data analysis

Immunocytochemistry was performed as described in section 2.6 using previously characterised antibodies as listed in Table 2.1. Immunocytochemical analysis of cultures and LSM image acquisition was performed as described in chapter 2.8. Differential interference contrast (DIC) images were acquired on a Nikon inverted Eclipse TiE, using NIS Elements 6D software (Nikon Instruments, NY, USA). Images were captured with a CoolSNAP *HQ2* turbo 1934 CCD camera (Photometrics, Tucson, Arizona, USA). Data is represented as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Student's *t*-test. P-values < 0.05 were considered significant.

5.2.3 Cell viability assays

DHR was used as a marker of viable neurons in live cultures and cell viability was determined by the proportion of PI stained neurons (Chapter 2.7). For viability assays a

minimum of 100 neurons were counted for each replicate. Data represents averages of a minimum of four cultures, each assessed in duplicate. Statistical comparisons between cultures were made using the student's *t*-test.

5.2.4 ^{1}H-NMR spectroscopy

The metabolite composition of growth media obtained from WT and *Snca* -/- cortical-neuron cultures was assessed with ^{1}H-NMR spectroscopy (performed by Dr. James Horne, Central Science Laboratory, University of Tasmania). Media was taken at 15 DIV from a minimum of eight independent cultures for each genotype. Media samples were adjusted to pH 7.00 (±0.01) and deproteinated with acetonitrile according to the methods of Daykin and colleagues (2002). Briefly, media was mixed with acetonitrile in a ratio of 1:1, inverted (30 sec) and allowed to stand (5 min). Protein was separated by centrifugation (13000 *g*, 3 min) and 1 mL of the supernatant was evaporated to dryness in an evacuated centrifuge (Labconco, MO, USA). Dried samples were reconstituted in 650 µL NMR solution ($^{2}H_2O$ containing 2 mM DSS and 0.01% (w/v) sodium azide). Samples were analyzed with one dimensional ^{1}H nuclear overhauser effect spectroscopy (NOESY), using a Varian INOVA NMR spectrometer (Varian, CA, USA), at a ^{1}H resonance frequency of 400 MHz (9.4 Tesla). Spectra were acquired at 25ºC with a 90º pulse, pulse length 9.7 µs and sweep width of 10.5 ppm. A total of 128 transients were collected into 32 k data points. A pre-saturation pulse was set to the water frequency during the relaxation delay (1 sec) and mixing time (100 msec) to suppress the residual water peak.

Individual free induction decays were Fourier transformed and baseline corrected in NMR Manager 12.0 (Advanced Chemistry Development, Toronto, CAN). ^{1}H chemical shifts were referenced relative to DSS at 0.00ppm. The spectra were normalised using probabilistic-quotient normalisation (Dieterle et al., 2006) and bucketed with adaptive intelligent binning, both in Matlab (MathWorks, Massachussets, USA) using custom scripts (courtesy of Dr. Timothy DeMeyer, Ghent University, Belgium) The resulting spectra were mean-centered and orthogonal signal-corrected before being analysed using partial least squares discriminant analysis (PLS-DA), both in PLS Toolbox 6.2 (Eigenvector Research Inc., Wenatchee, USA). Statistical analysis of spectra was performed with the assistance of Dr. Lindsay Edwards, University of Tasmania.

The statistical significance of class classification by the PLS-DA model was determined with permutation testing, as outlined by van der Voet (1994). Briefly, spectra were randomly reassigned to either WT or *Snca-/-* groups, irrespective of their original class classification. PLS-DA was performed on these 'false' classifications, and this process was repeated 50 times to build a distribution of results, given no genuine difference between the classes. This distribution was used to calculate the P-value for the 'true' class classification.

Features of interest in the NMR spectra were associated with their respective metabolites using database searches and correlation spectroscopy. In this regard, definitive assignment of metabolites depended on their correlation with resonances from associated protons in different spectral regions.

5.3 Results

5.3.1 Phenotypic characterisation of cortical neurons grown *in vitro*

The extracellular metabolite profiles of cortical neurons derived from WT and *Snca* -/- mice were analysed in order to further characterise the role of alpha-synuclein in neuronal function. Neurons were grown in a serum-free media (Neurobasal TM media with B-27 supplement; Chapter 2.4) to selectively facilitate neuronal growth and inhibit survival of non-neuronal cells (Brewer et al., 1993; Brewer, 1995), thereby minimising metabolite uptake by glia. The relative purity of these cultures was assessed with double immunolabelling for the neuron and glial specific markers, microtubule associated protein 2 (MAP2) and glial-fibrillary acidic protein (GFAP), respectively (Figure 5.1A-C). According to these criteria neurons constituted 93.2% of cells and 6.8% (± 0.8) expressed GFAP. There was no difference ($P>0.05$) in the proportion of glia (GFAP positive cells) between WT (6.8 ± 1.1) and *Snca* -/- (6.9 ± 1.4) cultures (Figure 5.1D).

The metabolic profiles of distinct brain regions are characterised by the cell populations and pharmacological attributes (Tsang et al., 2005). The cellular composition of cortical neurons was therefore examined in the current culture paradigm and found to comprise a heterogeneous population. GABAergic interneurons (calretinin and parvalbumin immunopositive; Figure 5.2A,B) constituted less than 5% of neurons in mature (15 DIV) cultures (Figure 5.2C). Within this subpopulation, there was a relative lack of parvalbumin immunopositive interneurons, with this population accounting for less than 0.5% of all

neurons (Figure 5.2B,C). Pre-synaptic GABAergic input was confirmed by the presence of the vesicular GABA transporter (VGAT) (Figure 5.3A,B). Glutamatergic projection neurons comprised the majority of the cell population, as indicated by the expression of neurofilament-medium protein (NF-M), pyramidal morphology and pre-synaptic expression of excitatory glutamate transporters (VGLUT) (Figure 5.3C,D).

Alpha-synuclein is localised to the synapses of neurons, where it functions in the regulation of synaptic vesicle storage and release. An *in vitro* model of mature (15 DIV) neurons was established to identify downstream-metabolic effects of alpha-synuclein function. Neuronal development was morphologically characterised by neurite extension and synapse formation (Figure 5.4A). Maturity was defined by the presence of dendritic spines, the absence of growth cones on neurite projections and by the expression of the pre-synaptic marker synaptophysin (Figure 5.4B,C).

5.3.2 Alpha-synuclein expression did not affect the viability of cultured cortical neurons

Apoptosis and necrosis cause the release of distinct pools of metabolites (Heijne et al., 2005; Rainaldi et al., 2008; Shen et al., 2011). The affect of alpha-synuclein on cell viability was therefore assessed in the current culture paradigm. During development (7 DIV) there was no difference ($P>0.05$) in the proportion of PI-stained neurons between WT (10.4% ± 1.2) and *Snca* -/- (8.3% ± 0.7) cultures. Between 7 and 15 DIV there was a 2% decrease ($P=0.03$) in cell viability, although the proportion of PI stained neurons remained independent ($P>0.05$) of genetic variation.

5.3.3 Endogenous alpha-synuclein expression changes metabolism in cortical neurons

The effect of alpha-synuclein on metabolite uptake and secretion in mature cortical neurons was determined using ^{1}H NMR spectroscopy profiles of cell culture media (exometabolomics). NMR spectrum from each media sample was classified according to genotype, and a data-reduction technique (PLS-DA) was used to identify areas of the spectra accounting for differences between WT and *Snca-/-* neurons. This technique effectively groups together spectral features that are correlated with each other *across samples* into latent variables. In this experiment, a single latent variable was sufficient to account for 98.8% of the variation between samples (Figure 5.5).

The most dominant features of the latent variable corresponded to peaks in the spectra at 1.3, 4.1 and 2.9 ppm. These were subsequently identified as proton resonances from $_{L}$-lactate, (1.3 and 4.1 ppm) (Figure 5.6, 5.7), and the pH buffer HEPES (2.9 ppm) (Figure 5.7). Furthermore, the classification of media derived from *Snca -/-* and WT cultures, was highly significant ($P<0.01$) according to the permutation test (Chapter 5.2.4), suggesting that the variation between these classes was not a product of over-fitting.

5.4 Discussion

Alpha-synuclein gene silencing techniques have provided insight into its role in synaptic activity and neuronal function. However, these models provide little evidence for an association between the normal function of alpha-synuclein and its role in neurodegeneration. The current study aimed to further characterise the role of endogenous alpha-synuclein in the metabolic activity of primary cortical neurons. Although there was no difference in the cellular composition or neuronal viability of cultures derived from wild-type and alpha-synuclein knockout mice, gene depletion was associated with differential uptake and excretion of metabolites into culture media, as determined by ^{1}H NMR. These differences correlated to increases in glycolytic metabolites in growth media of *Snca -/-* cortical cultures, indicating a dependence on anaerobic energy production.

Previous characterisations of alpha-synuclein knockout mice have found no obvious behavioural abnormalities or gross morphological defects (Abeliovich et al., 2000; Cabin et al., 2002). These reports have specifically focussed on the role of alpha-synuclein in the nigrostriatal dopaminergic neurons, but report few functional deficits and no decrease in the viability of these neurons. The current study characterised cortical neuron populations derived from alpha-synuclein knockout mice. The proportion of inhibitory and excitatory neurons did not differ in respect to genotype and alpha-synuclein did not influence neuron maturation, or the rate of neurite development (Figure 5.2, 5.3). Furthermore, alpha-synuclein expression had no affect on neuronal viability under normal culture conditions

(Chapter 5.3.2). These data are in accordance with previous reports indicating functional redundancy of this protein (Abeliovich et al., 2000; Anwar et al., 2011). Significantly, the lack of phenotypic difference between these neuronal populations enables independent analysis of metabolic differences, which is not influenced by cell-type variation.

Despite the lack of clarity surrounding the normal function of alpha-synuclein, previous chapters of this thesis have established a role for this protein in the neuronal response to oxidative stress. This data implies direct involvement of alpha-synuclein in opposing the neurotoxic mechanisms of PD and DLB (Chapters 3 & 4). The current study further indicates that alpha-synuclein may facilitate metabolic pathways to indirectly confer neuroprotection. This study identified significant differences in the metabolic properties of wild-type and alpha-synuclein knockout neurons.

A PLS-DA model comprising a single latent variable was sufficient to correctly classify growth media derived from wild-type and *Snca* -/- cultures (Figure 5.5), ($P<0.01$)). This variable accounted for 98.8% of between-class variation, indicating that alpha-synuclein expression produced a highly consistent metabolic response, affecting related metabolic pathways.

Media samples derived from *Snca* -/- cultures had a significantly higher concentration of $_{L}$-lactate. As there was no lactate in 'fresh' growth media (Neurobasal TM with B-27 supplement; Appendix A; B), this result signifies increased $_{L}$-lactate production in cultured neurons that lacked alpha-synuclein. The implication of glycolytic metabolism is

strengthened by the increased resonances from one of the HEPES protons, in media from *Snca -/-* neurons. Specifically, it is likely that this proton was attached to a functional group in the HEPES molecule that aids its pH-buffering characteristics. The increased signal may therefore result from a higher proton output from *Snca -/-*, relative to WT neurons, consistent with increased lactic acid production via glycolytic metabolism. These deductions are based on the assumption that the protons are products of lactic acid dissociation, although this simplistic interpretation remains contentious (Robergs et al., 2004).

The current data are consistent with previous reports that alpha-synuclein indirectly facilitates oxidative phosphorylation by maintaining the linked-activity of and NADH and cytochrome c reductase (complex I/III) (Ellis et al., 2005; Devi et al., 2008). Ellis and colleagues (2005) also report decreased levels of cardiolipin in *Snca -/-* whole brain. Cardiolipin is involved in maintaining physical association between ETC complexes and the electrochemical gradient across the inner mitochondrial membrane (Chicco and Sparagna, 2007), these results therefore indicate a role for alpha-synuclein in ATP synthesis through oxidative phosphorylation. The current results further suggest that a reduction in aerobic energy production in *Snca -/-* neurons drives obligate glycolytic ATP synthesis.

Neurons require vast amounts of ATP to continually repolarise neurite membranes following action potential propagation. The brain has an effective respiratory coefficient of one, indicating that its energy demand is met by the oxidative metabolism of glucose

(Siesjo, 1984). This premise is supported by the limited glycolytic capacity of neurons, attributed to low activity of Pfkfb3 (6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase-3) (Herrero-Mendez et al., 2009; Bolanos and Almeida, 2010). However, recent evidence suggests that neurons undergo glycolysis to maintain ATP levels in response to hypoxia (Malthankar-Phatak et al., 2008), manganese toxicity (Zwingmann et al., 2003) and during normal activation (Mangia et al., 2009).

The JNK signaling pathway can also impair mitochondrial respiration and induce glycolysis in primary cortical neurons (Hanawa et al., 2008; Zhou et al., 2008). JNK phosphorylation inhibits pyruvate dehydrogenase, which catalyses the conversion of pyruvate to acetyl-CoA for entry into the tricarboxylic acid (TCA) cycle (Zhou et al., 2008). Alpha-synuclein may therefore facilitate mitochondrial import of pyruvate and oxidative metabolism through its inhibitory interactions with JNK (Hashimoto et al., 2002).

Mitochondrial dysfunction, and specifically impaired activity of the ETC are primary mechanisms of cell death in PD, DLB and Parkinsonism. However, direct evidence for the involvement of alpha-synuclein in mitochondrial function is limited. The current study supports a role for this protein in facilitating oxidative phosphorylation and further implicates energy impairment as a primary mechanism of neurotoxicity. Metabolic deficits may initiate downstream processes involved in neuropathology. Specifically, glycolysis has been linked to induction of oxidative stress in primary neurons (Herrero-Mendez et al., 2009). Furthermore, glycolytic metabolism shunts glucose away from the pentose

phosphate pathway, which produces NADPH used for the reduction of glutathione (GSH). Based on the current results, it can be inferred that lack of alpha-synuclein activity promotes glycolysis and impairs the regeneration of GSH, which may ultimately increase neuronal susceptibility to oxidative stress (Herrero-Mendez et al., 2009).

Neuronal activation *in vivo* has been linked to an increase in glycolysis in both neurons and astrocytes under normal conditions. Mangia and colleagues (2009) have shown that prolonged stimulation causes a net increase in glucose uptake, lactate release and a reciprocal decrease in lactate metabolism in neurons (Mangia et al., 2009). Alpha-synuclein may indirectly modulate neuronal activity through inhibition of dopaminergic and glutamatergic transmission (Nemani et al., 2010), thereby preventing conversion to anaerobic metabolism.

The metabolic consequences of gene deletion can provide insight into the function of a given protein. Comparisons of the metabolic fluxes in gene knockout models have the potential to define the pathways of gene function. However, these techniques are limited in their ability to pinpoint the site of activity and direct interactions of a given protein. Results from the current study indicate that loss of alpha-synuclein drives an increase in glycolysis. Further analysis is required in order to establish whether this effect is a consequence of increased energy demand, or impaired mitochondrial function. Significantly, mitochondrial function is both genetically and pharmacologically implicated in PD, DLB and Parkinsonism. Direct comparison of the metabolic profiles of alpha-synuclein and Parkin, or DJ-1 knockout models will aid in determining whether a single defective pathway

causes these conditions, or if these effects represent a common consequence of convergent pathological mechanisms.

6 Mutant (A53T) alpha-synuclein sensitises cortical neurons to oxidative stress through a toxic gain of function

6.1 Introduction

Point mutations within five independent genes have been linked to Mendelian inheritance of PD (Gasser et al., 2011; Chapter 1.1.5; Table 1.1). The majority of these genes (*PRKN*, *PINK1*, *DJ-1*) encode proteins involved in mitochondrial physiology or the cellular response to oxidative stress (Hardy, 2010). It has therefore been proposed that dysfunction of these pathways underlies disease pathogenesis. Point mutations within the *SNCA* gene are also linked to autosomal dominant inheritance of PD and DLB, although cellular consequences of these mutations have been difficult to determine, particularly as the normal function of wild-type alpha-synuclein remains unclear. However, current studies indicate that expression of mutant alpha-synuclein can detrimentally alter mitochondrial physiology and contribute to free radical production (Parihar et al., 2009). In contrast, endogenous expression of wild-type protein facilitates mitochondrial function and attenuates the effects of oxidative stress (Ellis et al., 2005; Alves Da Costa et al., 2006; Chapters 3-5). The current study aimed to determine whether expression of mutant (A53T) alpha-synuclein abolishes the neuroprotective function of wild-type protein, and clarify its role in neuronal susceptibility to *in vitro* models of cellular toxicity. This chapter specifically examines the hypothesis that mutant protein impairs mitochondrial function and promotes free radical production through a toxic gain of function.

The current study utilised a transgenic mouse model of alpha-synuclein pathology characterised by mutant (A53T) alpha-synuclein expression in all CNS neurons under the mouse prion promoter (PrP). Mutant (A53T) alpha-synuclein homozygous, and hemizygous mice display a progressive decline in motor function, corresponding to the development of intracellular alpha-synuclein aggregates. The composition of these aggregates is distinguished from typical Lewy bodies by lack of immunoreactivity to neurofilament and inconsistent labelling for ubiquitin (Giasson et al., 2002; Lee et al., 2002). The age of symptomatic onset in these mice varies according to the number of inherited alleles confirming that loss of motor function correlates with transgene expression, rather than chromosomal disruption. Significantly, PrP driven expression of wild-type human alpha-synuclein is not associated with neuropathology or motor dysfunction (Giasson et al., 2002).

Several studies have characterised the role of mutant alpha-synuclein in impaired mitochondrial function (Martin, 2006; Parihar et al., 2009). Choubey and colleagues linked the overexpression of mutant (A53T) alpha-synuclein to loss of primary dopaminergic neurons through ATP depletion. These bioenergetic defects were driven by enhanced mitophagy, targeting both viable and depolarised mitochondria (Choubey et al., 2011). Expression of the A53T mutation also sensitises dopaminergic neurons to MPTP toxicity and drives the formation of oxidative dopamine metabolites (Yu et al., 2008). More recent studies indicate that expression of mutant or wild-type alpha-synuclein contributes to disruption of cellular calcium homeostasis (Hettiarachchi et al., 2009). Specifically, two independent studies have reported an increase in intra-mitochondrial calcium stores in,

both cultured neuronal cell lines expressing mutant (A53T) alpha-synuclein (Marongiu et al., 2009) and in isolated mitochondria incubated with aggregates of both mutant and wild-type protein (Parihar et al., 2008b). These studies suggest a role for alpha-synuclein in mitochondrial dysfunction but fail to define specific effects of mutant protein, relative to wild-type. Mitochondrial loading of calcium can lead to rupturing of the inner membrane, collapse of the $\Delta\psi_m$ and inhibition of ATP production (Parihar et al., 2008a). This study therefore aimed to establish whether A53T mutant alpha-synuclein sensitised neurons through a reduction in mitochondrial calcium buffering capacity.

6.2 Experimental procedures

6.2.1 Primary mouse cortical culture

Primary dissociated cortical cell cultures were prepared from individual embryonic (E14) mutant (A53T) alpha-synuclein transgenic and non-transgenic mice, as described in chapter 2.4. Prior to cortical dissection livers were removed from individual embryos for genotyping (Chapter 2.3)

6.2.2 Microscopy and data analysis

Live cell imaging, immunocytochemical analysis of cultures, and LSM image acquisition was performed as previously stated (Chapter 2.8). Data is presented as mean ± standard error of the mean (SEM) and represents averages from a minimum of four individual culture repeats, each assessed in duplicate. Statistical analysis was performed using the Student's *t*-test. P-values < 0.05 were considered significant.

6.2.3 Oxidative stress, mitochondrial impairment and excitotoxicity

Cell stress models were applied to cortical cultures at 15 DIV. Mild oxidative stress (six hours antioxidant free growth media) and excitotoxicity (25 μM glutamate, six hours), were induced as described in chapter 2.5. Inhibition of complex I was mediated by the addition of rotenone (5 nM for six hours). Changes in cell viability were determined with PI staining as described in chapter 2.7.

6.2.4 Isolation of mitochondria

Brain mitochondria were isolated from mutant (A53T) alpha-synuclein transgenic and non-transgenic mice between eight and ten weeks of age, as described in section 2.9. Liver mitochondria were isolated as described by Damiano and coworkers (2006), with the following modifications. Mutant (A53T) alpha-synuclein transgenic and non-transgenic mice were anesthetised and perfused with isolation medium (IM) as described in chapter 2.9. The superficial lobe of the liver was removed and placed into ice cold IM. Tissue was homogenised in a glass Dounce homogeniser in 10% (w/v) IM, and centrifuged 10 min at 600 *g*. The supernatant was filtered through 40 µm gauze and centrifuged for 11 min at 4236 *g*. The pelleted material was resuspended in BSA-free IM, and both centrifugation steps were repeated in this medium to obtain a mitochondrial enriched fraction. Protein concentration was determined with the BioRad dye-binding assay (Chapter 2.9) and isolated mitochondria were stored on ice until use.

6.2.5 Activity dependent mitochondrial H_2O_2 production from complex I/III

Mitochondrial H_2O_2 production was measured in isolated mitochondria according to the methods of Whiteman and colleagues (2009). To specifically compare the contribution of complex I/III activity to total mitochondrial H_2O_2, isolated mitochondria (20 µg) were incubated in a reaction buffer containing IM, 0.1 mM ADP, the complex I substrate NADH (5 mM ß-NADH in 35 mM KH_2PO_4 buffer (pH 7.2)), 1 mM malic acid, 0.1 U/mL HRP and 50 µM Amplex ultraRED. The reaction was run as described in chapter 2.10.

6.2.6 Measurement of mitochondrial Ca^{2+} loading capacity

Mitochondrial calcium capacity was measured with the ratiometric, membrane impermeable calcium indicator, Fura-6F (Molecular Probes), according to the methods of Damiano and colleagues (2006) with minor alterations. Briefly, mitochondria were isolated from brain and liver of adult mutant (A53T) alpha-synuclein transgenic and non-transgenic mice between 8-10 weeks of age (Chapter 2.9). Isolated mitochondria were washed once in IM, centrifuged to pellet and resuspended in an incubation medium (100 µL for brain and 1 mL liver) consisting of 125 mM KCl; 20 mM HEPES, 2 mM KH_2PO_4; 2 mM $MgCl_2$; 5 mM succinic acid; 1 µM rotenone, pH was adjusted to 7.2 with Tris base (2-Amino-2-hydroxymethyl-propane-1,3-diol). Mitochondrial protein content was determined by the Bio-Rad dye-binding assay. Mitochondria (6.5 µg) were added to reaction buffer, comprised of incubation medium containing 1 µM oligomycin, 0.2 mM ADP and 0.3 µM Fura-6F, to a final volume of 100 µL. Bolus additions of 2 nM $CaCl_2$ were made every 4 min (with three sec shaking, every 2 min) and the ratio of Fura-6F fluorescence (340/380 nm excitation, 510 nm emission) was recorded at 25 sec intervals on a FLUOstar optima platereader (BMG labtech, Ortenberg, GER). The 340/380 Fura-6F ratio displayed linear sensitivity for the external Ca^{2+} concentration [external Ca^{2+}], which was not effected by addition of mitochondria with ruthenium red (2 µM). A coefficient for the relationship between [external Ca^{2+}] and the Fura-6F ratio was obtained by subsequent additions of 2 nM bolus $CaCl_2$ into mitochondrial-free reaction buffer. Total calcium uptake by mitochondria was calculated by subtraction of the minimum [external Ca^{2+}] value from the peak [external Ca^{2+}] value after each bolus $CaCl_2$ addition. The total calcium capacity is represented by the mean sum of these values (± SEM). Results were normalized to a negative control containing 2 mM EGTA.

