Analysis of Mutations in Alpha-Synuclein and the Protective Effect of Heat Shock Proteins in a Model of Alpha-Synuclein-induced Toxicity

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To my parents Georgios and Agni and
my sisters Stephania and Dimitra.

"I know nothing except the fact of my ignorance"
Socrates

Abstract

Genetic studies have revealed three mutations (A30P, A53T and E46K) in α-synuclein (α-Syn) that cause Parkinson's disease (PD) in a small number of pedigrees with autosomal dominant inheritance. For the purpose of this thesis an *in vitro* model has been developed by stably over-expressing wild type (wt), A30P or A53T mutant α-Syn in ND7 neuronal cells. Wt α-Syn can enhance cell death in response to ischaemia/reoxygenation or staurosporine treatment whilst protecting against serum removal and dopamine-induced cell death in this system. In contrast, both mutant forms of α-Syn enhance cell death. The above stresses were used to induce primarily apoptotic cell death, implicated in PD pathology. Hence, the PD-associated mutations convert α-Syn from a protein which could modulate cell death differently in different circumstances to forms which are deleterious in response to various stresses.

Subsequently, the neuroprotective effect of various heat shock proteins (hsps) in the above system was studied, utilising a Herpes Simplex Virus-based gene delivery system. For the first time, it was demonstrated that in an *in vitro* mammalian model of α-Syn-induced toxicity over-expression of hsp27 protects, under all the stresses tested, both wt and mutant α-Syn expressing cells, as assessed by multiple apoptotic/necrotic death assays. Interestingly, A30P α-Syn expressing cells were markedly protected by caspase-8 and caspase-9 inhibition as well as by hsp27 over-expression. No synergy between hsp27 and the caspase inhibitors was observed. In addition, hsp70 conferred protection only to wt α-Syn expressing cells exposed to ischaemia whereas hsp56 had no protective role in this system. Hence, hsp27 was neuroprotective by interfering with the enhanced caspase-dependent cell death resulting from mutant A30P α-Syn over-expression. Finally, studies of the mitochondrial status in this system were performed to further explore the site of action of hsp27. Hsp27 reduced significantly the mitochondrial membrane potential loss in stressed A30P mutant α-Syn cells and this correlates well with their enhanced cell survival. These findings suggest that hsp27 has a novel neuroprotective role against mutant α-Syn toxicity and this is achieved by interfering with the caspase cascade and mechanisms modulating the mitochondrial membrane potential.

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Declaration

All the work presented in this thesis is the work of Alexandra Zourlidou.

Publications

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Abbreviations

AD	Alzheimer's disease
AD-PD	autosomal dominant PD
AIF	apoptosis inducing factor
Akt	protein kinase B
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
Apaf-1	apoptotic protease-activating factor 1
AR	androgen receptor
AR-PD	autosomal recessive PD
ATP/ADP/AMP	adenosine trisphosphate/disphosphate/monophosphate
bp	base pairs
BSA	bovine serum albumine
CCCP	carbonyl cyanide chlorophenylhydrazone
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator
CHIP	carboxyl terminus of the Hsc70 -interacting protein
CIAP	calf intestine alkaline phosphatase
CMV	cytomegalovirus
CMV-IE	cytomegalovirus immediate-early
DA	dopamine, 3,4-dihydroxyphenethylamine
ddH_2O	double distilled water
DIABLO	direct IAP binding protein
DJBP	DJ-1 binding protein
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
dUTPs	deoxy-uridine triphosphates
DRPLA	dentatorubropallidoluysian atrophy
E	early gene

E.coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	diaminoethanetetra-acetic acid, disodium salt
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FADD	Fas-associated protein with death domain
FCS	fetal calf serum
FGM	full growth medium
FITC	fluorescein isothiocyanate
FKBP	FK506 binding protein
GAD	glutamic acid decarboxylase
gad	gracile axonal dystrophy
GDNF	glial derived neurotrophic factor
GFP	green fluorescent protein
GR	glucocorticoid hormone receptor
HBSS	Hank's balanced salt solution
HD	Huntington's disease
HMBA	hexamethylene bisacetamide
HNE	4-hydroxy-2-trans-nonenal
HO1	haem oxygenase 1
HSE	heat shock element
HSF	heat shock factor
Hsp	heat shock protein
HSV-1	herpes simplex virus type 1
ICP	infected cell protein
IE	immediate-early gene
$InsP_3$	inositol 1,4,5-trisphosphate
Kb	kilobase
kDa	kiloDalton
L	late gene
L-15	medium Leibovitz's L-15 medium
LAT	latency associated transcript
LAT P	latency associated transcript promoter
LB	Lewy body