6.3 Results

6.3.1 Mutant (A53T) alpha-synuclein sensitises neurons to oxidative stress and complex I inhibition

The A53T alpha-synuclein mutation sensitises dopaminergic neurons to MPTP. To determine if this effect is specific to pathways involving dopamine oxidation, or representative of a non-specific vulnerability to mitochondrial dysfunction, cortical neurons derived from mutant (A53T) alpha-synuclein transgenic and non-transgenic derived cortical neurons were subjected to an *in vitro* model of complex I inhibition (5 nM rotenone; six hours). Loss of membrane integrity (Propidium Iodide; PI staining) was used as an early quantitative measure of cell death (Chapter 2.7). Expression of the A53T mutation sensitised cortical neurons to loss of cell viability ($P<0.05$) following complex I impairment. This was evidenced by a 17.4% (± 1.39) increase ($P<0.05$) in PI stained neurons in cultures derived from mutant (A53T) alpha-synuclein transgenic embryos, compared with a 12.7% (± 1.69) increase in those from non-transgenic littermates, following rotenone treatment (Figure 6.1A)

The vulnerability of A53T transgenic and non-transgenic neurons to oxidative stress was confirmed by treatment of mature cortical cultures in antioxidant-free media (six hours). Mutant alpha-synuclein expression was associated with a marked decrease in cell viability ($P<0.05$). Specifically, cultures derived from mutant (A53T) alpha-synuclein transgenic embryos had a higher proportion ($P<0.05$) of PI positive neurons, (42.06% ± 6.09 increase

from control), relative to those derived from non-transgenic (25.29% ± 4.24 increase from control) littermates (Figure 6.1B).

6.3.2 Mutant (A53T) alpha-synuclein does not effect the rate of mitochondrial free-radical production

To further characterise the contribution of mutant alpha-synuclein to mitochondrial dysfunction and free-radical production, the rate of hydrogen peroxide production was compared in mitochondria isolated from adult A53T alpha-synuclein transgenic and non-transgenic mice. In the current study, NADH and malic acid were added to the reaction buffer to determine the effect of mutant alpha-synuclein expression on mitochondrial free radical production through complex I/III activity. There was no difference ($P>0.05$) in the rate of H_2O_2 production in somatic mitochondria isolated from whole brains of A53T transgenic and non-transgenic mice.

6.3.3 Mutant (A53T) alpha-synuclein does not activate intrinsic apoptotic pathways

Previous results suggest alpha-synuclein acts downstream from an increase in mitochondrial free radical production to prevent apoptosis (Chapter 4). The involvement of mutant alpha-synuclein in the activation of cell death pathways was therefore assessed in the current culture paradigm in response to mild oxidative stress. Oxidative toxicity caused induction of mitochondrial-mediated apoptosis, characterised by cytochrome c release (Figure 6.2A) and caspase-3 activation (Figure 6.2B). However, mutant alpha-synuclein expression did not augment ($P>0.05$) cytochrome c release (non-transgenic 10.1% ± 1 increase from control; A53T alpha-synuclein transgenic 15.3% ± 2 increase from control)

or caspase-3 activity (non-transgenic 20.4% ± 3 increase from control; A53T transgenic 11.2% ± 3 increase from control) following treatment.

6.3.4 Toxicity associated with mutant (A53T) alpha-synuclein is specific to cellular pathways of oxidative stress

The above data indicates that mutant alpha-synuclein does not contribute to mitochondrial free-radical production or activation of specific apoptotic pathways. This study therefore aimed to determine if the increased vulnerability of cortical neurons derived from mutant (A53T) alpha-synuclein transgenic embryos represents a generalised increase in cellular susceptibility to insult, which may be associated with induction of calpain-mediated cell death or necrosis. The current culture model was used to compare the effect of A53T synuclein transgene expression on the response of cortical neurons to glutamate excitotoxicity (25 μM; six hours). Mutant (A53T) alpha-synuclein expression did not influence the loss of cell viability ($P>0.05$) in non-transgenic (29.7% ± 3) compared with transgenic (31.7% ± 5) cortical neurons.

6.3.5 Mutant (A53T) alpha-synuclein did not affect total mitochondrial Ca^{2+} capacity

Alpha-synuclein mutations are associated with increased intramitochondrial Ca^{2+} levels, which can drive subsequent increases in nitric oxide levels, free radical production and cytochrome c release (Hettiarachchi et al., 2009). The current study aimed to determine whether these factors underlie the sensitivity of neurons expressing the A53T alpha-synuclein mutation. Calcium uptake capacity was determined in mitochondria isolated

from brain and liver of mutant (A53T) alpha-synuclein transgenic mice, and their non-transgenic littermates. A representative trace of changes in the Fura-6 fluorescence ratio following $CaCl_2$ additions is given in Figure 6.3, and is linearly correlated to the [external Ca^{2+}]. Following the bolus addition of a 2 nM $CaCl_2$, mitochondria rapidly accumulated Ca^{2+}, which is reflected by a decrease in the Fura-6F fluorescence. Maximum Ca^{2+} capacity is defined by a lack of downward deflection in the Fura-6F ratio following $CaCl_2$ addition. There was no difference in the total calcium Ca^{2+} capacity between A53T alpha-synuclein transgenic mice (6.98 μM ± 1.07 Ca^{2+}/mg mitochondrial protein), and their non-transgenic (6.08 μM ± 0.93 Ca^{2+}/mg mitochondrial protein) littermate controls. Mutant (A53T) alpha-synuclein expression was not associated with differences in the Ca^{2+} capacity of liver mitochondria (Data not shown).

6.4 Discussion

Genes associated with the inheritance of Parkinson's disease indicate mitochondrial dysfunction and oxidative stress as primary causes of neurodegeneration. However, the role of alpha-synuclein mutations in these mechanisms is uncertain. Previous studies, and the proceeding chapters of this thesis, have indicated that wild-type alpha-synuclein protects neurons from oxidative stress. The current study aimed to determine if protein mutations counteract these functions, or cause toxicity through independent biochemical pathways. Mutant (A53T) alpha-synuclein compromised the neuronal response to oxidative stress and complex I inhibition (Figure 6.1B), but was not associated with increased mitochondrial free radical production, alterations in mitochondrial calcium buffering capacity or induction of specific apoptotic pathways. These data indicate that mutant alpha-synuclein acts through an undetermined toxic gain of function pathway to impair the neuronal response to oxidative stress.

Overexpression of wild-type and mutant alpha-synuclein can impair mitochondrial function and augment free radical production through direct binding interactions (Hsu et al., 2000; Song et al., 2004; Devi et al., 2008; Parihar et al., 2009). There is evidence that this mitochondrial damage mediates the toxicity of mutant alpha-synuclein. However, few studies have provided evidence for differential roles of wild-type and mutant protein in these mechanisms. Despite increasing neuronal vulnerability to oxidative stress, results from the current study indicate that mutant alpha-synuclein does not contribute to mitochondrial free radical production. Rather the current findings suggest that mutant (A53T) alpha-synuclein acts downstream to sensitise neurons to oxidative toxicity.

Previous chapters of this thesis have established a role for wild-type alpha-synuclein in prevention of cytochrome c release and caspase-3 activation (Figure 4.4; 4.5). Induction of apoptosis was assessed in cortical neurons to ascertain whether the toxicity caused by mutant alpha-synuclein expression was due to a loss of this protective function. There was, however, no association between A53T mutant alpha-synuclein and induction of specific apoptosis pathways. These data therefore indicate that mutant alpha-synuclein increases neuronal susceptibility to oxidative stress through a molecular pathway independent from loss of wild-type function.

Mutant alpha-synuclein is proposed to mediate neuronal toxicity through impaired function of multiple cellular pathways including protein degradation (Liu et al., 2005) and perturbed calcium homeostasis (Calì et al., 2011). The current study specifically investigated the effect of these mutations on mitochondrial calcium capacity. Mutant alpha-synuclein can increase cellular membrane permeability to calcium, which has been implicated in its contribution to neurodegeneration (Furukawa et al., 2006). Specifically, the A53T mutation drives the formation of oligomeric species, which enhance the plasma ion permeability (Danzer et al., 2007) and can insert into cellular membranes and enable calcium influx (Lashuel et al., 2002). The effect of mutant alpha-synuclein on extracellular calcium influx is minimal however, and its contribution to pathology remains controversial (Calì et al., 2011). Recent evidence suggests that deregulation of intracellular calcium storage and release may have a more significant role in neurodegeneration. Mitochondria are a primary site for buffering excess intracellular calcium. Studies by Parihar and colleagues indicate

that mitochondrial association of both mutant and wild-type alpha-synuclein increase intramitochondrial calcium stores (Parihar et al., 2008b), which can drive nitric oxide synthesis, oxidative stress and mitochondrial membrane permeability transition (Parihar et al., 2008a; Parihar et al., 2008b). Results from the current study indicate that calcium-buffering capacity was not affected by somatic overexpression of mutant (A53T) alpha-synuclein and negate a role for this protein in impaired mitochondrial calcium homeostasis. However, Parihar and colleagues directly incubated mitochondria with high doses of alpha-synuclein and discrepancies between the current, and previous data may reflect differences in the concentration of this protein. These data therefore suggest that the relative concentration of alpha-synuclein is a stronger determinant of mitochondrial calcium dysfunction than mutations to the protein.

Further definition of the differential effects of wild-type and mutant alpha-synuclein would require direct comparisons between overexpression of wild-type and mutant protein on the sensitivity of neurons to these stress models. The current data does however, indicate that expression of mutant (A53T) alpha-synuclein sensitises primary cortical neurons to oxidative toxicity. This increase in neuronal vulnerability was not associated with a loss of the protective function of the wild-type protein. From these results, it may be deduced that mutant alpha-synuclein is likely to mediate neurotoxicity through a toxic gain of function, which is independent of effects on mitochondrial free radical production or calcium buffering. These data highlight the complex role of alpha-synuclein in disease pathogenesis, and the differential contributions of wild-type and mutant protein. These

findings have significant implications for the use of mutant models to establish the role of alpha-synuclein in sporadic PD cases.

7 Final Discussion

Two critical articles were published in 1997, which identified alpha-synuclein as the principal component of Lewy bodies (Spillantini et al., 1997) and linked mutations in this protein to genetic inheritance of PD (Polymeropoulos et al., 1997). There have since been major advances in understanding the contribution of alpha-synuclein to disease pathogenesis. Many studies have employed models of gene overexpression and mutation to investigate the role of this protein in cell pathology and have uncovered molecular pathways through which alpha-synuclein modifies neuronal viability. However, the involvement of endogenous wild-type alpha-synuclein in these mechanisms had remained unclear. This ambiguity was confounded by a lack of understanding surrounding the normal function of the protein. Therefore, the central aim of this thesis was to clarify the function of endogenous wild-type alpha-synuclein and its role in the neurodegenerative mechanisms of PD and DLB. The final chapter of this thesis examined the hypothesis that mutant alpha-synuclein induces neurotoxicity through a toxic gain of function mechanism, which is independent of its normal role.

7.1 The contribution of alpha-synuclein to selective neurodegeneration in PD and DLB

Although the cause of pathology in PD and DLB is unknown, neurodegeneration involves dysfunction of several cellular pathways including mitochondrial metabolism, protein degradation and free radical scavenging. These are discussed in detail in the introductory chapter of this thesis, where the potential role of dopamine oxidation in the specific loss of

dopaminergic neurons in PD is emphasised. However, data within this study (Chapter 3.3) do not support the notion that dopamine metabolism sensitises neurons to oxidative stress. Rather, they indicate that a complex interaction of factors contribute to neurodegeneration. These results are further supported by problems with the dopamine oxidation hypothesis (reviewed in Chapter 1.4.1; Ahlskog, 2005).

Despite inconsistent evidence for the role of dopamine in PD pathology, defective sequestering of this neurotransmitter provides a plausible mechanism for its contribution to selective cell loss. Overexpression of alpha-synuclein directly increases cytoplasmic dopamine content and drives cellular toxicity (Bisaglia et al., 2010), presumably through an increase in the generation of its oxidative metabolites. Endogenous expression of alpha-synuclein is conversely associated with inhibition of DAT activity, reduced dopamine uptake and a decrease in its oxidation (Wersinger and Sidhu, 2003). This effect is in concordance with the neuroprotective properties of endogenous alpha-synuclein (reported in Chapters 3 & 4). However, the parallel responses of both cortical and dopaminergic neurons (Chapter 3.3) indicate that alpha-synuclein expression mediates protection through a mechanism independent of dopamine metabolism. This association supports other independent studies, which indicate a neuroprotective effect of endogenous alpha-synuclein upregulation across multiple cell types (Vila et al., 2000; Manning-Bog et al., 2002; Leng and Chuang, 2006).

Although previous studies (Manning-Bog et al., 2002; Quilty et al., 2006) imply an association between alpha-synuclein levels and neuroprotection, they have failed to

directly examine whether increased intracellular protein is a result of transcriptional upregulation or impaired protein degradation. Quantitative PCR studies report increases in alpha-synuclein gene expression in primary neurons following MPTP administration (Vila et al., 2000) and valproic acid treatment (Leng and Chuang, 2006). Given that protein expression is energetically expensive, these data indicate that alpha-synuclein upregulation is an endogenous response mediated by cell survival pathways. In the current study (Chapter 3.3.4) there was no association between increased intracellular alpha-synuclein and differential localisation of ubiquitin following chronic oxidative stress treatment. These data suggests normal function of the UPS and provides further support for transcriptional upregulation of this protein. Ultimately these results indicate that induction of alpha-synuclein expression is an innate response of heterogeneous neuron populations under stress.

7.1.1 The role of UPS impairment in alpha-synuclein aggregation

Proteasomal dysfunction is a pathogenic feature of PD (McNaught and Jenner, 2001; McNaught et al., 2003), and pharmacological inhibition of the proteasome is directly linked to alpha-synuclein aggregation and dopaminergic degeneration (McNaught et al., 2002b; McNaught et al., 2004; Rideout et al., 2005). Although UPS impairment is likely to contribute to disease progression, its contribution to alpha-synuclein pathology is unclear. Data within this thesis (Chapter 3.3.4-5) suggest that both chronic oxidative stress and proteasome impairment induce the accumulation of ubiquitinated (presumably aggregated) alpha-synuclein. However, these toxic mechanisms induce different cellular localisation of alpha-synuclein aggregates, suggesting that oxidative stress is able to induce abnormal

alpha-synuclein aggregation through a mechanism independent of UPS impairment. The cellular consequences of these differential responses are not known, although nuclear localisation of alpha-synuclein has previously been associated with toxicity (Xu et al., 2006; Monti et al., 2007).

7.1.2 The alpha-synuclein load hypothesis

The differential effects of oxidative stress and proteasome impairment may be clinically irrelevant, given the strong evidence for both (presumably concomitant) pathogenic processes in disease (McNaught and Jenner, 2001; Jenner, 2003; McNaught et al., 2003; Wersinger and Sidhu, 2006). Furthermore, chronic oxidative stress indirectly impairs protein degradation through accumulation of damaged proteins and saturation of the UPS (Elkon et al., 2004; Branco et al., 2010). However, the link between these mechanisms provides a plausible basis for the notion that increases in intracellular alpha-synuclein precedes its concentration dependent aggregation. This hypothesis is diagrammatically outlined in Figure 7.1 (acquired from Cookson, 2006) and evidence for this theory has been detailed by both Cookson (2006) and McGeer and McGeer (2008). The alpha-synuclein load hypothesis is founded on a proposed correlation between its concentration and aggregation (Kim and Lee, 2008), but is verified by clinical evidence. Specifically, genetic multiplications of the *SCNA* gene are a direct cause of PD and the age of onset in these cases positively correlate to the gene copy number (Singleton et al., 2003; Chartier-Harlin et al., 2004). Furthermore, mutations to proteins associated with functions and/or cellular clearance of alpha-synuclein [namely parkin (Shimura et al., 2001) and indirect functions of UCH-L1 (Kabuta and Wada, 2008)] are linked with familial disease (Kitada et

al., 1998) and impairment of both the UPS and the lysosomal autophagy pathway lead to accumulation of alpha-synuclein (Ebrahimi-Fakhari et al., 2011). Collectively these data provide clinical and experimental support for the alpha-synuclein load hypothesis.

7.1.3 Two-hit hypothesis for the role of alpha-synuclein in neurodegeneration

The alpha-synuclein load hypothesis proposes a potential mechanism through which accumulation of this protein can drive toxicity, but fails to explain the selective nature of cell loss in PD and DLB. This subject is addressed in the current study, which provides significant insight into the regulation of intracellular alpha-synuclein levels among different cell populations. These data indicate that the basal expression of alpha-synuclein varies among subpopulations of dopaminergic and cortical neurons (Chapter 3 and 4) and determines the susceptibility of cortical neurons to oxidative stress and rotenone (Chapter 4.3.6; Figure 4.4). However, the current results also show that an additional insult following chronic oxidative stress (either acute oxidative toxicity or proteasome impairment) drives the accumulation of ubiquitinated alpha-synuclein aggregates in neurons expressing high levels of the protein (Chapter 3.3.4-5). These data suggest a two-hit hypothesis for the role of alpha-synuclein in neurodegeneration. This theory is outlined in Figure 7.2 and involves an initial neuroprotective upregulation of alpha-synuclein, which may be mediated by the activation of cell stress response pathways in response to mild toxic stress. According to this model, genetic predisposition or additional toxicity can induce a secondary toxic aggregation of alpha-synuclein. The current study implies this secondary process (Chapter 3.3.4-5), may be a direct result of further oxidative toxicity

(Hashimoto et al., 1999b) or indirectly mediated by an increased intracellular load of the protein (Figure 1.4) (Uversky, 2007; Kim and Lee, 2008).

7.1.4 Summary of the involvement of alpha-synuclein in selective vulnerability

The first specific aim of this thesis was to determine the role of endogenous alpha-synuclein in the selective vulnerability of neurons exposed to oxidative stress. The results within chapter three do not indicate that dopaminergic neurons have increased sensitivity to oxidative stress; instead they suggest that alpha-synuclein expression indirectly dictates susceptibility through neuroprotective functions. The current results provide further evidence that alpha-synuclein upregulation is involved in neuronal oxidative stress preconditioning, but this endogenous response may predispose neurons to toxic consequences of alpha-synuclein aggregation. Specifically, a secondary insult was shown to drive the formation of ubiquitinated alpha-synuclein accumulations. These results support a two-hit hypothesis for the role of alpha-synuclein in neurodegeneration.

7.2 Hero or villain? The cellular mechanisms which define the two faces of alpha-synuclein

The second aim within this thesis was to establish the mechanism by which alpha-synuclein confers protection against oxidative stress in cultured primary neurons (Aim 2; Chapter 4). Current data indicate that alpha-synuclein prevents the activation of apoptosis in *in vitro* models of oxidative stress and rotenone-mediated complex I inhibition (Chapter 4.3.1; Figure 4.4.1-3). These models were used to clarify the contrasting roles of alpha-synuclein in cytosolic and mitochondrial free radical production, respectively.

Specifically, alpha-synuclein is implicated in neuronal survival against oxidative toxicity (Hashimoto et al., 2002; Manning-Bog et al., 2003; Quilty et al., 2006; Monti et al., 2007), but conversely augments neurotoxicity of the complex I inhibitor MPTP/MPP+ (Klivenyi et al., 2006; Fountaine and Wade-Martins, 2007; Fountaine et al., 2008). The current data (Chapter 4.3.1; Figure 4.4) exclude a role for alpha-synuclein in the toxicity process of complex I inhibition, and instead found that it prevents induction of intrinsic apoptotic pathways in cortical neurons exposed to low-dose rotenone as well as oxidative stress (Chapter 4.3). The differential effects of alpha-synuclein against MPP+ and rotenone toxicity are likely to reflect the displacement of dopamine by MPP+, leading to its cytoplasmic accumulation and production of oxidative metabolites. The notion that MPP+ induces toxicity independent of its actions on complex I is supported in a recent study by Choi and colleagues (2011). Theses results have significant implications for the uses of MPTP as a model of PD, particularly in regard to mitochondrial pathology.

The lack of association between alpha-synuclein expression and excitotoxicity (Chapter 4.3.1) provides critical evidence that alpha-synuclein specifically intercepts pathways of oxidative toxicity. Results in chapter four indicate that alpha-synuclein prevented induction of intrinsic apoptosis pathways upstream from cytochrome c release (Chapter 4.3.6-7). These data are in accordance with previous reports that alpha-synuclein modulates apoptosis through interaction with pro-apoptotic proteins acting immediately upstream of the mitochondrial permeability transition. Specifically, indirect actions of alpha-synuclein regulate the activation of Bak and Bax, which permeabilise the outer mitochondrial membrane (Kaul et al., 2005). During apoptosis, noxious stimuli activate BH3-only proteins, which antagonistically bind to anti-apoptotic Bcl2 members thereby

allowing the insertion of Bak and Bax into the mitochondrial membrane (Degterev and Yuan, 2008). Alpha-synuclein is shown to bind to non-phosphorylated Bad, a pro-apoptotic inhibitor of Bcl-2 (Ostrerova et al., 1999; Kroemer et al., 2007) and directly inhibit its activity (Kaul et al., 2005a).

The mechanisms of neuroprotection described above are maintained across all pathways of intrinsic apoptosis. However, the aim of this study was to determine the involvement of alpha-synuclein in specific pro-apoptotic pathways activated by oxidative stress. These molecular cascades mediate apoptosis through downstream activities of the Bcl-2, and BH3-only protein families. The proposed chaperone activity of alpha-synuclein (Ostrerova et al., 1999; Chandra et al., 2005), suggests that it has a role in these cell stress response pathways. As these neuronal survival mechanisms involve the expression of pro-survival genes (Calabrese et al., 2010), upregulation of alpha-synuclein (Chapter 3.3) is therefore likely to reflect a role in neuronal preconditioning. This possibility is supported by current evidence linking high endogenous expression of the protein, with neuronal resistance to additional toxicity (Chapter 3.3.2) as well as the independent data from Monti and colleagues (2007).

The involvement of alpha-synuclein in preconditioning fits with a proposed role for the ceramide-mediated cell stress response pathway in PD (France-Lanord et al., 1997; Arboleda et al., 2009). Specifically, alpha-synuclein may indirectly modify the generation of ceramides from their sphingomyelin precursors through negative regulation of palmitic

acid uptake (Golovko et al., 2005) and downstream induction of ceramide synthesis (reviewed in Bras et al., 2008).

The current study (Chapter 4.3.7) demonstrates that alpha-synuclein confers neuroprotection through modulation of MAPK activity. This may reflect a downstream effect of altered ceramide levels, or a direct interaction with components of the MAPK signalling cascade. The relative level of MAPK activation dictates its downstream consequences, particularly in respect to its involvement in cell survival and apoptotic pathways. At present, the role of MAPK signalling in the cellular response to oxidative stress is complex and unresolved. However, gene deletion studies provide evidence for the involvement of alpha-synuclein in MAPK pathways. Specifically, Kuhn and colleagues (2007) reported an increase in JNK1 transcription and a decrease in ERK1 (MAPK1) transcription in alpha-synuclein knockout mice, suggesting a pro-survival role for alpha-synuclein in the regulation of MAPKs (Kuhn et al., 2007). Previous reports show that alpha-synuclein inhibits JNK and ERK pathways (Hashimoto et al., 2002). (Iwata et al., 2001a; Iwata et al., 2001b). These studies were conducted in stably transfected cell lines (GT1-7 and neuro2a cells, respectively), but found differential effects of alpha-synuclein expression on cell viability, which may reflect the tight physiological regulation of both the MAPK pathways and the expression of alpha-synuclein.