LTR	long terminal repeat
m.o.i	multiplicity of infection
Maneb	manganese ethylenepistithiocarbamate (fungicide)
MAP	mitogen-activated protein
MAPK	mitogen activated protein kinase
MCS	multiple cloning site
MDMA	3,4-methylenedioxy-methamphetamine (or ecstasy)
MFI	mean fluorescence intensity
MJD	Machado–Joseph disease
MMTV	murine mammary tumor virus
MPP^+	methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
mtDNA	mitochondrial DNA
MTG	Green fluorescent MitoTracker Green FM
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
Pael-R	parkin-associated endothelin receptor-like receptor
PAGE	polyacrylamide gel electrophoresis
Paraquat	1,1'-dimethyl-4,4'-5 bipyridinium
PBS	phosphate buffered saline
PD	Parkinson's Disease
PDGF	platelet-derived growth factor
PET	positron emission tomography
pfu	plaque forming units
PI	propidium iodide
PINK1	PTEN-induced kinase 1
PKC	protein kinase C
polyQ	polyglutamine
PS	phosphatidylserine
RA	retinoic acid
RM	rainbow protein marker
RNA	ribonucleic acid
ROS	reactive oxygen species

rpm	revolutions per minute
RSV	Rous sarcoma virus
SBMA	spinal and bulbar muscular atrophy
SCA1	spinocerebellar ataxia type 1
SD	standard deviation
SDS	sodium dodecyl sulphate
SFM	serum free medium
SIAH	human SINA(seven in absentia) homologue
SMAC	second mitochondrion derived activator of caspases
SOD-1	superoxide dismutase
SUMO	small ubiquitin-related modifier
TAE	tris-acetate-EDTA-buffer
TdT	terminal deoxynucleotidyl transferase
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TMRM	tetramethylrhodamine methyl ester
TPR	tetratricopeptide repeat
TUNEL	TdT-mediated dUTP Nick End Labelling
TWEEN 20	polyoxyethylene-sorbitan monolaurate
UCH-L1	ubiquitin carboxyl-terminal-hydrolase L1 (PGP9.5)
UPS	Ubiquitin-proteasome system
UV	ultraviolet
VMAT2	vesicular monoamine transporter 2
VP16	virion protein 16
wt	wild type
X-gal	4-Cl,5-bromo,3-indolyl-β-galactosidase
$\Delta\Psi m$	mitochondrial membrane potential (Delta psi)
α-Syn	alpha-synuclein
β-Syn	beta-synuclein
γ-Syn	gamma-synuclein

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CHAPTER 1

Introduction

1.0 Introduction

The work described in this thesis attempted to investigate the potentially toxic effects of α-synuclein (α-Syn) and its Parkinson's disease (PD) associated mutants, A30P and A53T, in an *in vitro* cellular model. Considering previous experimental evidence on the protective effect of heat shock proteins (hsps) it was subsequently tested if they play a neuroprotective role in this system and if so, what the mechanisms are of such neuroprotective effect. For this purpose, Herpes Simplex Virus type 1 (HSV-1) based vectors, originally developed for gene therapy studies in the central nervous system, were utilised in order to efficiently over-express hsps in mammalian neuronal cells.

Therefore the Introduction of this thesis focuses on the following aspects, which are directly relevant to the work presented here:

a. Parkinson's disease genetics with references to the identified genes in hereditary PD and a detailed review on α-Syn in PD and the experimental models of PD.

b. The role of programmed cell death in PD, an overview of apoptotic cell death and the importance of the mitochondrion both in apoptosis and in PD.

c. Heat shock protein biology, their roles in the cell and in the context of neurodegeneration as well as the role of hsps as therapeutic targets for PD and other neurodegenerative disorders. A brief account is also given on the viral vectors used in this study and their potential use in the development of therapeutical strategies for PD or other diseases.

1.1 Parkinson's Disease

Parkinson's disease (PD) is a common, slowly progressive, age related neurodegenerative movement disorder affecting about 1% of the human population over 65 years of age and 4%-5% of the population over the age of 85. PD is sporadic in the majority of cases but familial cases also exist, reaching only about 10% of all diagnoses. After loss of 70%-90% of the dopaminergic neurons of the substantia nigra pars compacta, leading to reduced dopamine levels in the striatum, clinical symptoms begin to manifest. These are resting tremor, rigidity, bradykinesia, gait dysfunction and postural instability. The pathological hallmark of PD is the presence of eosinophilic intracytoplasmic proteinaceous inclusions called Lewy Bodies (LBs) or when these inclusions are in the neuronal processes are called Lewy neurites (LNs) (Fearnley and Lees, 1991). The pattern of distribution of LBs and LNs are strongly correlated to severity of neurodegeneration. In addition, there is a pronounced loss of dopaminergic neurons in the substantia nigra pars compacta, resulting in dopamine depletion in the striatum, into which these neurons project. In addition, neuronal death and LBs can also be found in the locus ceruleus, nucleus basalis of Meynert, hypothalamus, cerebral cortex, cranial nerve motor nuclei and central and peripheral components of the autonomic nervous system (Olanow and Tatton, 1999). LBs and LNs are found in both sporadic and familial PD, with some inconsistency in LB formation in some cases of early onset autosomal recessive PD due to parkin mutations.

The exact aetiology of PD is unclear. However, several environmental factors, as well as various - originally underestimated - genetic factors, which are now a great focus of PD research, are linked to PD. There are various epidemiological and experimental studies supporting an environmental influence for PD. For instance, two case-control studies (examining 57 and 144 PD patients respectively) reported an association between pesticide exposure and increased risk for PD (Hertzman et al., 1990 and Gorell et al., 1998).Gorell et al. (1998) demonstrated that there was a significant association of PD risk with herbicide or insecticide exposure or farming as an occupation but this risk

is not increased with rural living or drinking of well water. More recently, pesticides like rotenone and herbicides like paraquat have been directly implicated in PD (Olanow and Tatton, 1999; Sherer et al., 2001).Interestingly, exposure to rotenone (Betarbet et al., 2000) or paraquat (McCormack et al., 2002) has produced useful animal PD models. Finally, a very compelling evidence for an environmental factor and PD is related to the toxin 1,2,3,6-methyl-phenyl-tetrahydropyridine (MPTP) which was taken by drug addicts and developed a syndrome resembling PD, both clinically and pathologically (Langston et al., 1983).

On the other hand, fewer cases of PD with known genetic influence have been identified but the mechanisms of pathogenesis have only recently begun to be explored and will be described later. The current knowledge suggests the probability that both genetic and environmental factors are likely to contribute to the development of PD and that more than one cause may be operative in a single patient. Genetic studies of the rare families with hereditary PD have identified loci and genes involved in monogenic forms of the disease providing valuable knowledge on the disease molecular pathogenesis, which is essential for the development of strategies for prevention and cure of PD.

1.2 The Genetics of Parkinson's disease

There are several disease-causing genes that have been implicated in the aetiology of some forms of PD, multiple sclerosis, epilepsy, motor neuron disease, Alzheimer's disease, resulting from parametric and non-parametric linkage analysis and the candidate gene approach (Wood, 1997; Bell and Lathrop, 1996; Robberecht, 2000; Bertram and Tanzi, 2001). The evidence in favour of a genetic predisposition in PD comes from epidemiologic surveys, twin studies, analysis of dominant families with typical PD with LB pathology and candidate gene studies (reviewed by Vaughan et al., 2001).

Twin studies in particular are very useful in defining the contribution of genes to disease cause, but there has been some conflicting evidence for PD. The largest twin study (Tanner et al., 1999) screened 19,842 white male twins

for PD and compared the estimated disease concordance rates between monozygotic and dizygotic twin pairs. Monozygotic twins are genetically identical and dizygotic twins share about 50% of their genes. If there is a genetic cause to the disease the concordance rates in the dizygotic twins would be greater. The study found similar overall concordance rates and suggested that genetic factors do not play a role in late onset PD. But when they analysed 16 twin pairs with diagnosis before the age of 50, they found that there is a relative risk of six, i.e. if one twin was diagnosed with PD before the age of 50, the other twin was six times more likely to develop PD in monozygotic twin pairs than in dizygotic twin pairs, suggesting that genetic factors are important in earlier onset PD.