Results within this thesis indicate that alpha-synuclein can also modify neuronal susceptibility to oxidative stress preceding the activation of cell response pathways. Metabolomic screening of media derived from alpha-synuclein knockout and wild-type

mice identified increases in the release of glycolytic metabolites in alpha-synuclein null neurons (Chapter 5.3.3). These results indicate a functional role for alpha-synuclein in facilitating mitochondrial energy production through oxidative phosphorylation, and are consistent with previous reports that alpha-synuclein indirectly facilitates the linked-activity of and NADH cytochrome c reductase (complex I/III) (Ellis et al., 2005; Devi et al., 2008). These data may have significant implications for the vulnerability of neurons exposed to oxidative stress. Specifically, glycolytic activity can induce oxidative toxicity in primary neurons, and further compromise the reduction of GSSH (Herrero-Mendez et al., 2009). Loss of alpha-synuclein function may therefore reduce available GSH stores and lead to the accumulation of H_2O_2 within the mitochondria and cytosol. Although this is a plausable explaination for the increased susceptability of alpha-synuclein knockout neurons toward oxidative stress, independent data within this thesis indicates no reduction in either antioxidant capacity (chapter 4.3.5), or free radical production (Chapter 4.3.3-4).

In addition to its effects on the ETC, Ellis and colleagues (2005) also report decreased levels of cardiolipin in whole brain from alpha-synuclein knockout mice. Cardiolipin is involved in maintaining physical association between ETC complexes, the electrochemical gradient across the inner mitochondrial membrane and the membrane association of cytochrome c (Chicco and Sparagna, 2007). These data therefore suggest a role for alpha-synuclein in oxidative phosphorylation and may imply a secondary function in prevention of apoptosis through cytochrome c release. However, Pennington and colleagues have conversely reported that alpha-synuclein reduces ATP synthesis through a reduction in complex I activity. These findings correlate to significant differences in the mitochondrial

proteasome of SH-SY5Y cells overexpressing alpha-synuclein (Pennington et al., 2010). The discrepancies between these data and the current results are likely to reflect the levels of alpha-synuclein expression. The current, and previous data provide substantial evidence that alpha-synuclein modulates mitochondrial energy production, which is likely to be mediated through an involvement in the synthesis and import of mitochondrial proteins.

7.2.1 Summary of neuroprotective mechanisms

Data reported in chapters four and five address the second aim of this thesis, which was to establish the mechanism by which alpha-synuclein confers neuroprotection. These studies indicate a functional role for alpha-synuclein in facilitating mitochondrial energy production (Chapter 5) and direct modulation of the neuronal response to oxidative stress (Chapter 4). Both of these effects concur with previous studies (da Costa et al., 2000; Alves Da Costa et al., 2002; Ellis et al., 2005; Quilty et al., 2006; Monti et al., 2007), and collectively indicate that alpha-synuclein can modulate neuronal viability through multiple mechanisms. Such divergent functions may derive from alpha-synuclein's role as a molecular chaperone, able to bindof alpha-synuclein to bind to multiple proteins and lipids (Mcfarland et al., 2008; Ruipérez et al., 2010) Chaperone activity may also mediate the synaptc activities of alpha-synuclein (Chandra et al., 2005; Nemani et al., 2010), the synthesis and import of mitochondrial proteins (Ellis et al., 2005; Pennington et al., 2010). The proposed role of alpha-synuclein upregulation in neuronal preonditioning and neuroprotection further supports a chaperone function (Vila et al., 2000; Manning-Bog et al., 2002; Quilty et al., 2006; Monti et al., 2007).

The current indications of a neuroprotective effect of endogenous alpha-synuclein would be strengthened if the resistance of wild-type neurons were re-established following restoration of this protein into *Snca* null neurons. However, current methods of gene delivery fail to control the dose of gene transcription. Because high intracellular concentrations of alpha-synuclein may drive its toxic aggregation (Cookson, 2006; Kim and Lee, 2008) such experiments may not simulate the effects of endogenous protein.

7.3 Neurotoxicity of alpha-synuclein is conferred through a toxic gain of function

The contribution of alpha-synuclein load in cell health remains contentious. However, results within this study provide evidence of a two-hit hypothesis for the involvement of alpha-synuclein in neurodegeneration (Chapter 3; Figure 7.2). Proposed mechanisms by which alpha-synuclein can infer neurotoxicity are discussed in the introductory chapter (Chapter 1.4) and those relative to the current results involve the role of protein aggregation. Specifically, these aggregates act through a toxic gain of function mechanism to compromise membrane integrity and disrupt cytoplasmic ion homeostasis (Danzer et al., 2007; van Rooijen et al., 2010b, van Rooijen et al., 2010a), although the full consequences of alpha-synuclein aggregation are not resolved.

In addition to multiplications, three rare mutations within the *SNCA* gene cause early onset familial PD. The causal link between these mutations and disease is unclear and is further complicated by the ambiguous function of wild-type protein. However, other genes involved in familial disease have provided some insight into pathogenic mechanisms of familial disease. Several proteins (namely DJ-1, PRKN and PINK1) highlight the role of

mitochondrial dysfunction in disease related neurodegeneration. These pathways are further implicated through pharmacological induction of Parkinsonism and PD, by MPTP and rotenone. However, there is little evidence directly linking alpha-synuclein and LRRK2 in mitochondrial pathology. It has been suggested that mutations to these proteins may induce a toxic gain of function, which acts through mitochondrial pathways. However the association between these gene mutations and disease alternatively suggest the contribution of divergent molecular pathways. Therefore, the fourth aim within this thesis was to establish the neurotoxic mechanisms of mutant (A53T) alpha-synuclein. Studies within chapter six tested the hypothesis that the A53T alpha-synuclein mutation incurred a toxic gain of function, which sensitises neurons to oxidative stress through mitochondrial dysfunction. Although the precise molecular pathway of neurotoxicity was not defined in this study, the results indicated that expression of mutant A53T alpha-synuclein augments oxidative stress (Chapter 6.3.1) downstream from mitochondrial free radical production (Chapter 6.3.2).

There is strong evidence to suggest that overexpression of wild-type alpha-synuclein is associated with a toxic gain of function. The current data further support a toxic function of mutant (A53T) protein (Chapter 6). Direct comparisons of mutant alpha-synuclein expression and wild-type overexpression suggest these may share a common gain of toxic function common mechanisms of neurotoxicity (Miller et al., 2007; Parihar et al., 2008b; Cho et al., 2011; Reynolds et al., 2011). This supports clinical data linking both gene mutations and multiplications to disease.

The lack of association between alpha-synuclein mutations and mitochondrial dysfunction imply that this protein acts through pathways independent of DJ-1, Parkin and PINK1. Although there is evidence that these proteins influence a common molecular pathway, the toxic mechanisms of alpha-synuclein may be more closely related to the functions of LRRK2 (Cookson and Bandmann, 2010; Greggio et al., 2011). This has significant implications for elucidating the mechanisms of PD and DLB. These data specifically support the hypothesis that these conditions represent a single clinical outcome of divergent pathogenic mechanisms.

7.4 Implications and limitations

Alpha-synuclein is central to the pathogenesis of PD and DLB. A full understanding of its role in neuronal function and in degeneration may therefore provide critical insight into the mechanisms of these diseases. It is hoped that this knowledge will form a solid basis for the future development of effective therapeutics or methods of disease prevention. Data within this thesis has clarified both the normal function of alpha-synuclein and identified potential ways in which alpha-synuclein may contribute to the selective cell loss associated with both PD and DLB.

A critical observation within the current thesis is the notable difference in the tolerance of primary neurons derived from mice and rats, to the current *in vitro* model of oxidative stress. Differences in the susceptibility of differential rodent species and mouse strains have previously been reported regarding the MPTP lesion model of Parkinson's disease (Riachi et al., 1998; Mitra et al., 1994; Hamri et al., 1999). The mechanisms underlying

these differences are unclear, although the increased maturation rate and decreased longevity in culture indicate differences in the metabolic activity of mouse and rat neurons. These differences may be amplified by the isolation of neurons, and their growth *in vitro*.

Finding from chapter three specifically indicate that alpha-synuclein mediates the neuronal survival mechanisms activated by oxidative stress. This pro-survival response is conserved among a heterogeneous subpopulation of both cortical and dopaminergic neurons. The neuroprotective effect of alpha-synuclein expression may signify its involvement in preconditioning neurons to oxidative stress, and fit with its proposed role as a molecular chaperone (Souza et al., 2000a).

Results within this thesis support a two-hit hypothesis for the role of alpha-synuclein in neurodegeneration (outlined in Figure 7.2). Specifically, mild, chronic oxidative stress was shown to induce a neuroprotective upregulation of this protein, which predisposed neurons to toxicity through alpha-synuclein aggregation upon additional environmental insult. Both oxidative stress and impairment of protein degradation are associated with normal aging, and are augmented in disease. Based on the two-hit hypothesis it be proposed that the combinations of these pathogenic processes could alpha-synuclein upregulation and may increase the cellular concentration beyond a threshold, which induces its aggregation. These results therefore have significant implications for understanding disease pathogenesis and indicate the involvement of alpha-synuclein in the relationship between age and disease onset.

The alpha-synuclein load hypothesis proposes that the toxicity of this protein results from a toxic gain of function, induced by its aggregation. The results in this thesis further imply that a similar mechanism underlies the sensitisation of mutant (A35T) alpha-synuclein expressing neurons to oxidative stress. Significantly, the current results suggest that the molecular pathways of alpha-synuclein toxicity are distinct from those of other mutations implicated in PD and DLB. Specifically, DJ-1, Parkin and PINK1 are implicated in mitochondrial dysfunction. Based on these conclusions, the use of alpha-synuclein mutant mice as models of PD pathology would need to be supplemented by an alternative model involving mitochondrial dysfunction.

The correlation between alpha-synuclein expression level and neurotoxicity remains contentious and may be clarified by accurate titration of intracellular protein concentration and its direct effect on cell viability. However, current limitations of gene transfection impede this assessment. Other restraints of the current studies include the use of cell culture models to examine the effects of oxidative stress. Specifically, the chemical reactions which occur under the hyperoxic conditions of cell culture significantly differ from those in a true homeostatic physiological environment (Halliwell et al., 2000). Cultured neurons are under a basal level of oxidative toxicity, which may select for the survival of resistant populations, and initiate cell stress response pathways.

Although *in vitro* models (specifically, primary cell culture and isolated organelles) provide a systematic platform to study mechanisms of toxicity they do not recapitulate the complex interactions and signalling cascades, which occur at the level of an organism. The

use of *in vivo* models is therefore necessary to verify the current *in vitro* data. However, etiological models of PD are complicated by imperative differences between humans and rodents, including life span, structural and functional organisation of the motor system and biochemical properties of the SN (such as the absence of neuromelanin in rodents) (Dauer and Przedborski, 2003).

7.5 Conclusions

In summary, the studies within this thesis have clarified the contribution of alpha-synuclein to normal neuronal function and the mechanisms of PD and DLB. The results have highlighted complexities surrounding the contentious role of alpha-synuclein in both neuroprotection and toxicity. Based on these findings, a sound hypothesis for the role of this protein in the pathogenesis of PD and DLB has been proposed. Significantly, this two-hit hypothesis validates past studies, which have detailed a role of alpha-synuclein in both neuroprotection through chaperone activity and in neurotoxicity through a toxic gain of function mechanism. Results within this thesis will provide a basis and direction for further investigations into the relationship between intracellular alpha-synuclein levels, and the selective nature of neurodegeneration in PD and DLB.

8 References

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9 Appendix

Appendix A

Composition of Neurobasal TM Medium

Component	Final concentration (mg/L)
Inorganic salts	
$CaCl_2$ (anhydrous)	200
$Fe(N0_3)_3 . 9H_2O$	0.1
KCI	400
$MgCI_2$ (anhydrous)	77.3
NaCl	3000
$NaHC0_3$	2200
$NaH_2PO_4 . H_2O$	125
$ZnSO_4 \cdot 7H_2O$	0.19
Other components	
D-Glucose	4500
HEPES	2600
Phenol red	8.1
Na pyruvate	25
Amino acids	
L-Alanineb	2
L-Arginine' HCI	84
L-Asparagine' H_2O	0.83
L-Cysteine	1.21
L-Glutamine[a]	73.5
L-Glutamate	
Glycine	30
L-Histidine HCl . H_2O	42
L-Isoleucine	105
L-Leucine	105
L-Lysine HCI	146
L-Methionine	30
L-Phenylalanine	66
L-Proline	7.76
L-Serine	42
L-Threonine	95
L-Tryptophan	16
L-Tyrosine	72

$_{L}$-Valine	94
Vitamins	
D-Calcium pantothenate	4
Choline chloride	4
Folic acid	4
i-Inositol	7.2
Niacinamide	4
Pyridoxal HCl	4
Riboflavin	0.4
Thiamine HCl	4
Vitamin B_{12}	0.34

[a]Not supplied in liquid medium; needs to be added.

Appendix B

Components of B27 Supplement

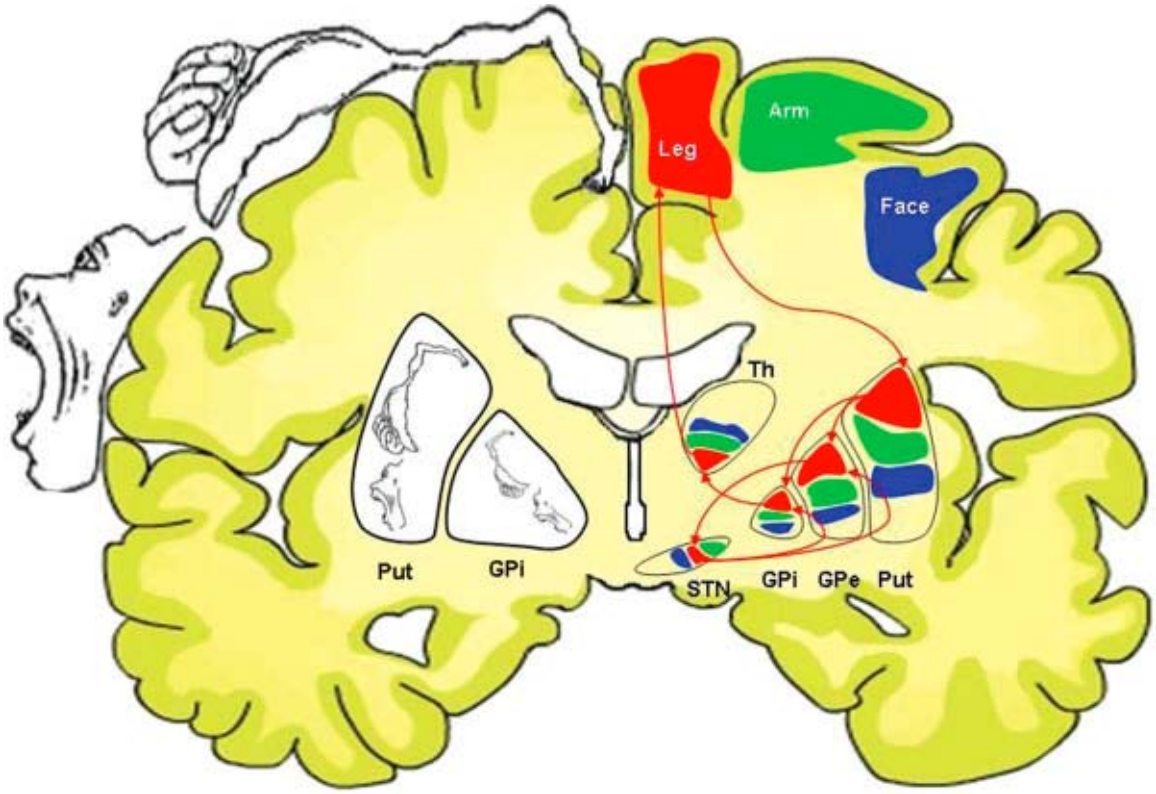
Components
Vitamins
Biotin
DL Alpha Tocopherol Acetate
DL Alpha-Tocopherol
Vitamin A (acetate)
Proteins
BSA, fatty acid free Fraction V
Catalase
Human Recombinant Insulin
Human Transferrin
Superoxide Dismutase
Other Components
Corticosterone
$_{D}$-Galactose
Ethanolamine HCl
Glutathione (reduced)
$_{L}$-Carnitine HCl
Linoleic Acid
Linolenic Acid
Progesterone
Putrescine 2HCl
Sodium Selenite
T3 (triodo-I-thyronine)

Appendix C

Components of A0-B27 Supplement

Components
Vitamins
Biotin
Vitamin A (acetate)
Proteins
BSA, fatty acid free Fraction V
Human Recombinant Insulin
Human Transferrin
Other Components
Corticosterone
$_{D}$-Galactose
Ethanolamine HCl
$_{L}$-Carnitine HCl
Linoleic Acid
Linolenic Acid
Progesterone
Putrescine 2HCl
Sodium Selenite
T3 (triodo-I-thyronine)

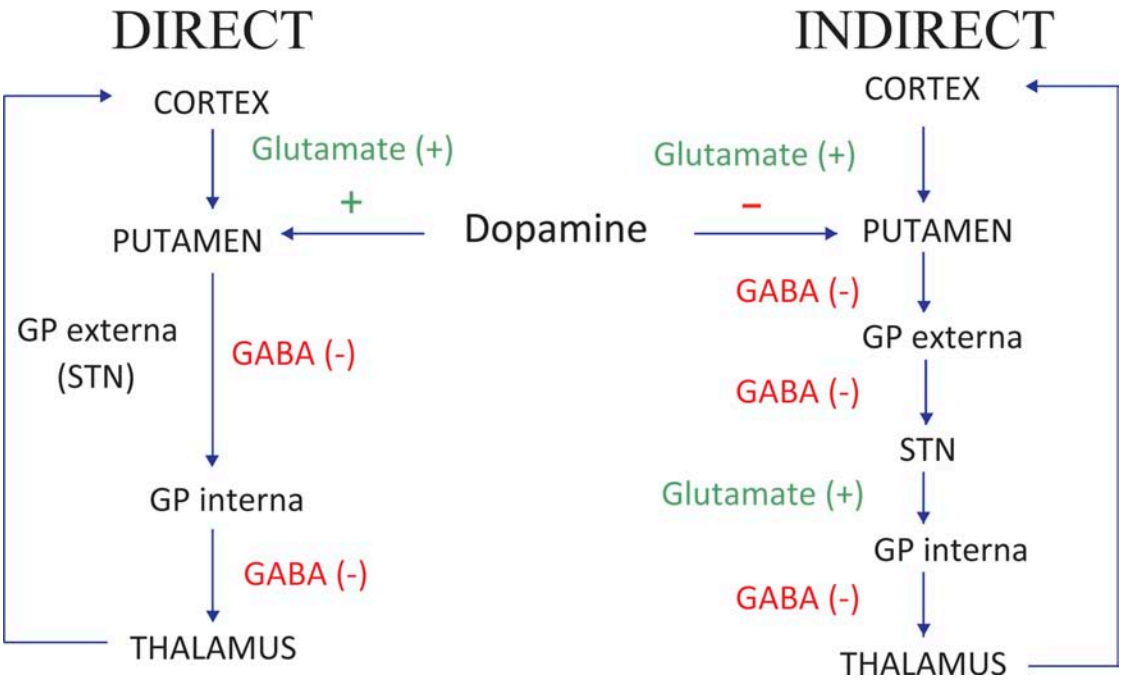
Figure 1.1 Functional organisation of the basal ganglia



Cortical inputs reach the basal ganglia through corticostriatal projections onto medium spiny neurons, which subsequently innervate the globus pallidus pars externa (GPe) and the subthalamic nucleus (STN). The cortical projections exert an opposite (inhibitory/excitatory) effect onto the GPe and globus pallidus pars interna. The GPe projects to the striatum and STN and inhibits their activity.

Figure reproduced from Obeso and colleagues (2006).

Figure 1.2 The effect of dopamine on the direct and indirect striatopallidal pathways



Dopamine release into the putamen regulates movement initiated by the motor cortex via the direct inhibitory, and indirect pathways. Dopamine regulates striatal activity by inhibiting the indirect pathway and facilitating the direct pathway.

Figure 1.3 Lewy bodies in tissue from patients with Parkinson's disease (A,C) and dementia with Lewy bodies (B). A pigmented neuron in the substantia nigra containing a 'classical' Lewy body (**A**) stained with hematoxylin and eosin. Hematoxylin and eosin staining of an atypical cortical Lewy body (**B**) in the temporal cortex of a patient with Dementia with Lewy bodies. A typical Lewy body in a pigmented neuron in the substantia nigra labeled with anti-a-synuclein (**C**). Images from Wakabayashi and colleagues, 2007.
Scale bar = 10 μM

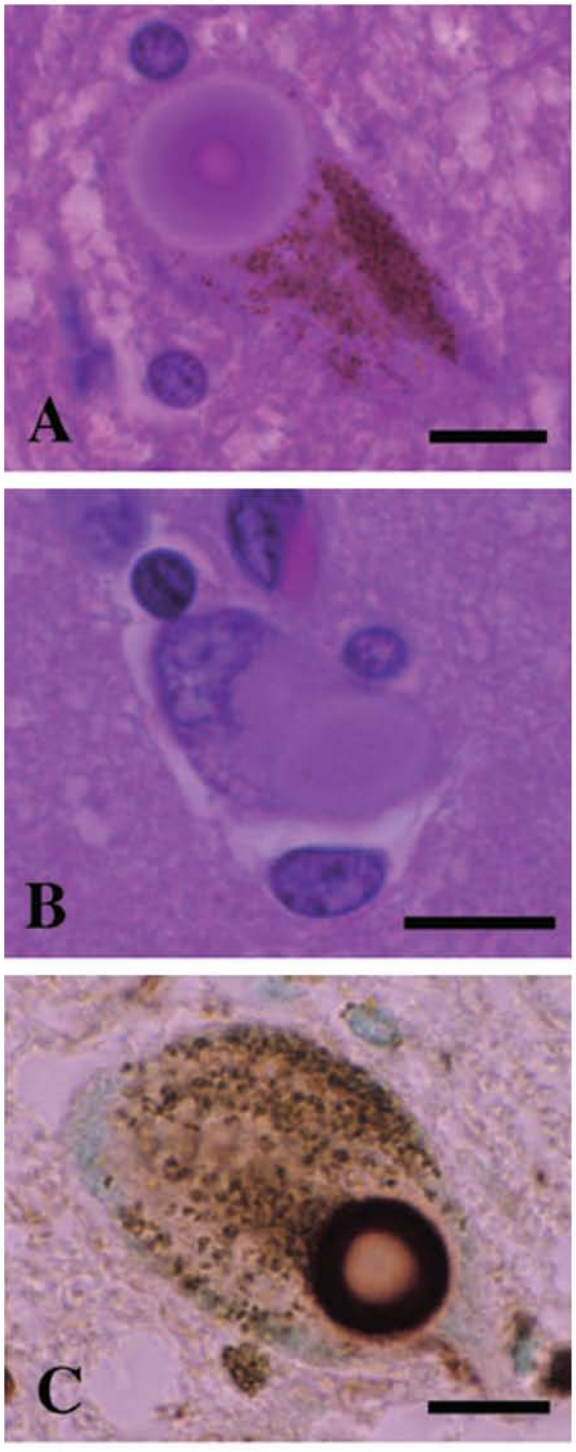

A
B
C

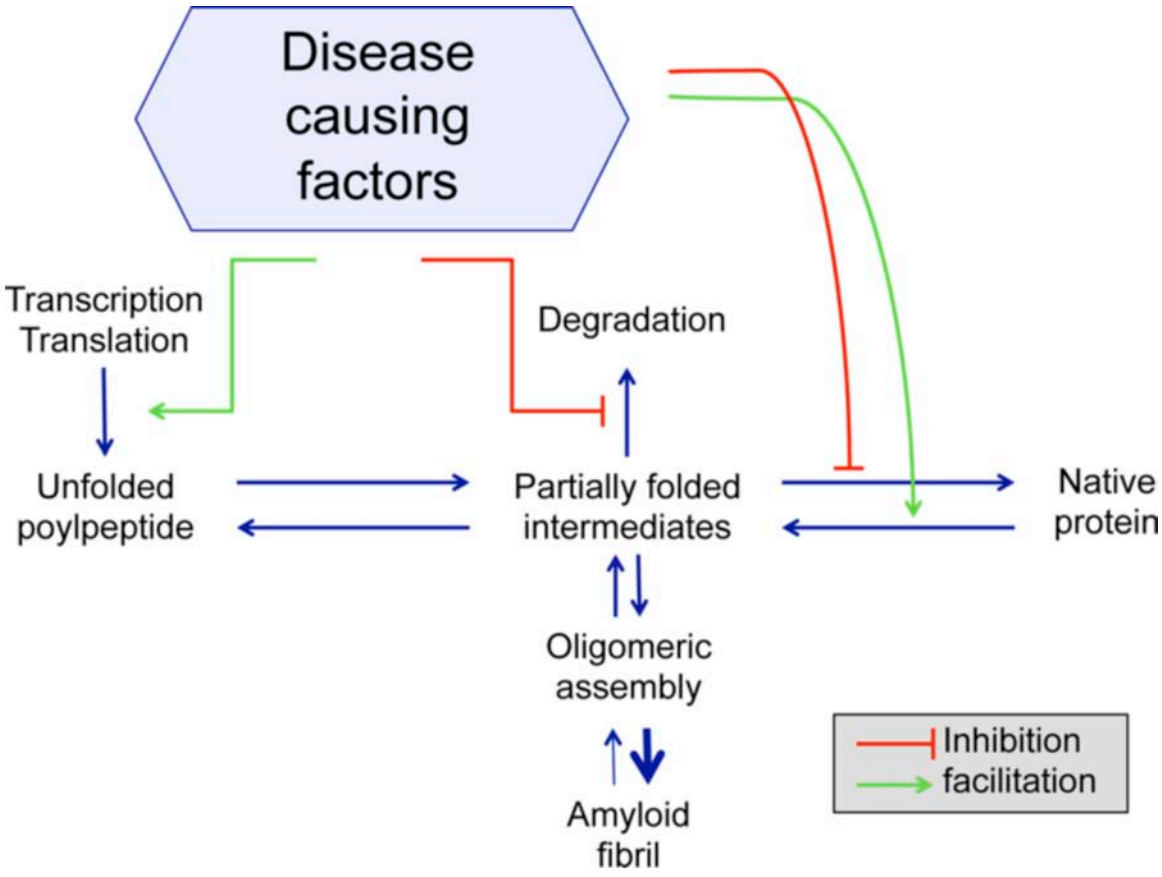
Table 1.1 Genes associated with Mendelian inheritance of PD and Parkinsonism