However, the above study has limitations due to the fact that the estimation of concordance rates was based on clinical diagnosis alone (concordance was stratified by zygosity and age at diagnosis). In PD an individual that is partially lesioned has no clinical manifestation of the disease but will develop PD symptoms later on, therefore a twin that might be affected and is presymptomatic will be counted as unaffected. Piccini et al. (1999) overcame this by using positron electron tomography (PET) and fluorodopa to assess dopaminergic function in affected and unaffected monozygotic and dizygotic twins and revealed higher concordance for PD in monozygotic than in dizygotic twins (Piccini et al., 1999). Therefore further longitudinal studies using both clinical and PET information are necessary, before we could claim definitely that genetic factors are more relevant in early onset PD or that the variable rate of disease progression in monozygotic twins is due to differential environmental effects, providing a clue for gene-environment interaction in PD.

The current understanding we have of the complexity of PD genetics is based on nine distinct loci responsible for rare Mendelian forms of this disease (Table 1.1) and will be described in separate sections. In addition, following the discovery of mutations causing rare forms of familial PD, several "genetic models" of PD have been developed, for example α-Syn and parkin models, and they will be reviewed in the respective sections of this chapter.

__Table 1.1__ **Loci and Genes linked to Parkinson's Disease**

Locus	Chromosomal Location	Gene	Inheritance and Families	Clinical features	LBs
PARK 1	4q21.3	*α-Synuclein*	AD / 13	Early onset, fast progression	+
PARK 2	6q25.2-27	*Parkin*	AR/>60	Juvenile onset dystonia	+/-
PARK 3	2p13	Unknown	AD/6	Late onset PD	+
PARK 5	4p14	*UCH-L1*	AD/1	Typical PD	?
PARK 6	1p35-p36	*PINK1*	AR/9	Early/late onset, slow progr.	?
PARK 7	1p36	*DJ-1*	AR/2	Early onset	?
PARK 8	12p11.2-q13.1	*LRRK2**	AD/>30	Typical PD, variable onset	+/-
PARK 9	1p36	Unknown	AR	Kufor-Rakeb Syndrome	?
PARK 10	1p32	Unknown	Susceptibility gene	Late onset PD	?

AD=Autosomal dominant, AR=Autosomal recessive, LBs=Lewy bodies; the information is from Warner and Schapira (2003) and was updated using sources cited in the respective sections of the genes.* Paisan-Ruiz et al. (2004).

The first breakthrough in gene mapping for familial PD occurred when Polymeropoulos et al. (1996) reported linkage of autosomal dominant PD to chromosome 4q21-23 in an Italian-American family (Contursi family) with typical clinical and pathological features of PD. Further analysis identified a single base change at position 209 from G to A resulting in an alanine to threonine substitution at position 53 of the α-Syn protein (A53T α-Syn) (Polymeropoulos et al., 1997), which was later found to be a very rare cause of familial PD. Vaughan et al. (1998) found that none of 230 European patients with familial PD had the G209A mutation. In addition to the A53T mutation the A30P mutation was subsequently identified in one German family (Kruger et al., 1998). There has also been a recent discovery of a PD family with α-Syn gene triplication (Singleton et al., 2003) and this is further discussed later. Apart from being mutated in a few familial cases of PD, α-Syn is a major component of LBs, the neuropathological hallmarks of PD found in both familial and sporadic cases. The above findings have led researchers to intensely investigate the role of this protein in PD pathogenesis. Since α-Syn is the major focus of this thesis, it will be discussed in detail later in this chapter, following Parkin, UCH-L1, DJ-1 and PINK1.

1.3 Genes Linked to Inherited Parkinson's Disease

1.3.1 Parkin

The genetic locus for autosomal recessive PD (AR-PD) was mapped to chromosome 6q25.2-27 in Japanese families (Matsumine et al., 1997). Various other families were identified from Europe, America, the Middle East and North Africa. This genetic locus (PARK2) encodes parkin protein, which has been identified as an E3 ubiquitin-ligase (Shimura et al., 2000). The clinical features include an earlier disease onset (under the age of 40), tremor, rigidity and dystonia that are more slowly progressive. The pathological features are similar to sporadic PD except for the notable but inconsistent absence of LBs. Studies of parkin mutants provide conflicting results regarding the anatomic distribution of LBs and neuronal loss because there are parkin cases with AR-PD with or without LBs (van de Warrenburg et al., 2001; Farrer et al., 2001). For instance, van de Warrenburg et al. (2001) showed that a 75 year-old parkin patient had selective dopaminergic neuronal loss in the substantia nigra pars compacta without LBs but with degeneration of parts of the spinocerebellar system, whereas Farrer et al. (2001) showed that a 52 year-old heterozygote for parkin mutations had a marked loss of dopaminergic neurons in the substantia nigra and numerous LBs, which were also found in the locus ceruleus and the nucleus basalis of Meynert.

Interestingly, there were no pathogenic mutations in 95 isolated cases and 23 cases with probable autosomal recessive PD with onset after the age of 45 (Oliveri et al., 2001), but overall at least 60 different parkin mutations (frequent point mutations and exon rearrangements) were found in patients with early onset PD. Periquet et al. (2001), using intragenic and closely flanking markers, demonstrated a founder effect accounting for several point mutations in parkin but not for exon rearrangements that were detected in families of various geographical origins. The largest amount of data on parkin mutations has come from the 'European Consortium on Genetic Susceptibility in PD', as they have sequenced the coding region of the parkin gene in 73 European families with early onset AR-PD and revealed a great variety of parkin

mutations (Lucking and Brice, 2000). In total, 19 different deletions or multiplications of exons were detected as well as 16 different point mutations in this particular study (frameshift, nonsense and missense).The parkin mutations were frequent (79%) in sporadic PD patients that develop symptoms before the age of 20, suggesting a causal role. In contrast, parkin mutations occurred in only 3% of patients with sporadic PD that develop symptoms over the age of 30 and therefore suggest that parkin is not causally important in this patient group.

Parkin is a 465 amino acid protein that has been identified as an E3 ubiquitin-protein ligase involved in the attachment of ubiquitin to the proteins that are targeted for proteasomal degradation (Shimura et al., 2000). E3 ligases often demonstrate substrate selectivity and several studies identified parkin substrates such as CDCrel-1, a synaptic vesicle protein (Peng et al., 2002), synphilin-1 (Chung et al., 2001), α-Syn *via* an *O*-glycosylated form in human brain (Shimura et al., 2001), parkin-associated endothelin receptor-like receptor (Pael-R) (Imai et al., 2001), and cyclin E (Staropoli et al., 2003). However, several other proteins have been identified as parkin substrates (see review by Kahle and Haass, 2004), leading to the hypothesis that loss-of-function mutations of parkin might impair toxic or aggregated protein removal *via* the proteasome. Interestingly, parkin also mediates the clearance of some polyglutamine repeat proteins (Tsai et al., 2003).

As reviewed by Feany and Pallanck (2003), there are three relatively recently published papers beginning to address some important issues while also raising new questions. They demonstrate a role of parkin in protecting neurons from diverse insults: α-Syn toxicity, proteasomal inhibition, Pael-R accumulation, and kainate-induced excitotoxicity (Petrucelli et al., 2002; Yang et al., 2003; Staropoli et al., 2003). It is important to note that they provide significant evidence on parkin's protective role and also the first evidence that Pael-R is selectively toxic to dopaminergic neurons. This selective toxicity, like the α-Syn-mediated toxicity, is influenced by parkin. Interestingly, parkin was also shown to be *S*-nitrosylated *in vitro* and *in vivo* in PD brains, which leads to

inhibition of its E3 ligase activity and its protective function (Chung et al., 2004).

In addition to its protective role in the above systems, parkin over-expression *in vitro* has been shown to protect against cell death in other systems, as for example in the study by Darios et al. (2003). Cell death mediated by ceramide, but not by some other stimuli, was suppressed by parkin over-expression. Protection was mediated by the E3 ubiquitin ligase activity of parkin, since disease-causing mutant parkin did not protect. Parkin also delayed mitochondrial swelling, cytochrome c release and caspase-3 activation during the death stimulus ceramide, while parkin was shown to be associated with the mitochondrial membrane (Darios et al., 2003).

Although wt parkin over-expression is generally protective, loss of parkin in various models has also been reported. In view of important recent findings from the generation of various fly (Greene et al., 2003; Pesah et al., 2004) and mouse models (Goldberg et al., 2003; Itier et al., 2003; Palacino et al., 2004; Von Coelln et al., 2004), there are some proposed models that summarise the pathways regulated by parkin and the effects of loss of parkin function (Feany and Pallanck, 2003; Kahle and Haass, 2004) but more work has do be done before we have a clearer understanding of the role of parkin in PD.