Gene	Protein	Function	Inheritance	Lewy Body Pathology	First description
SNCA	alpha-synuclein	Unclear; synaptic vesicle recycilng	AD	Always present	(Polymeropoulos et al., 1997)
LRRK2	dardarin/ Leucine-rich-repeat kinase 2	Cytosolic kinase/ Mitochondrial function	AD	Mostly present	(Zimprich et al., 2004)
PRKN	parkin	E3 ubiquitin ligase	AR	Usually absent	(Kitada et al., 1998)
PINK1	PTEN-induced putative kinase 1	Mitochondrial kinase	AR	Present (two cases analyzed by autopsy)	(Valente et al., 2004)
DJ-1	Daisuke-Junko-1	Redox sensitive chaperone/ mitochondrial kinase	AR	Unknown	(Bonifati et al., 2003)

AD = autosomal dominant
AR = autosomal recessive

Table adapted from Hardy and colleagues, (2006).

Figure 1.3 Potential mechanisms of alpha-synuclein fibril formation



Protein aggregation involves the oligomeric assembly of partially folded intermediates and is therefore a concentration-dependent process. As these partially folded intermediates are in concentration equilibrium with unfolded polypeptides, as well as with the native protein, an increase in the steady state level of protein promotes protein aggregation. The level of alpha-synuclein is affected by both its synthesis and breakdown, which is controlled by various genetic and environmental factors contributing to the development of PD.

Figure adapted from Kim and Lees (2008).

Table 2.1 Primary antibodies used for immunocytochemistry

Name	Antigen	Clone	Dilution	Manufacturer
Alpha-synuclein	Alpha-synuclein	Rp	1:1500	Millipore
Alpha-synuclein	Alpha-synuclein	Sp	1:1000	Millipore
Alpha-synuclein	Alpha-synuclein	Mm	1:1000	BD transduction laboritories
Calretinin	Calretinin	Rp	1:2000	SWANT
Caspase-3	Active (cleaved) caspase-3	Rp	1:200	Millipore
Cytochrome c	Cytochrome c	Mm	1:1000	BD Pharmingen
GFAP	Glial fibrillary acidic protein	Rp	1:5000	DAKO
MAP2	Microtubule-associated protein 2	Mm	1:1000	Millipore
NF-M	150kD neurofilament triplet medium molecular weight subunit	Rp	1:1000	Invitrogen
Parvalbumin	Parvalbumin	Rp	1:2000	SWANT
Synaptophysin	Synaptophysin	Rp	1:200	Millipore
TH	Tyrosine hydroxylase (TH)	Rp	1:20000	Chemicon
TH	Tyrosine hydroxylase (TH)	Mm	1:10000	Chemicon
Ubiquitin	Ubiquitin	Rp	1:1000	DAKO
VGAT	Vesicular GABA transporter	Mm	1:200	Synaptic systems
VGLUT	Vesicluar glutamate transporter	Rp	1:1000	Synaptic systems

Rp = Rabbit polyclonal
Mm = Mouse monoclonal
Sp = Sheep polyclonal

Table 2.2 Alexafluor secondary antibodies

Alexafluor Antibody conjugate	Species/Reactivity
Alexafluor 488	goat anti-mouse
Alexafluor 488	goat anti-rabbit
Alexafluor 488	donkey anti-sheep
Alexafluor 546	goat anti-mouse
Alexafluor 546	goat anti-rabbit
Alexafluor 594	goat anti-mouse
Alexafluor 594	goat anti-rabbit
Alexafluor 633	goat anti-rabbit

Figure 2.1 *Example of neuron viability assay using Propidium Iodide and Dihydrorhodamine1,2,3*

Cell viability was measured in live cortical cultures with Dihydrorhodamine 1,2,3 (DHR; **A, B** green), a reactive oxygen species (ROS) indicator, which is oxidized to flourescent cationic rhodamine 123 in the mitochondria. Propidium Iodide (PI; **A, B** red) was used as an early indicator of cell death. This non-polar compound exhibits red fluorescence upon binding to DNA in the nucleus, which is dependant on compromised cellular membrane integrity. An example of non-treated cortical neurons probed with DHR and PI is given in (**A**), and (**B**) is a representative image of cortical neurons treated with antioxidant free media for 3 hours. Both images were acquired from wild-type cultures.

Scale bar = 50 μm

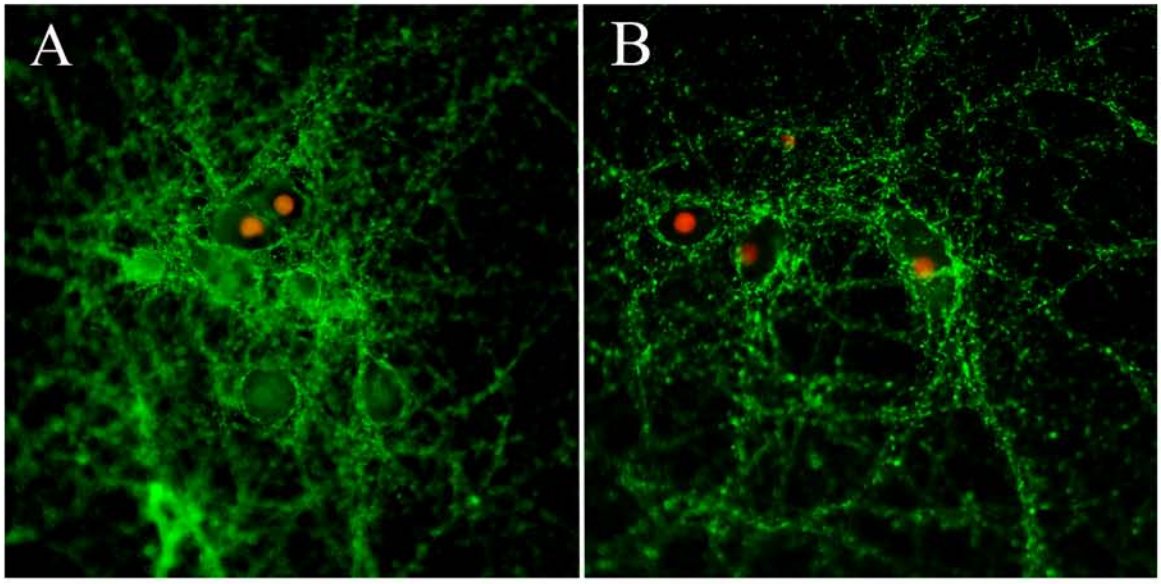

A
B

Figure 2.2 *Definition of 'low' and 'high' alpha-synuclein expressing neurons*

Raw image of mature cortical neurons, with high (**A,** arrow) and low (**A,** arrow head) level immunoreactivity for alpha-synuclein (**A**). The same image, after black and white inversion in Adobe Photoshop (**B**), and with a threshold mask applied at an intensity of 70 (based on 8-bit image). This intensity threshold was used to discriminate all alpha-synuclein expressing neurons. The same image with a threshold mask applied at an intensity of 140 (**D**), which was used to discriminate all neurons expressing high levels of alpha-synuclein.

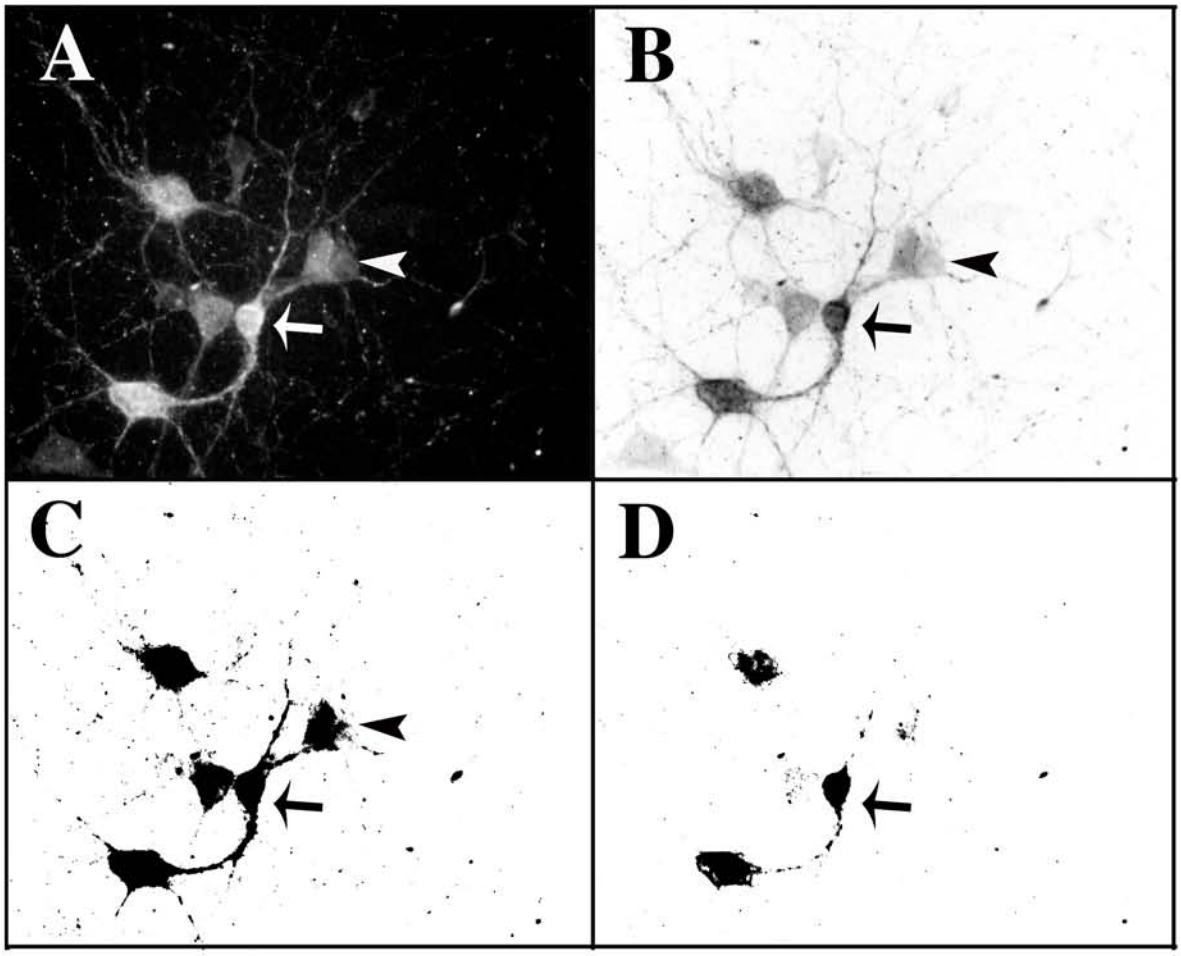

A
B
C
D

Figure 3.1 *Chronic oxidative stress induced alpha-synuclein upregulation*

The level of alpha-synuclein (green) immunoreactivity in dopaminergic (TH immunopositive, red) neurons was compared in non-treated (**A**) mesencephalic cultures, and chronic oxidative stress treated cultures (**B**). Chronic exposure to oxidative stress caused an increase in the proportion of dopaminergic neurons expressing high levels of alpha-synuclein (**A**;**B,** green, arrows), relative to non-treated cultures (**A**). This correlated with a decrease in neurons expressing low levels of alpha-synuclein (arrow head), in treated cultures.

Scale bar = 50μm

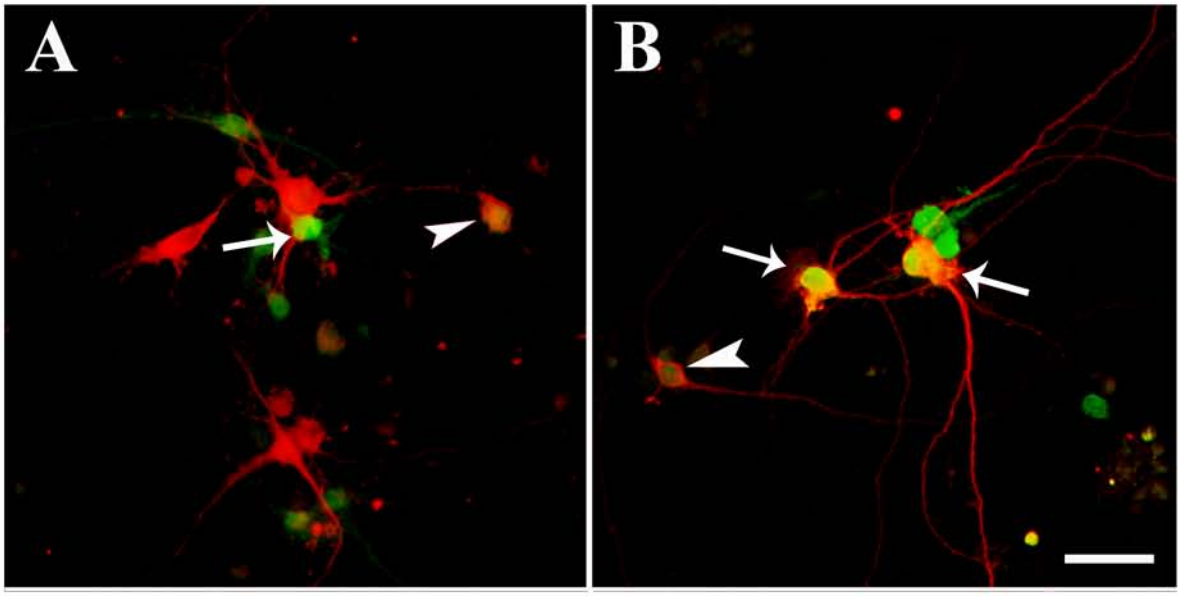

A
B

Figure 3.2 *Alpha-synuclein was upregulated in response to chronic oxidative stress and high levels of expression correlated with resistance to apoptosis following additional insult*

Chronic oxidative stress caused an increase in the proportion of dopaminergic and cortical neurons expressing high levels of alpha-synuclein. Acute hydrogen peroxide treatment (20 μM; 6 hours) did not alter levels of alpha-synuclein expression (**A**). Dopaminergic and cortical neurons expressing high levels of alpha-synuclein, after chronic exposure to oxidative stress, were less likely to have condensed apoptotic nuclear morphology following additional acute hydrogen peroxide treatment (**B**). Data is represented as the relative proportion of neurons with condensed nuclear morphology after treatment.

* = $P < 0.05$, Mean ± SEM

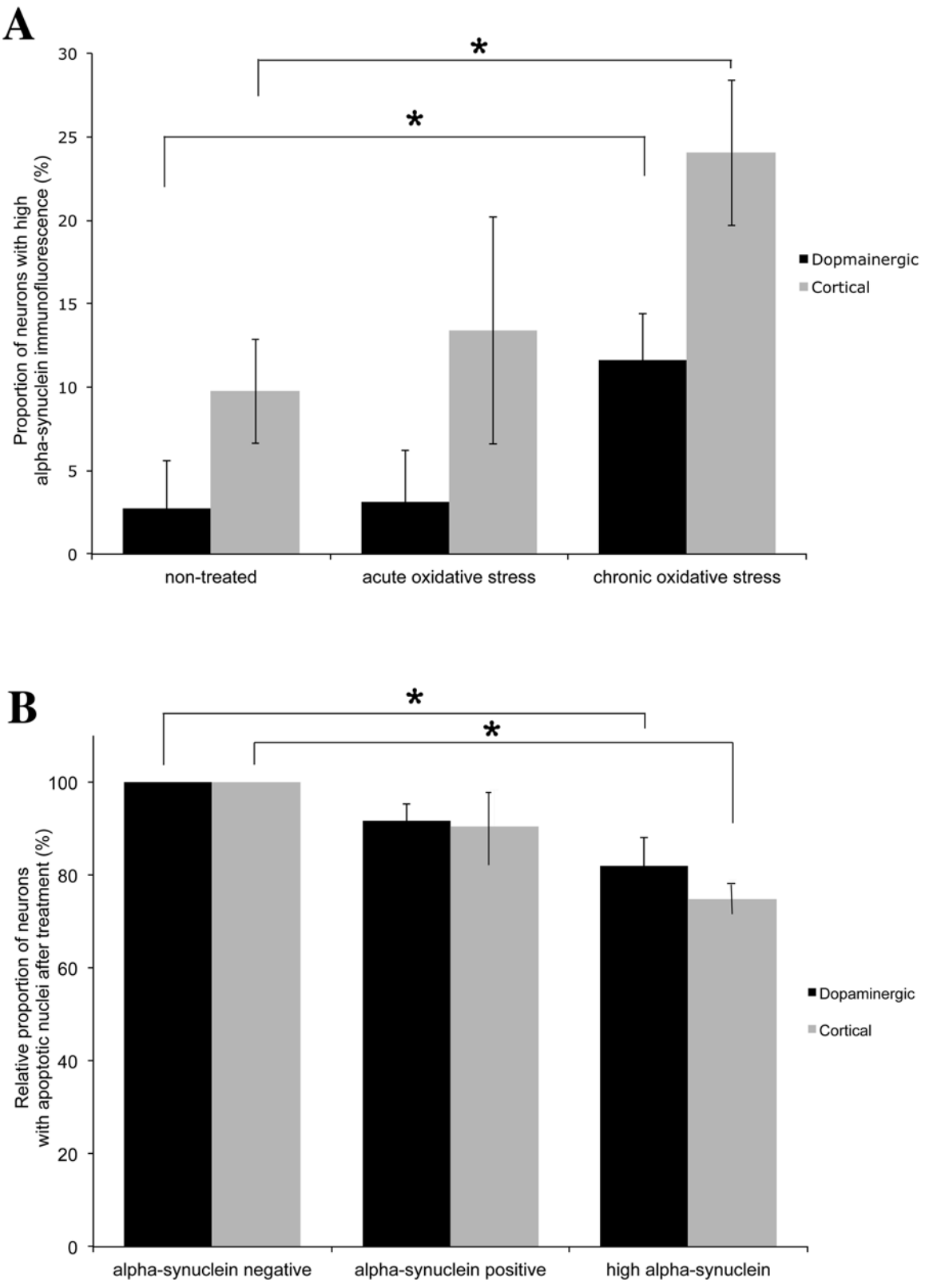

A
*
*
30
25
20
15
10
5
0
Proportion of neurons with high alpha-synuclein immunofluorescence (%)
Dopmainergic
Cortical
non-treated
acute oxidative stress
chronic oxidative stress
B
*
*
100
80
60
40
20
0
Relative proportion of neurons with apoptotic nuclei after treatment (%)
Dopaminergic
Cortical
alpha-synuclein negative
alpha-synuclein positive
high alpha-synuclein

Figure 3.3 *Alpha-synuclein expression was associated with neuroprotection*

Alpha-synuclein expression was correlated with cell health in dopaminergic neurons exposed to chronic oxidative stress. High levels of alpha-synuclein (**B**;**D,** green) expression were negatively associated with apoptotic nuclear morphology (nuclear condensation, indicated by staining for nuclear yellow) (**C**;**D,** blue) in dopaminergic neurons (TH immunopositive) (**A**;**D,** red). The triple labelled image (**D**) shows neurons with high alpha-synuclein expression and normal nuclear morphology (arrowheads) and neurons with low alpha-synuclein levels and condensed apoptotic nuclei (arrows).

Scale bar = 50μm

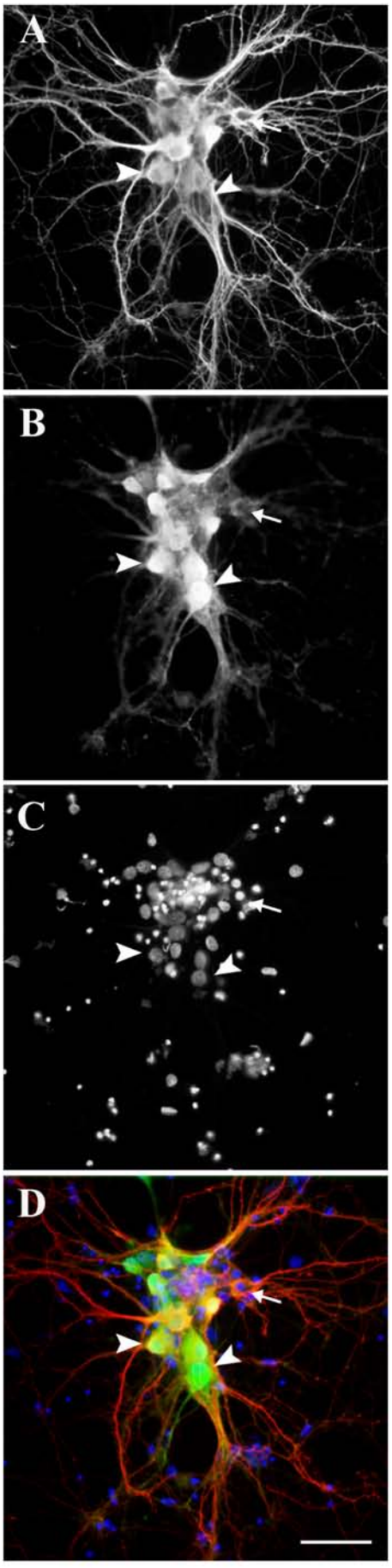

A
B
C
D

Figure 3.4 O*xidative stress and proteasome inhibition induced accumulation of alpha-synuclein puncta in cultured cortical neurons*

The localisation of alpha-synuclein immunoreactivity was compared between non-treated cortical cultures (**A;B**) and those treated with chronic oxidative stress (**C;D**), chronic and acute oxidative stress (**E;F**) and proteasome impairment with lactacystin (**G;H**). In non-treated cultures alpha-synuclein immunolabelling was diffuse within the soma (**A**), with synaptic labelling within the processes (**B**). The intensity of alpha-synuclein immunolabelling within the soma (**C**) and synaptic puncta (**D**) increased after exposure to chronic oxidative stress. Chronic and subsequent acute oxidative stress was associated with punctate accumulations of alpha-synuclein within the soma (**E,** arrow). The increased intensity of labelling at the synaptic puncta was maintained (**F**). Lactacystin treatment caused a different and distinct pattern of alpha-synuclein immunolabelling. Specifically alpha-synuclein immunolabelling accumulated in the perinuclear space (**G,** arrow). Proteasome impairment caused no increase in alpha-synuclein immunolabelling within the neurites (**H**) relative to non-treated cultures.

Scale bar A-H = 50μm

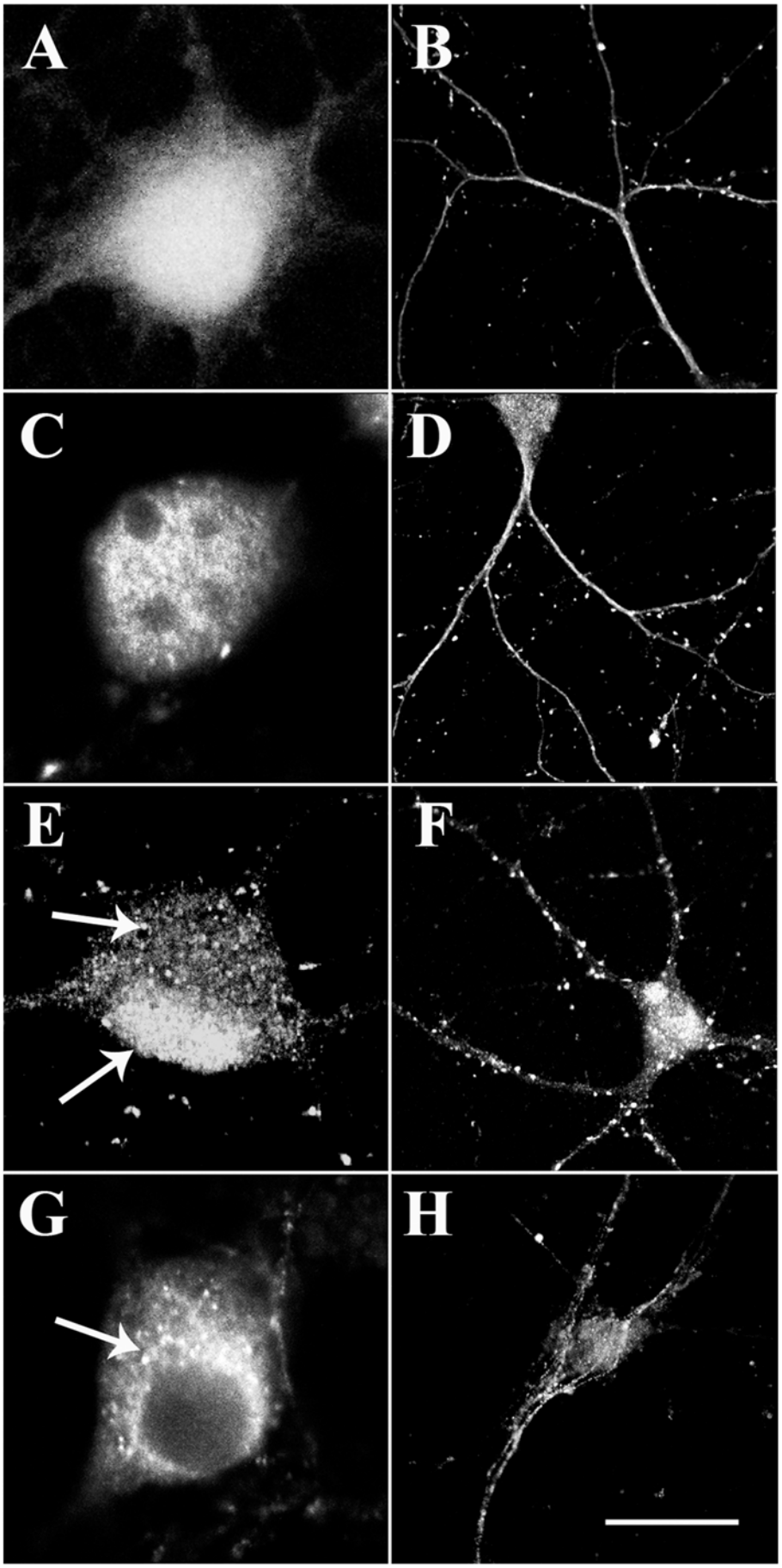

A
B
C
D
E
F
G
H

Figure 3.5 *Oxidative stress altered the expression and localisation of alpha-synuclein in cultured dopaminergic neurons*

The localisation of alpha-synuclein immunoreactivity (green) was compared in (**A;B**) non-treated mature (21 DIV) cultured dopaminergic (TH immunopositive (red)) neurons (**C;D**), those treated with chronic oxidative stress and (**E;F**) those treated with both chronic and acute oxidative stress. Somatic alpha-synuclein immunolabelling (green, **A;B**) was diffuse in non-treated dopaminergic neurons (**B**). Chronic oxidative stress caused upregulation of alpha-synuclein (**C;D**) in a subset of dopaminergic neurons (**D**), although alpha-synuclein immunolabelling remained diffuse. Chronic oxidative stress and additional acute oxidative toxicity caused regional accumulation of punctate alpha-synuclein (**E**;**F**) within the soma of dopaminergic neurons (**E,** arrow).

Scale bar A-H = 5μm

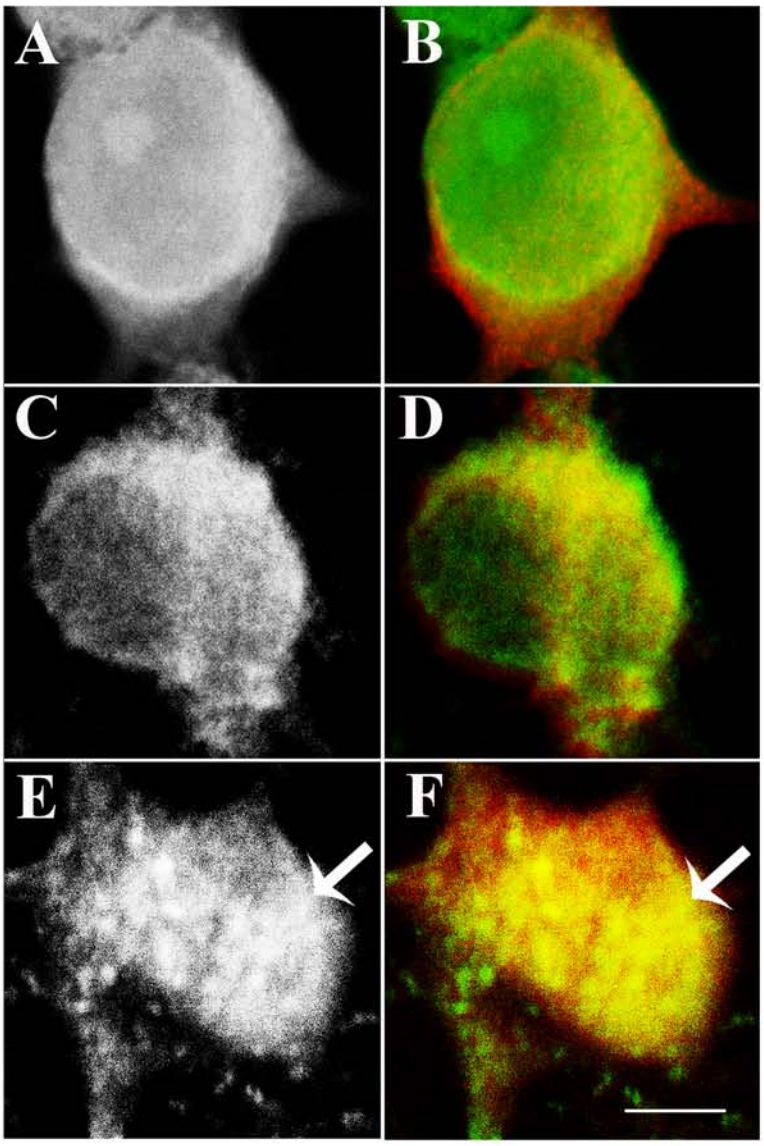

A
B
C
D
E
F

Figure 3.6 *Chronic oxidative stress and subsequent acute oxidative toxicity caused the formation of ubiquitinated aggregates of alpha-synuclein within the cell soma*

Colocalisation of alpha-synuclein and ubiquitin immunolabelling was assessed in non-treated (**A-D**) cortical neurons, and those treated with chronic oxidative stress (**E-H**), acute oxidative stress (**I-L**), chronic and subsequent acute oxidative stress (**M-P**) and proteasome impairment with lactacystin (**Q-T**). There was no colocalisation between alpha-synuclein (**B**;**D** green) and ubiquitin (**C**;**D,** red), immunolabelling in the soma of non-treated cortical neurons at 21 DIV (**A-D**). Chronic (**E-H**) or acute (**I-L**) oxidative stress caused an increase in the number of ubiquitin (**G-H**; **K-L,** red arrows) puncta within the soma of cortical neurons. These punctate accumulations did not colocalise with alpha-synuclein immunolabelling (**F**;**J,** arrowheads). Cultures treated with both chronic and subsequent acute oxidative stress (**M-P**) had punctate accumulations of alpha-synuclein (**N,** arrow). Some of these puncta colocalised (**P,** arrow) with ubiquitin (**O**), although not all ubiquitin accumulations were associated with alpha-synuclein (**O-P,** arrowhead). In cultures grown under conditions of chronic oxidative stress and then treated with lactacystin, there was also colocalisation between alpha-synuclein (**R**) and ubiquitin (**S**) within the soma of some neurons (**T,** arrow). However, this colocalisation did not appear to occur within the nucleus. Not all ubiquitin accumulations were colocalised with alpha-synuclein (**S-T,** arrowhead). For all merged images alpha-synuclein immunolabelling is shown in green and ubiquitin labelling in red. Arrows indicate examples of colocalisation and arrowheads indicate examples of non-colocalisation.

Scale bars: **A, E, I, M, Q** = 20μm: **B-D, F-H, J-L, N-P, R-T** = 5μm

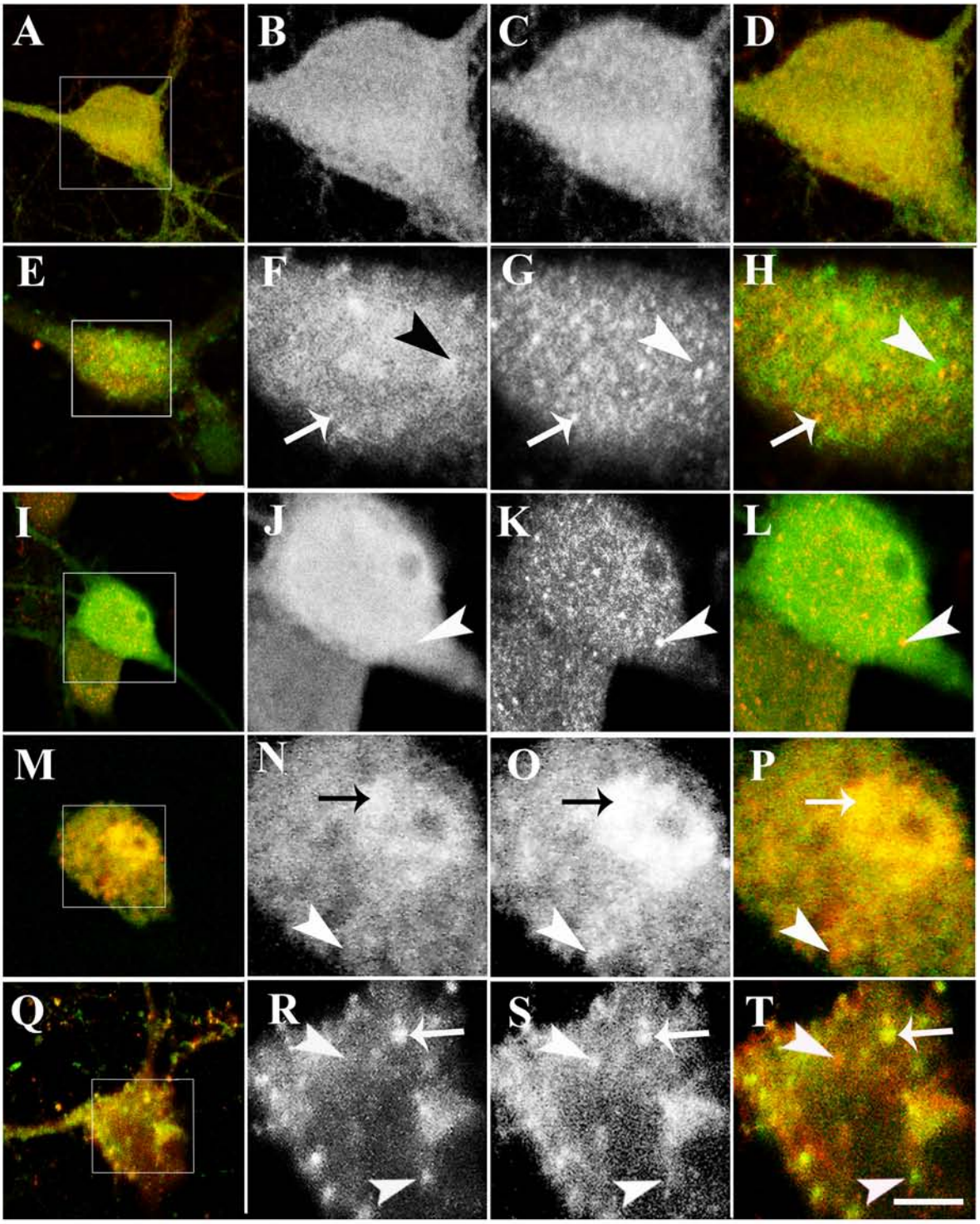

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T

Figure 3.7 *Chronic and subsequent acute oxidative toxicity caused the formation of ubiquitinated aggregates of alpha-synuclein within dopaminergic neurons*

Ubiquitin labelling was compared in non-treated dopaminergic neuron cultures (**A-D**), and those treated with acute oxidative stress (**E-H**), chronic oxidative stress (**I-L**) and both chronic and additional acute (**M-P**) oxidative stress. Ubiquitin immunolabelling (**C**) was diffuse in untreated dopaminergic (TH positive; **A**;**D,** red) neurons (21 DIV). There was no colocalisation between alpha-synuclein (**B**;**D,** green) and ubiquitin (**C**;**D** blue). Mesencephalic cultures with either acute (**E-H**) or chronic (**I-L**) oxidative stress had increased ubiquitin puncta within the soma (**G**;**K,** arrow heads), but did not significantly colocalise with alpha-synuclein immunolabelling (**F;J,** arrows). There was colocalisation (**P,** arrow) between alpha-synuclein (**N**) and ubiquitin (**O**) in cultures treated with chronic and subsequent acute oxidative stress (**M-P**). **E, I** and **M** show TH immunoreactivity (red).

Scale bar = 5μm

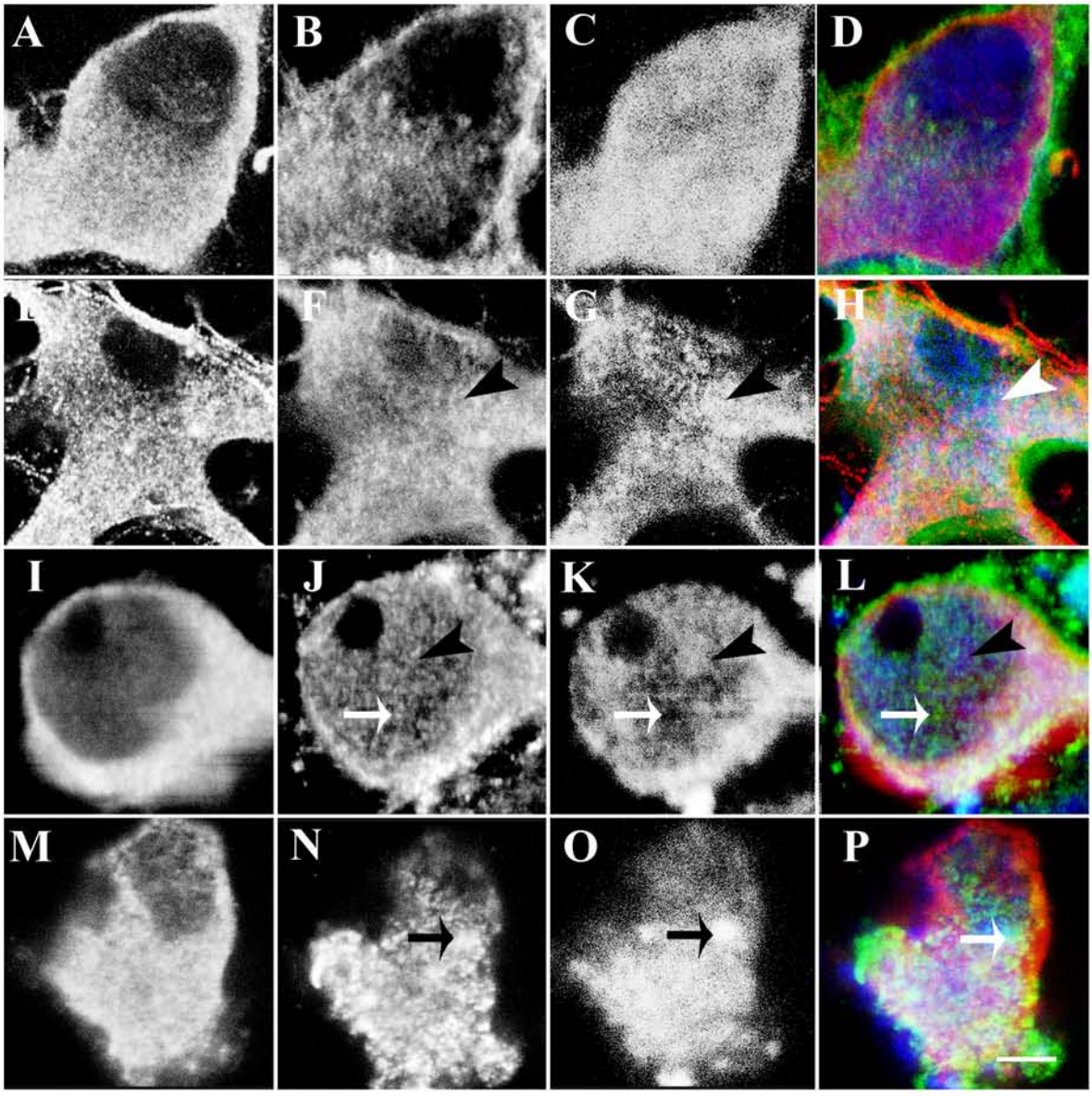

A
B
C
D
F
G
H
I
J
K
L
M
N
O
P

Table 3.1 Summary of protein expression and localisation after oxidative stress

Treatment	Alpha-synuclein Immunoreactivity	Ubiquitin Immunoreactivity	Ubiqutin and alpha-synuclein colocalisation
Non-treated	Diffuse within soma. Mostly diffuse within neurites, with some punctate expression.	Diffuse within soma. Mostly diffuse within neurites, with some punctate expression.	None
Chronic Oxidative Stress	Diffuse within soma. Increased punctate expression within neurites.	Mostly diffuse within the soma with some punctate expression in soma and neurites	None/Insignificant
Acute Oxidative Toxicity	Diffuse within soma. Limited punctate expression in the cytoplasm and neurites.	Increased punctate expression in the soma. Remained punctate in neurites.	None/Insignificant
Chronic + Acute Oxidative Stress	Punctate accumulations within the nuclear compartment. Some loss of expression in the neurites.	Accumulated immunoreactivity in the nuclear compartment, with punctate labelling in the surrounding cytoplasm. Decreased labelling of neurites.	Colocalisation within the nuclear compartment
Chronic Oxidative Stress + UPS inhibition	Punctate accumulations within the soma - extranuclear localisation. Punctate expression in the neurites.	Punctate accumulations within the soma and neurites.	Colocalsation within the extranuclear soma

Figure 4.1 *Endogenous alpha-synuclein conferred protection against oxidative stress and complex I inhibition but not excitotoxicity*

Neuronal viability was correlated to endogenous alpha-synuclein expression following application of *in vitro* models of cell stress. Cultures were treated with antioxidant free growth media, 5 μM rotenone or 25 μM glutamate for six hours. Neurons were identified by morphology and DHR staining. Loss of cell viability was calculated as the increase in the proportion of PI stained neurons compared to control cultures. WT neurons were more resistant to oxidative stress and complex I inhibition. * $P<0.05$; ** $P<0.01$ Mean ± SEM

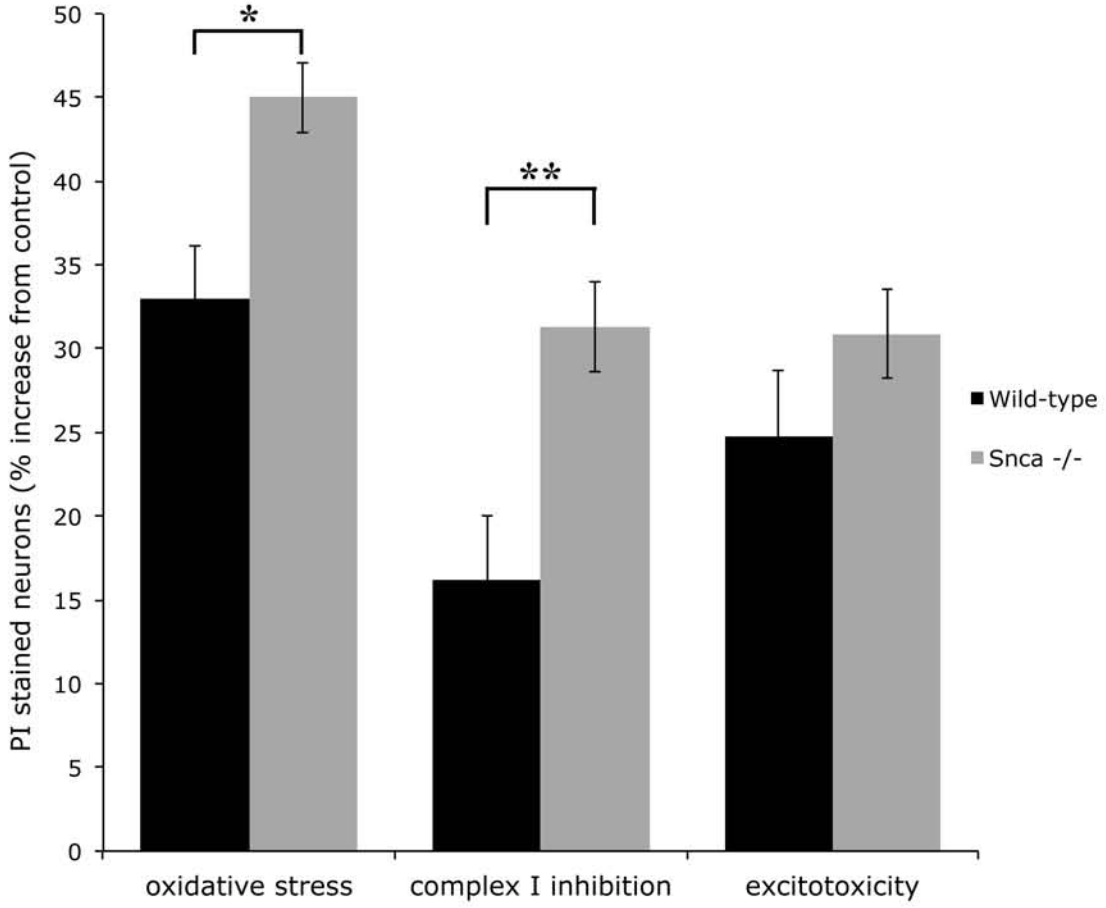

PI stained neurons (% increase from control)
50
45
40
35
30
25
20
15
10
5
0
*
**
oxidative stress
complex I inhibition
excitotoxicity
Wild-type
Snca -/-

Figure 4.2 *Endogenous alpha-synuclein conferred protection against low dose complex I inhibition*

The response of mature (15 DIV) cortical neurons derived from WT and *Snca* -/- mice was assessed following complex I inhibition. Cultures were treated with varying concentrations of rotenone (0-20 nM) for three hours. Loss of cell viability was calculated as the increase in the proportion of PI stained neurons compared to control cultures. *Snca* -/- neurons were more sensitive to low dose (5 nM) rotenone toxicity.

*P<0.05 Mean ± SEM

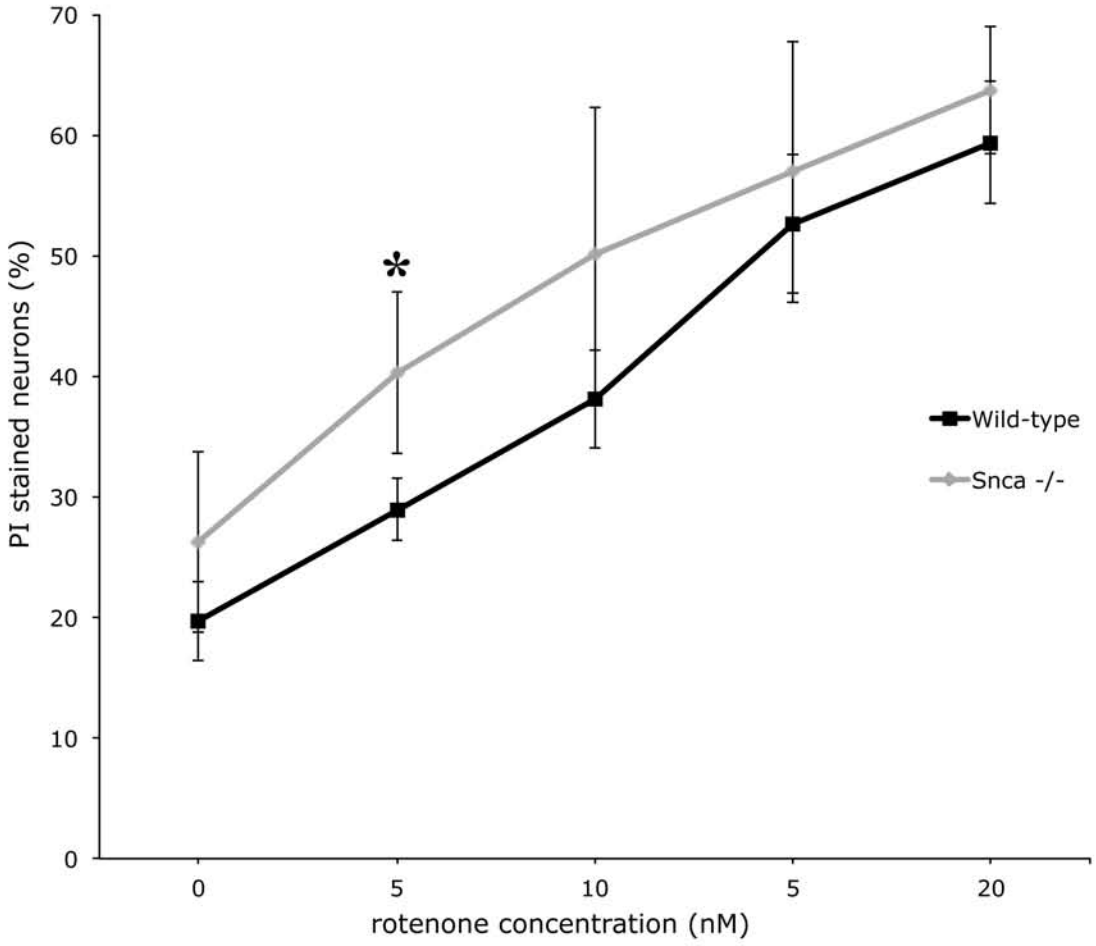

70
60
50
40
30
20
10
0
PI stained neurons (%)
*
Wild-type
Snca -/-
0
5
10
5
20
rotenone concentration (nM)

Figure 4.3 *Endogenous alpha-synuclein prevented cytochrome c release and caspases-3 activation*

Mature (15 DIV) WT cortical cultures were treated with antioxidant free growth media for six hours. Oxidative stress caused activation of caspase-3 and cytochrome c release. Results represent increase from control-treated cultures **(A)**. There was no difference in active caspase-3 activation between *Snca* -/- neurons and WT neurons at three hours treatment with antioxidant free media; however, the vulnerability of *Snca* -/- to oxidative stress was evident thorough loss of cytochrome c immunoreactivity **(B)**. WT cortical neurons were treated with antioxidant-free media for six hours and immunolabelled for alpha-synuclein and active caspase-3 or cytochrome c. The relative level of alpha-synuclein immunofluorescence correlated with resistance to caspase-3 activation (**C**), as determined by strong cytoplasmic and nuclear immunolabelling. Alpha-synuclein expression correlated with resistance to cytochrome c release (**D**).

* $P<0.05$ Mean ± SEM

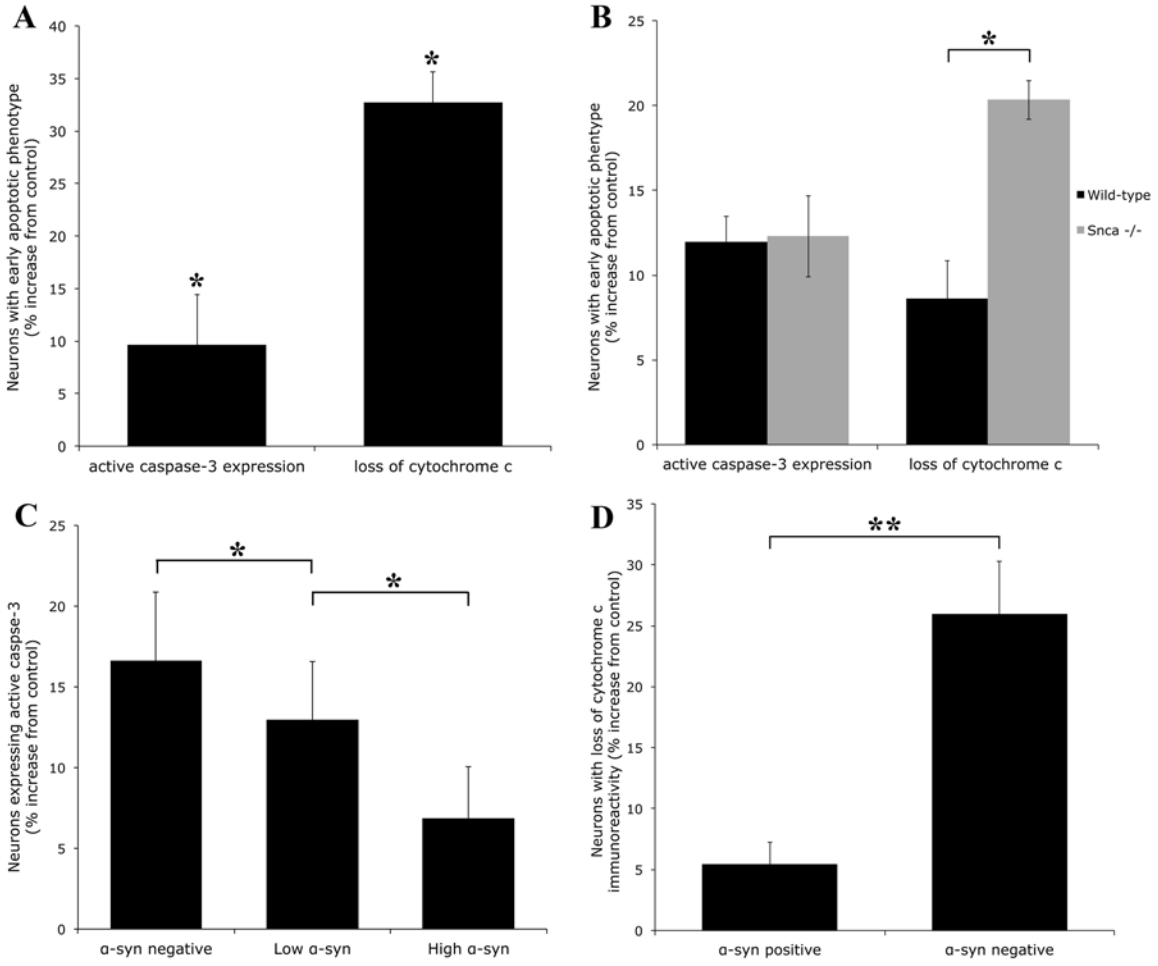

A
Neurons with early apoptotic phenotype (% increase from control)
40
35
30
25
20
15
10
5
0
*
*
active caspase-3 expression
loss of cytochrome c
B
Neurons with early apoptotic phentype (% increase from control)
25
20
15
10
5
0
*
Wild-type
Snca -/-
active caspase-3 expression
loss of cytochrome c
C
Neurons expressing active caspse-3 (% increase from control)
25
20
15
10
5
0
*
*
α-syn negative
Low α-syn
High α-syn
D
Neurons with loss of cytochrome c immunoreactivity (% increase from control)
35
30
25
20
15
10
5
0
**
α-syn positive
α-syn negative

Figure 4.4 *Endogenous alpha-synuclein prevents cytochrome c release and caspases-3 activation*

The relationship between alpha-synuclein expression and apoptosis was determined in WT cortical neurons after treatment with antioxidant-free media for six hours. The relative level of alpha-synuclein (**A;C**, green) immunofluorescence correlated with resistance to caspase-3 activation (**B**;**C,** red), as determined by strong cytoplasmic and nuclear immunolabelling. Alpha-synuclein (**D;F**, green) expression correlated with resistance to cytochrome c release (**E;F,** red), as indicated by a loss of cytochrome c immunoreactivity.

Scale bar = 50 μm.

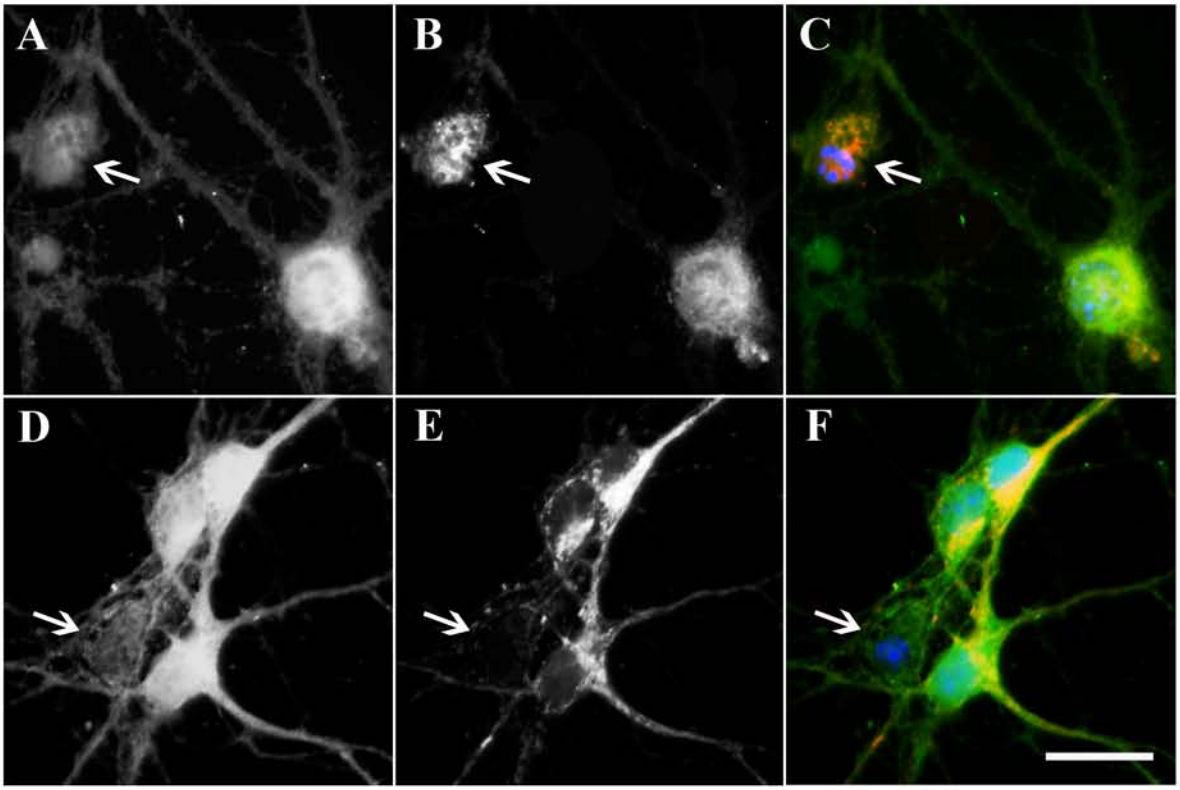

A
B
C
D
E
F

Figure 4.5 *The effect of endogenous alpha-synuclein expression on pro-apoptotic pathways of cytochrome c release*

Example of quantitation method used to determine the effect of alpha-synuclein expression on pro-apoptotic pathways. Mature (15 DIV) WT (**A**) and *Snca -/-* (**B**) cortical cultures were pre-labelled with Mitotracker Red and treated with anti-oxidant free growth media for six hours in the presence pro-apoptotic inhibitors (in this example the MAPK inhibitor PD98059). Cultures were immunolabelled with cytochrome c after fixation. Colocalisation of Mitotracker red and cytochrome c was determined using the RG2B colocalisation plugin on imageJ. Loss of mitochondrial and cytochrome c colocalization was calculated from the area of colocalised pixels as a percentage of the area of Mitotracker Red labelling, and was used as a determinant of cell viability.

Scale bar = 50 μm.

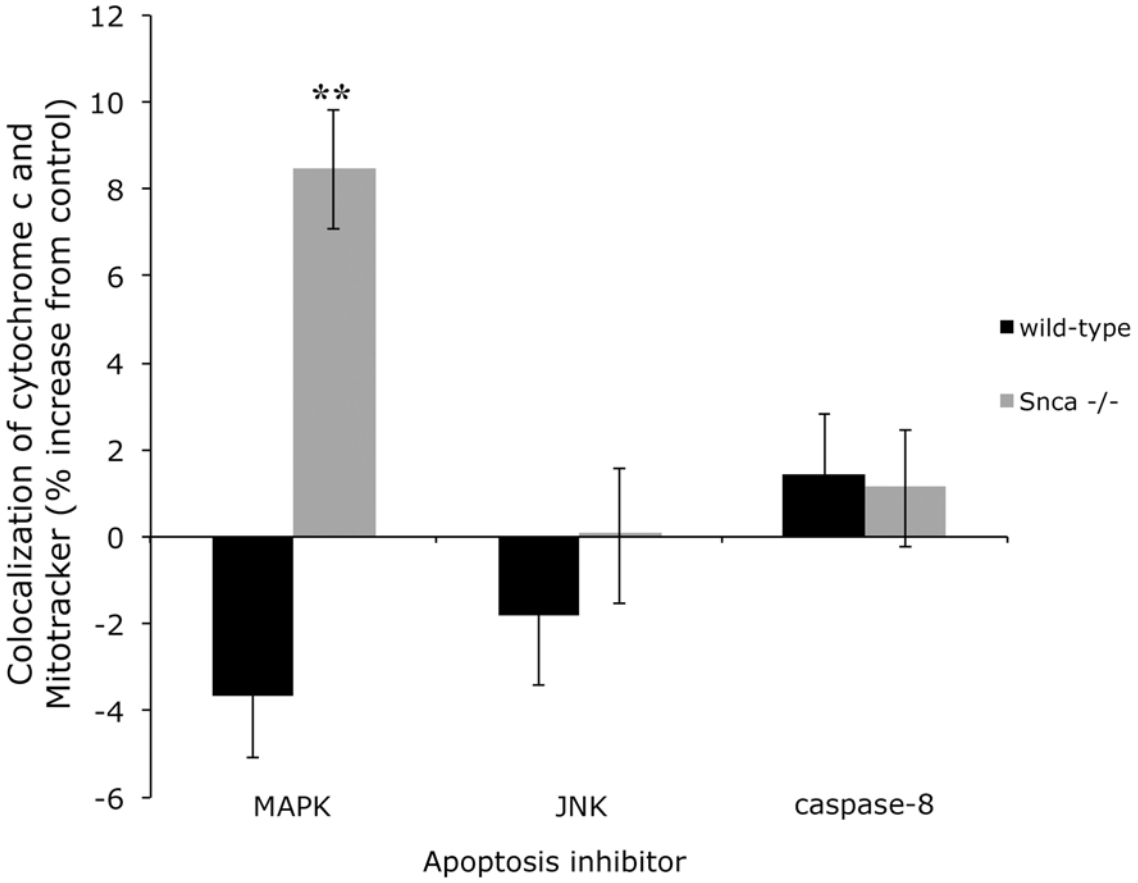

12
10
8
6
4
2
0
-2
-4
-6
**
Colocalization of cytochrome c and Mitotracker (% increase from control)
MAPK
JNK
caspase-8
Apoptosis inhibitor
wild-type
Snca -/-

Figure 4.6 *Alpha-synuclein acts through the MAPK pathway to confer neuroprotection* Mature (15 DIV) WT cortical cultures were treated with antioxidant free growth media for six hours in the presence of apoptosis inhibitors. Colocalisation of cytochrome c and Mitotracker red CMXRos was determined using an ImageJ RG2B colocalisation plugin, and used as a measure of cell viability. Results are represented as percent increase in colocalisation between these markers and are relative to DMSO vehicle control. Inhibition of MAPK increased mitochondrial colocalisation of cytochrome c in *Snca -/-* cultures.

**P<0.01 Mean ± SEM

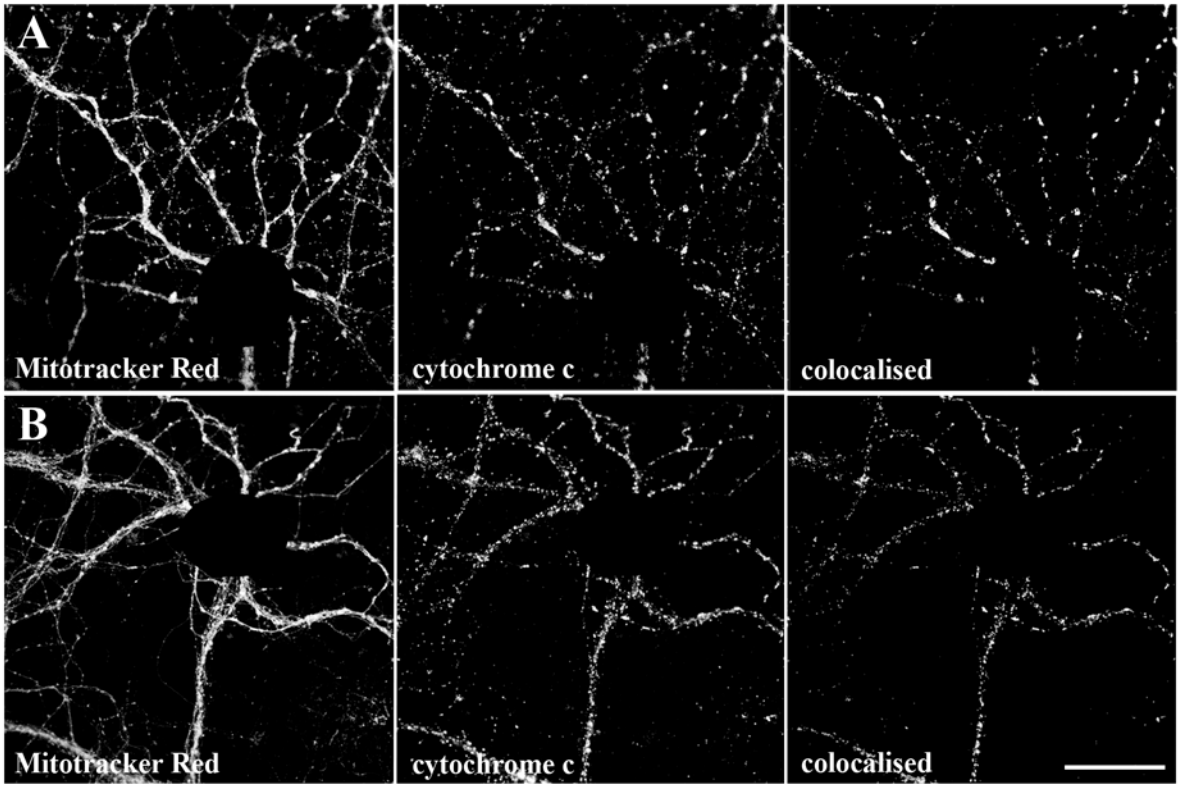

A
Mitotracker Red
cytochrome c
colocalised
B
Mitotracker Red
cytochrome c
colocalised

Figure 5.1 *Neuronal and glial composition of wild-type and Snca-/- cortical cultures*

Mature (15 DIV) cortical cultures derived from WT and *Snca* -/- embryonic mice were assessed for composition of neurons (MAP2 immunopositive **A**;**C**, green) and glial cells (GFAP immunopositive, **B**;**C**, red). Representative images are from *Snca* -/- cultures. Genetic variation did not influence the proportion of glia and neurons in cortical cultures (**D**).

Scale bar = 100 μm

Mean ± SEM

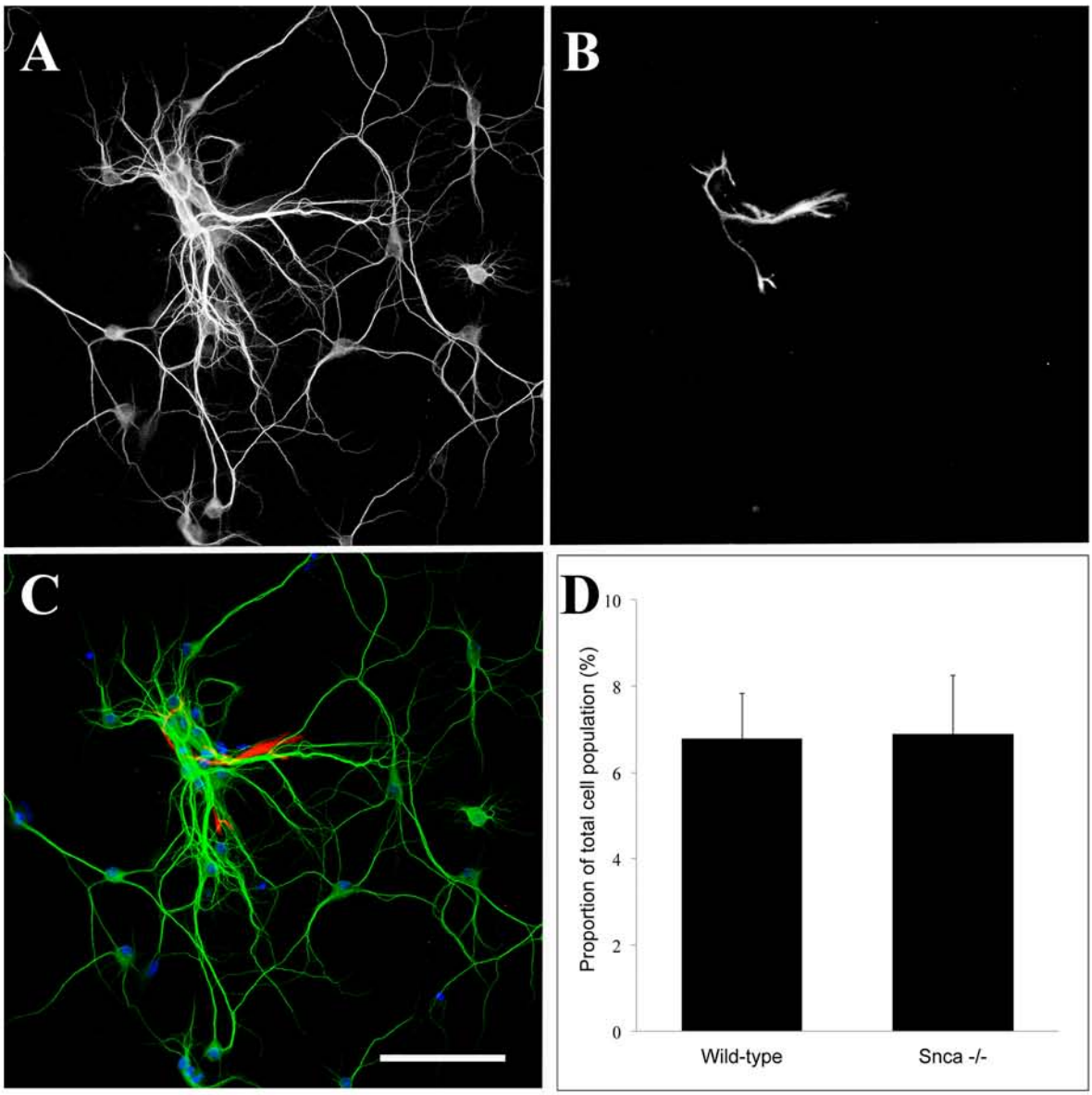

A
B
C
D
Proportion of total cell population (%)
0
2
4
6
8
10
Wild-type
Snca -/-

Figure 5.2 *Inhibitory and excitatory neuronal populations in wild-type and Snca-/- cortical cultures*

Mature (15 DIV) cortical cultures derived from WT and *Snca* -/- embryonic mice were assessed for presence of calretinin (**A**, red) and parvalbumin (**B**, red) immunopositive interneurons. Neurons were double immunolabelled with the dendritic marker MAP2 (**A**;**B**, green). Endogenous alpha-synuclein expression did not influence the proportion of calretinin or parvalbumin immunopositive neurons.

Scale bar = 50 μm

Mean ± SEM

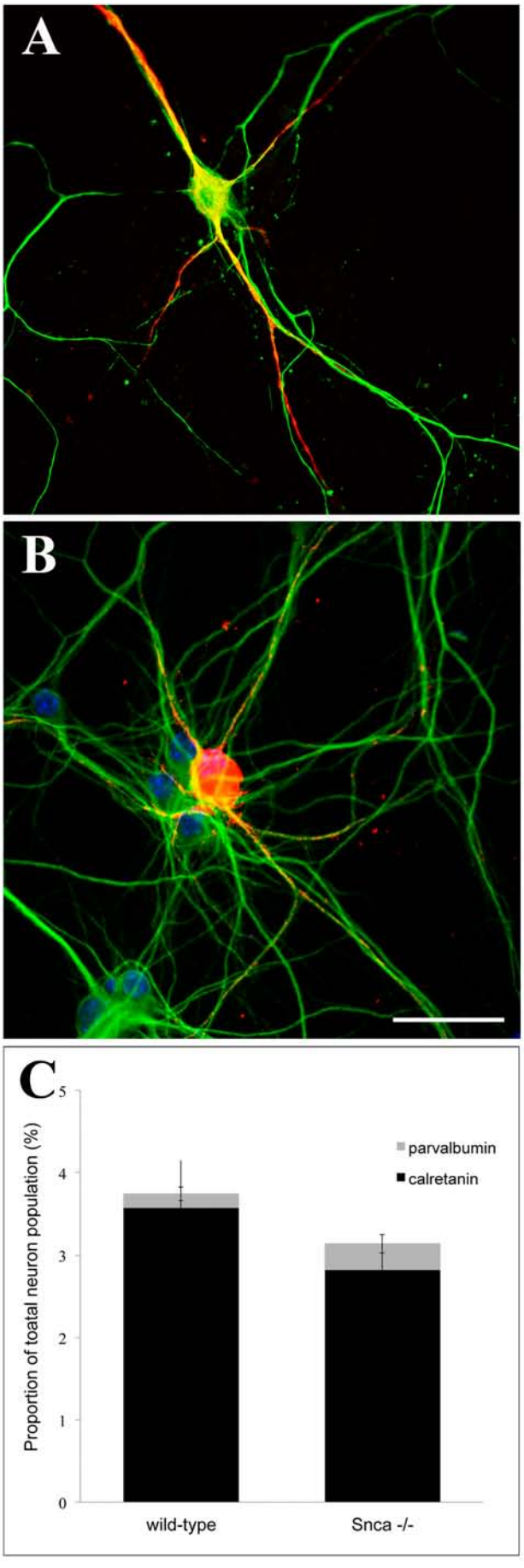

A
B
C
Proportion of toatal neuron population (%)
0
1
2
3
4
5
parvalbumin
calretanin
wild-type
Snca -/-

Figure 5.3 *Expression of excitatory synaptic receptors in mature cortical neurons*
Cortical neurons expressing pre-synaptic vesicular GABA transporter VGAT (**A**;**B**, green). There was a lack of co-localisation between neurites expressing VGAT and NF-M (**B**, red). Synaptic expression of the vesicular glutamate transporter VGLUT (**C**;**D**, green) on mature dendrites (MAP2 immunopositive) (**D**, red). Representative images are from WT cultures.
Scale bar = 50 μm

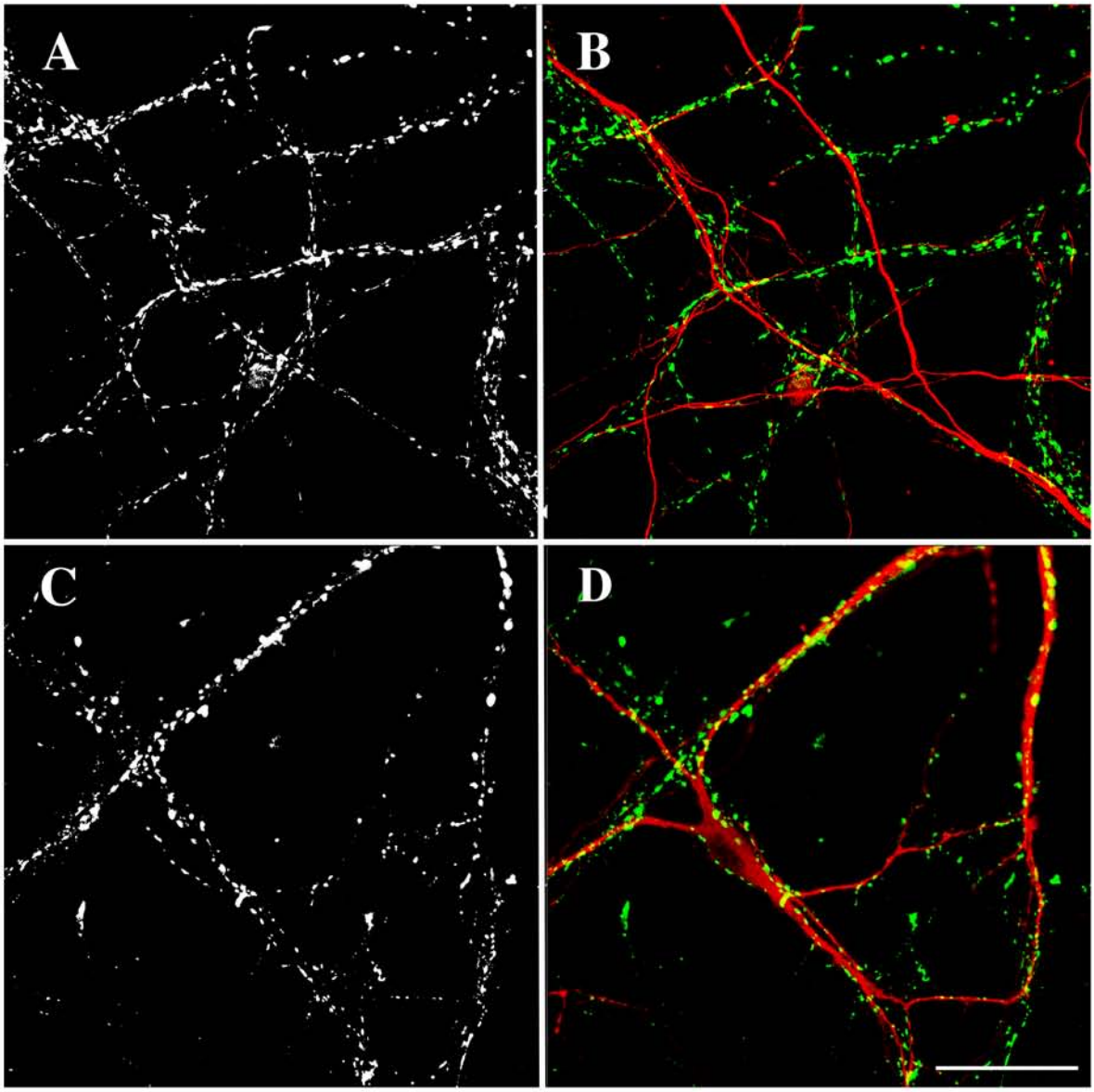

A
B
C
D

Figure 5.4 *Development and maturation of cortical neurons grown in vitro.*
Neuronal development (**A**) (4 DIV) characterised by the presence of growth cones (**A**, arrows) on extending neurites (**A**, arrowhead). At 15 DIV synaptophysin labelling (**B;C,** green) was distinctly punctate and localised along the dendrites, as determined with MAP2 immunolabelling (**C**, red). Representative images are from *Snca -/-* cultures.
Scale bars = 50 μm.

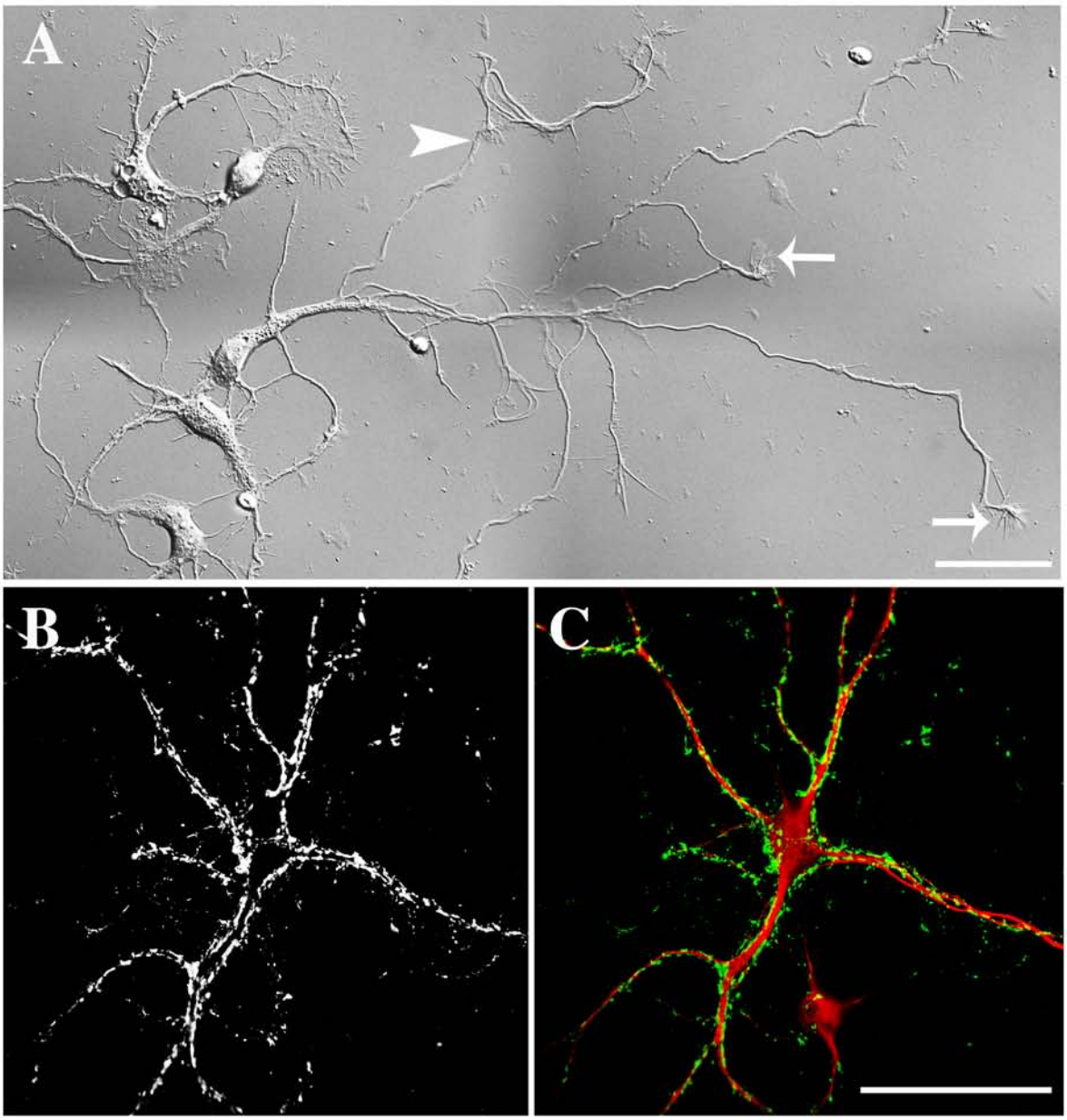

A
B
C

Figure 5.5 *Sample-wise plot of class classification of ^{1}HNMR spectra from media derived from wild-type and Snca -/- cortical neuron cultures*

Sample-wise comparison plots of spectra from media derived from WT (blue) and *Snca -/-* (red) cortical neuron cultures. Each point represents a different media sample. A single latent variable was sufficient to correctly class media samples according to the respective genotype of their origin. The x-value (Y predicted 1 (knockout)) indicates the PLS-DA classification of each sample according to the latent variable. $P<0.01$ Mean ± SEM

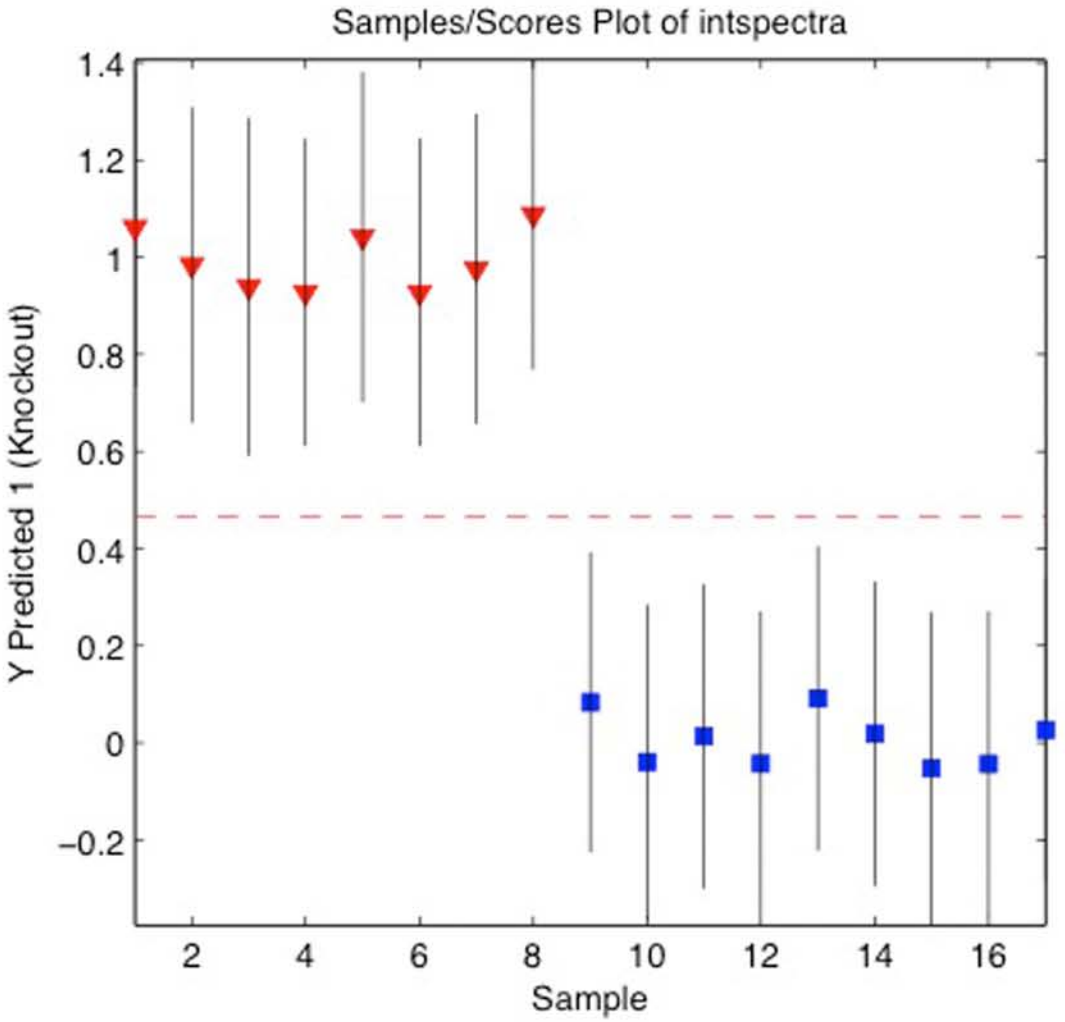

Samples/Scores Plot of intspectra
Y Predicted 1 (Knockout)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
−0.2
2
4
6
8
10
12
14
16
Sample

Figure 5.6 *Lack of alpha-synuclein increases lactate in culture media*

A section of ^{1}H-NMR spectra of media derived from WT (blue) and *Snca* -/- (red) cortical neuron cultures. There was an increase in duplet peaks resonances at 1.3 ppm in *Snca* -/-, relative to WT samples, corresponding to $_{L}$-lactate.

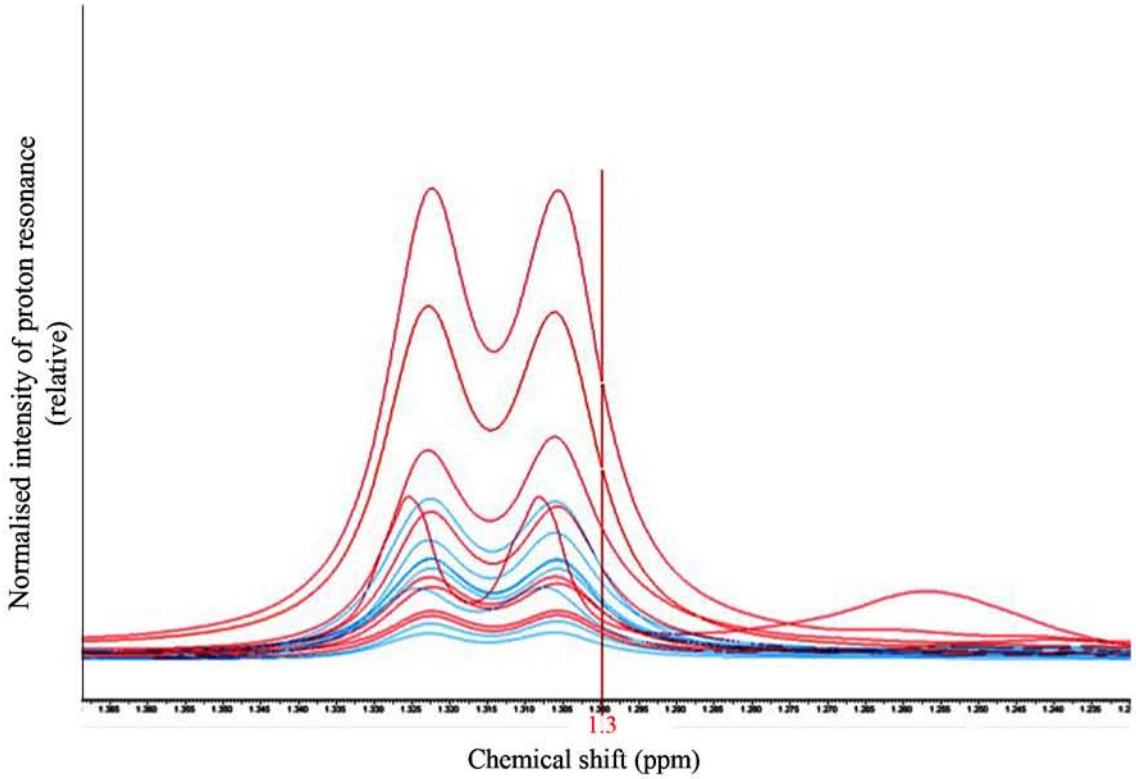
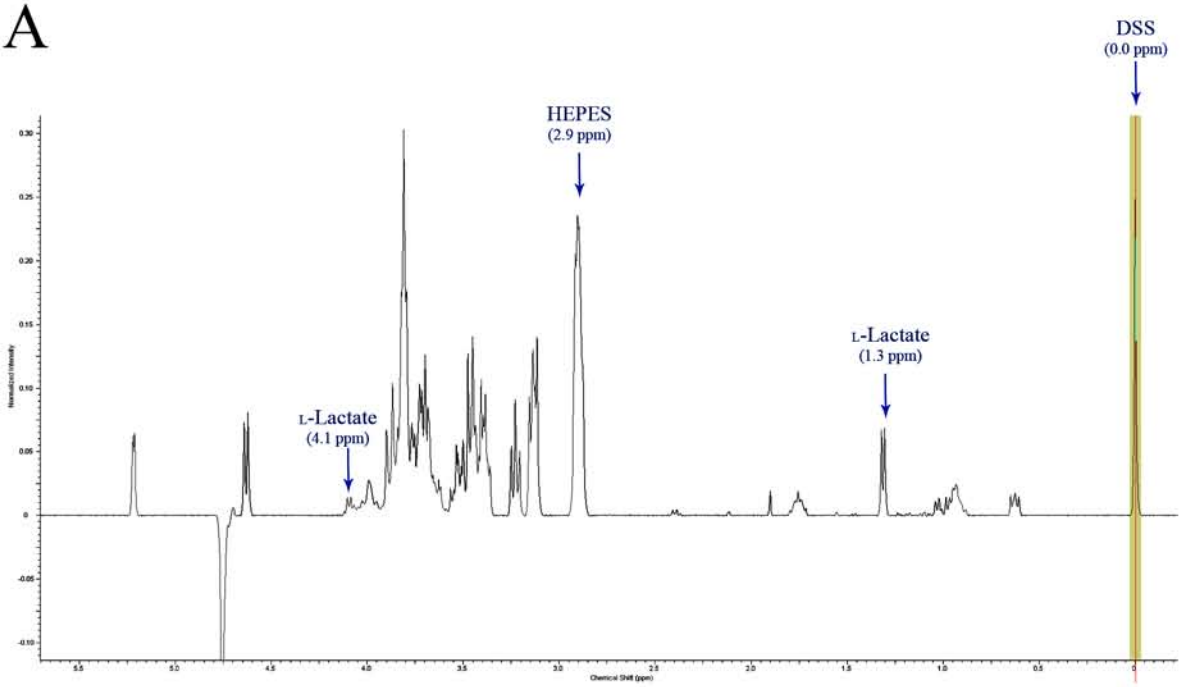

Normalised intensity of proton resonance
(relative)
1.3
Chemical shift (ppm)

Figure 5.7 *Representative ^{1}H NMR spectra of growth media taken from wild-type and Snca -/- cortical neuron cultures*

Representative ^{1}H NMR spectra of growth media from WT (**A**) and *Snca -/-* (**B**) cultures. Spectra were distinguished according to genotype by proton resonance intensities at peaks corresponding to $_{L}$-lactate (1.3 ppm and 4.1 ppm) and HEPES (2.9 ppm). All chemical shifts were reference to DSS (0 ppm).

A


DSS
(0.0 ppm)
HEPES
(2.9 ppm)
L-Lactate
(1.3 ppm)
L-Lactate
(4.1 ppm)

B

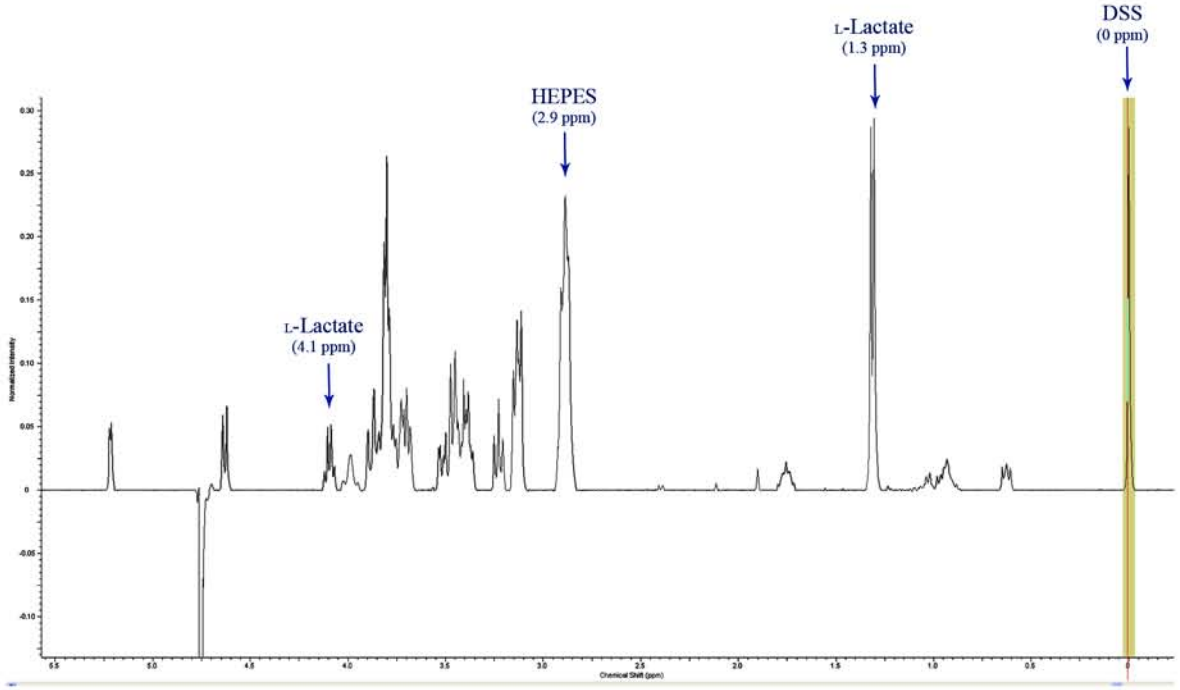

DSS
(0 ppm)
L-Lactate
(1.3 ppm)
HEPES
(2.9 ppm)
L-Lactate
(4.1 ppm)

Figure 6.1 *A53T alpha-synuclein expression sensitises neurons to oxidative stress and complex I inhibition.*

Response of mature (15 DIV) cortical neurons derived from hemizygous mutant (A53T+/-) alpha-synuclein transgenic mouse embryos and their non-transgenic littermates, to a mild *in vitro* model of complex I inhibition (5 μM rotenone; six hours). Expression of the mutant (A53T+/-) alpha-synuclein increased neuronal susceptibility to complex I inhibition, as indicated by PI staining (**A**). Response of mature (15 DIV) cortical neurons derived from hemizygous mutant (A53T+/-) alpha-synuclein transgenic mouse embryos and their non-transgenic littermates, to a mild *in vitro* model of oxidative stress (six hours antioxidant free media) (**B**). Expression of the mutant (A53T+/-) alpha-synuclein increased neuronal susceptibility to complex I inhibition, as indicated by the increase in PI stained neurons compared with non-treated control cultures.

* $P<0.05$

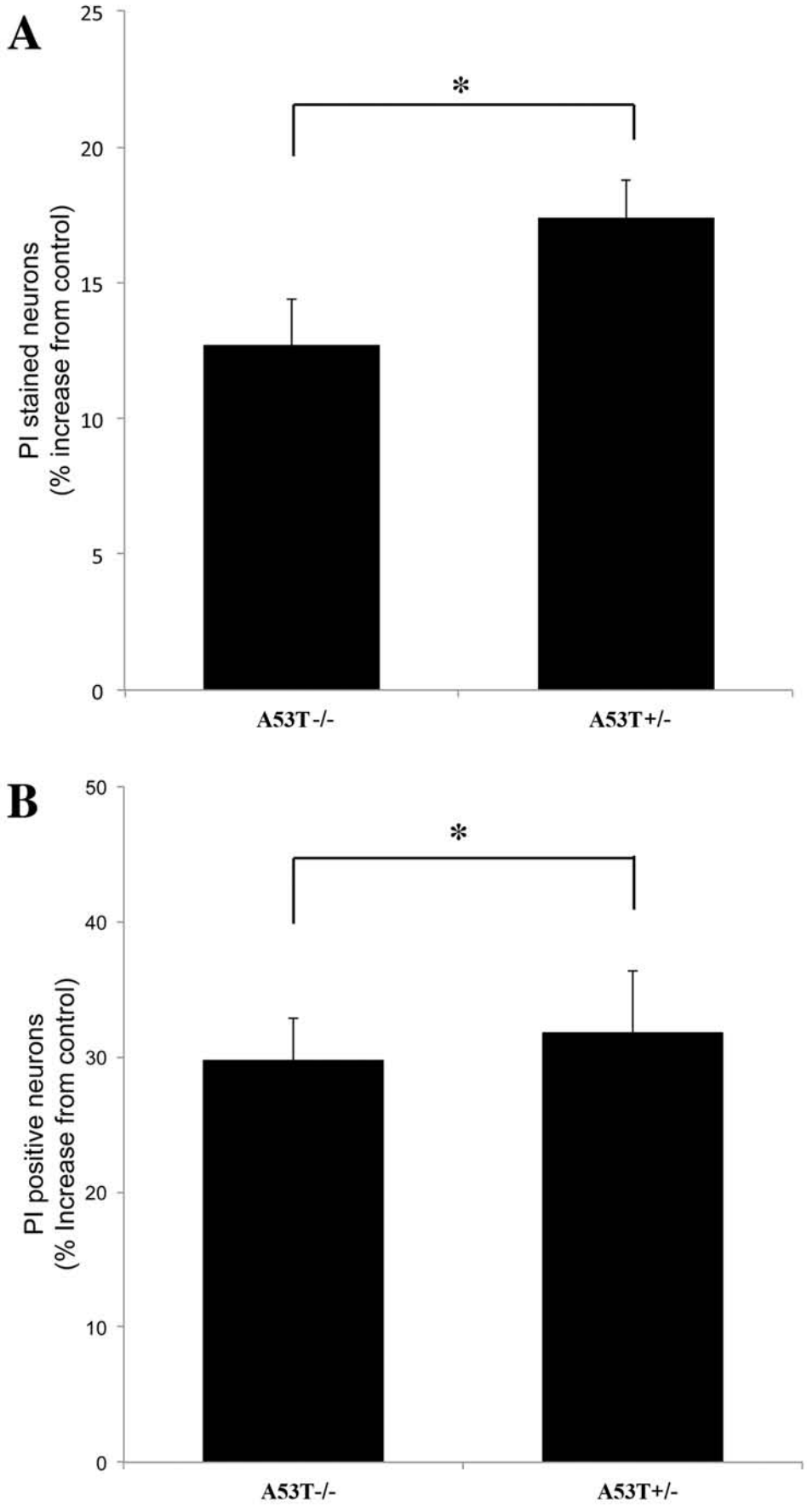

A
25
20
15
10
5
0
*
PI stained neurons
(% increase from control)
A53T -/-
A53T+/-
B
50
40
30
20
10
0
*
PI positive neurons
(% Increase from control)
A53T-/-
A53T+/-

Figure 6.2 *Oxidative stress induces apoptosis in cortical neuron cultures*

Representative immunolabelling for loss of cytochrome c (**A,** red). Arrow indicates a cortical neuron lacking cytochrome c immunoreactivity, corresponding with apoptotic nuclear morphology, as indicated by nuclear yellow (blue) staining. Representative immunolabelling for active caspase-3 expression (**B,** red), correlating with apoptotic nuclear morphology (nuclear yellow; blue).

Scale bar = 30 μm (**A**); 20 μm (**B**).

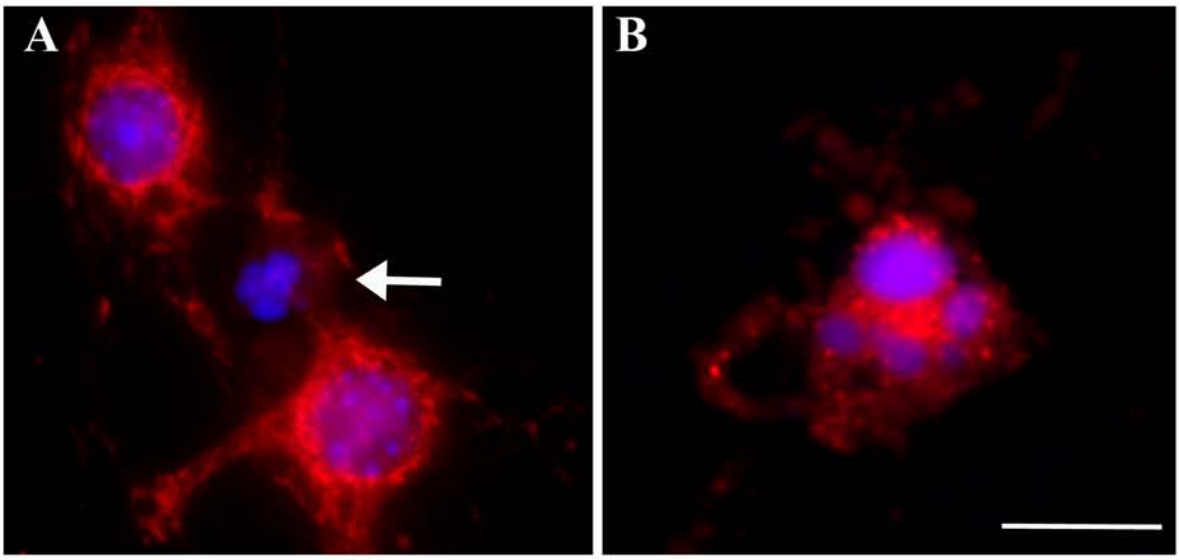

A
B

Figure 6.3 *Ca^{2+} uptake in isolated brain mitochondria.*

Mitochondrial Ca^{2+} uptake was monitored by changes in Fura-6F fluorescence ratio (340/380 nm excitation; 510 nm emission). The peaks correspond to bolus additions of 2 nM $CaCl_2$, and reductions in the Fura-6F ratio reflect mitochondrial Ca^{2+} uptake.

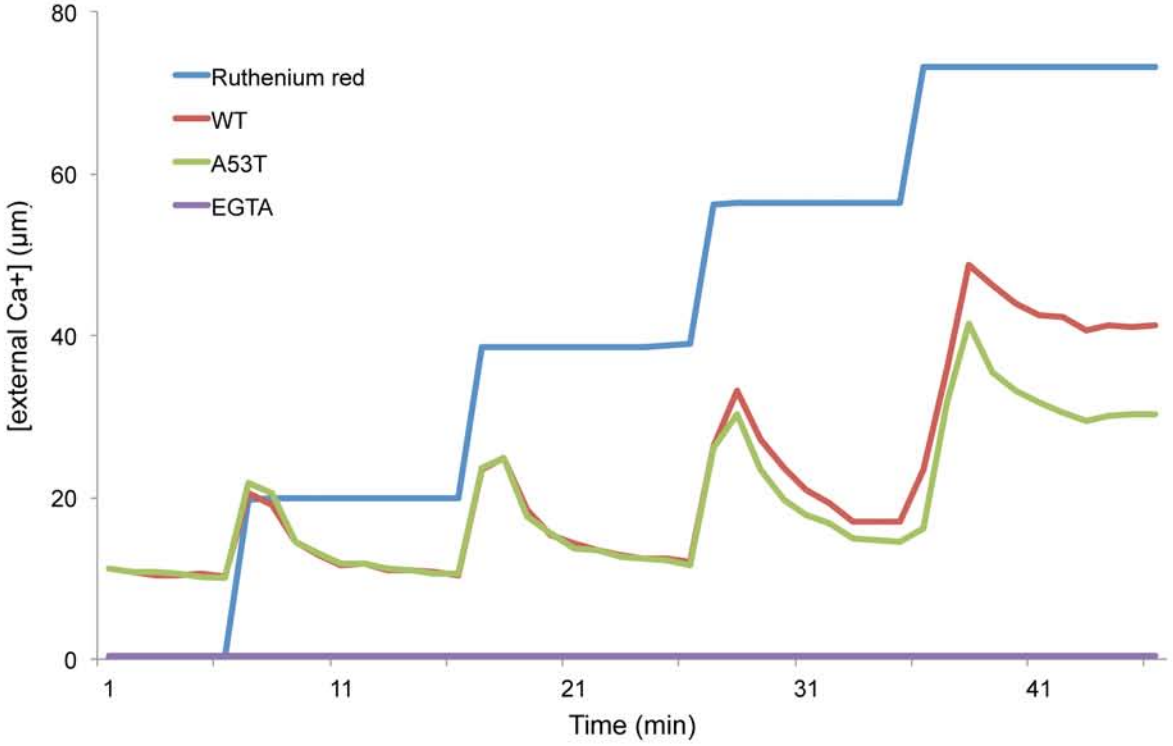

80
60
40
20
0
[external Ca+] (µm)
Ruthenium red
WT
A53T
EGTA
1
11
21
31
41
Time (min)

Figure. 7.1. *The relationship between alpha-synuclein expression level and cell survival*

Moderate expression levels of alpha-synuclein (correlating to intracellular concentration [α-synuclein]) promote cell survival, but high expression levels are associated with pathological events including inclusion body formation and neurodegeneration. There are two plausible consequences of high expression levels, shown in the current schematic as the two divergent lines. A current hypothesis is that more soluble forms of alpha-synuclein are toxic. These are represented as oligomers and pore-like annular structures and are associated with neuronal damage (black line). An alternative consequence of high alpha-synuclein is the deposition of fibrils into inclusion bodies (Lewy bodies shown here). While this may come at the cost of decreased function, this may also be permissive for neuronal survival (blue line).

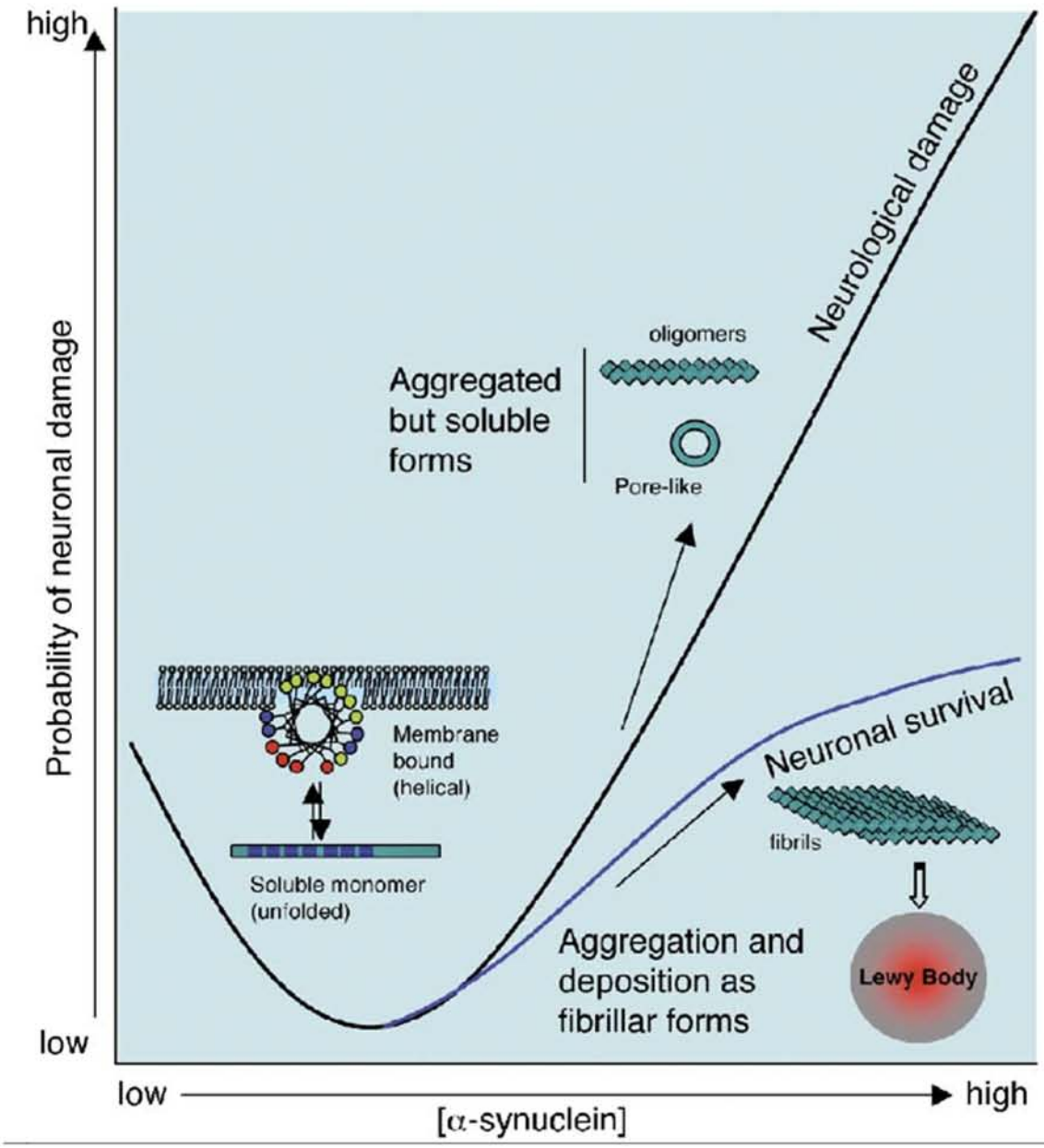
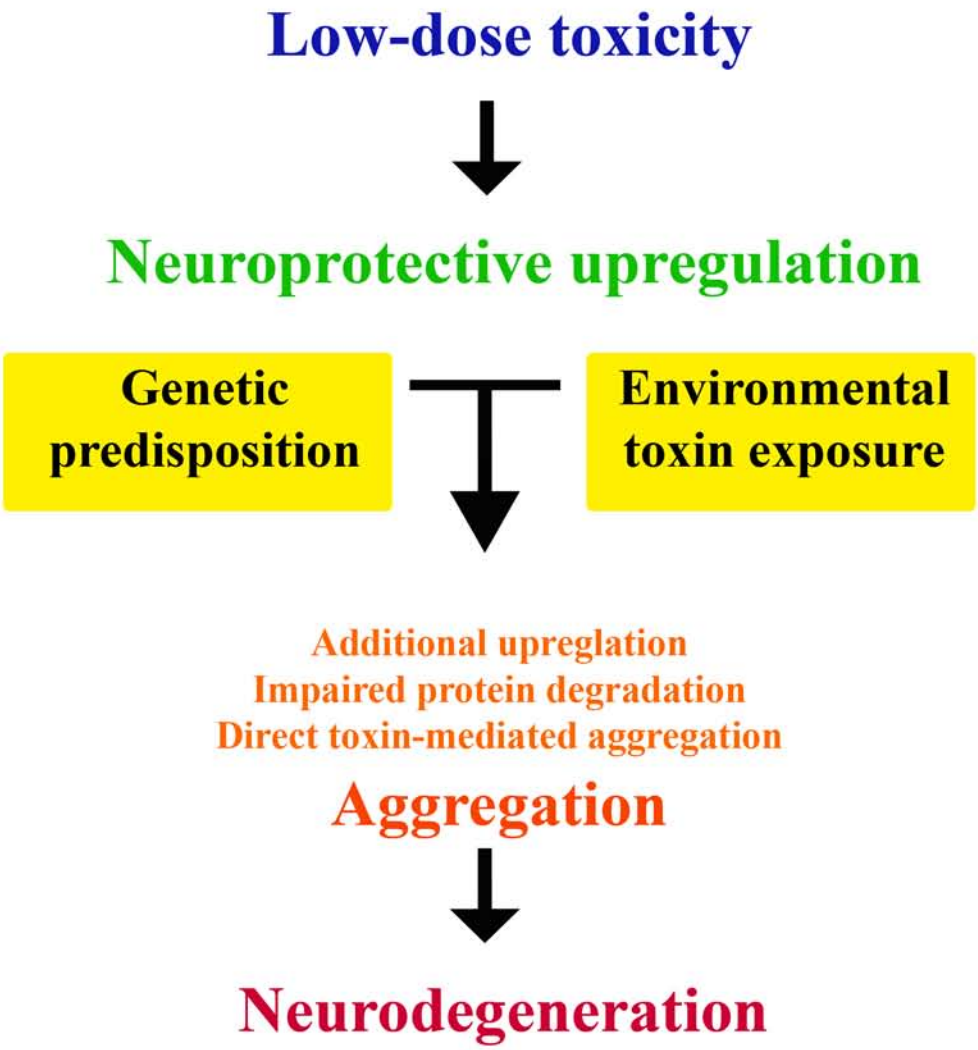

high
low
Probability of neuronal damage
Neurological damage
oligomers
Aggregated
but soluble
forms
Pore-like
Membrane
bound
(helical)
Soluble monomer
(unfolded)
Neuronal survival
fibrils
Aggregation and
deposition as
fibrillar forms
Lewy Body
low
high
[α-synuclein]

Figure 7.2 *Two-hit hypothesis for the involvement of alpha-synuclein in neurodegeneration*

Initial low dose neuronal stress induces a neuroprotective upregulation of alpha-synuclein. Additional toxicity, or genetic predisposition drive further accumulation of the protein through additional expression, or impaired protein degradation. This increase in the cellular load of alpha-synuclein triggers its aggregation which compromises cell health through a toxic gain of function mechanism.


Low-dose toxicity
Neuroprotective upregulation
Genetic predisposition
Environmental toxin exposure
Additional upreglation
Impaired protein degradation
Direct toxin-mediated aggregation
Aggregation
Neurodegeneration