Firstly, in the *Drosophila* model, parkin null mutants have reduced lifespan, apoptotic cell death in flight muscles, and male sterility (Greene et al., 2003), which can be attributed to mitochondrial dysfunction. The mouse models developed by Goldberg et al. (2003) and Itier et al. (2003) both show subtle behavioural deficits, some dysfunction of dopamine and glutamate neurotransmission and abnormalities in dopamine metabolism, but no loss of catecholaminergic neurons. Parkin null mice by targeted deletion of parkin exon 7 show a loss of noradrenergic neurons and a marked reduction of the norepinephrine-dependent startle response, whereas the nigrostriatal dopaminergic system does not show any defects (Von Coelln et al., 2004). On the other hand, one of the mouse models (Palacino et al., 2004) has high concentrations of striatal extracellular dopamine, normal parkin E3 ligase

substrate concentrations and dopamine receptor binding affinities, low synaptic excitability and mild, non-progressive motor deficit but no inclusion formation nor dopaminergic neuron loss. In addition, these mice have deficits in mitochondrial function and decreased levels of proteins involved in protection from oxidative stress (Palacino et al., 2004).

In summary, parkin null mice do not have dopaminergic cell loss and it is therefore essential to develop an animal model that recapitulates the selective loss of dopaminergic neurons, which is the case in humans. Nevertheless, they indicate a role for parkin in dopaminergic neurotransmission and/or mitochondrial function. Von Coelln et al. (2004) suggested that the mild phenotype in parkin knockout mice compared to the human disease may be due to (a) a redundancy in the mouse but not in the human E3 ligase enzyme family (b) the short lifespan of mice, which may not live long enough to accumulate toxic amounts of potentially toxic parkin substrate in contrast to humans or (c) loss of parkin activity may prerequisites a toxic stimulus or cellular stress to induce a PD phenotype.

Overall, many questions remain unanswered regarding parkin and its functional role in both sporadic and familial PD. Recent data, revealing several parkin substrates, parkin interacting proteins (see review by Kahle and Haass, 2004) and parkin modifications such as nitrosylation (Chung et al., 2004), suggest that parkin has a complex role in PD pathology which involves functions additional to its property to ubiquitinate.

1.3.2 UCH-L1

UCH-L1 was linked to PD by an autosomal dominant point mutation (I93M) that was identified in two siblings with a strong family history of PD (Leroy et al., 1998). UCH-L1 is an abundant neuronal enzyme (1%–2% of brain protein) (Wilkinson et al., 1989) that catalyzes the hydrolysis of C-terminal ubiquityl esters and amides; peptide-ubiquityl amides are the preferred substrates (Larsen et al., 1996; Larsen et al., 1998). This activity is important for

cytoplasmic protein degradation because it allows recycling of ubiquitin. UCH-L1 frees ubiquitin by cleaving ubiquitylated peptides that are the products of the proteasomal degradation of polyubiquitylated proteins (Larsen et al., 1996; Larsen et al., 1998).

The PD I93M mutation decreases the *in vitro* hydrolytic activity of UCH-L1 but there is evidence suggesting that simple loss of hydrolytic function does not completely explain the PD phenotype in this family: the mutation is not 100% penetrant; the father of the two affected individuals who was the presumed carrier of I93M did not develop PD. The failure to detect PD associated I93M mutations in hundreds of other patients with familial and sporadic PD indicates that this gene mutation is responsible for only a few rare cases of PD. Post-mortem brain tissue from patients with this mutation is not yet available, so it is unknown whether LB formation exists in inherited PD. However, UCH-L1 is found in the LBs associated with sporadic PD (Lowe et al., 1990). It is possible that loss of UCH-L1 activity in PD might lead to reduced labeling and impaired clearance of abnormal proteins, and consequent neurodegeneration.

Although there are no reported genetic models for PD involving *UCH-L1,* there is the UCH-L1 deficient gad (gracile axonal dystrophy) mouse resulting from intragenic deletion in *UCH-L1* (Saigoh et al., 1999).The gad mice have an in-frame deletion including exons 7 and 8 of *UCH-L1* resulting in an allele that encodes a truncated UCH-1, lacking 42 amino acids containing a catalytic residue. However, these mice, lacking functional UCH-L1, do not develop a parkinsonian phenotype and the neuronal loss is in the gracile tract and not in the substantia nigra (Kurihara et al., 2001; Saigoh et al., 1999; Miura et al., 1993; Mukoyama et al., 1989). They develop sensory and motor ataxia, axonal degeneration and formation of spheroid bodies in nerve terminals.

Furthermore, a polymorphism in the UCH-L1 gene (S18Y) was discovered and was later found to be linked to a decreased susceptibility to PD (Satoh and Kuroda, 2001).This polymorphism is relatively rare in the European population (allele frequency is 14%–20%) but common in the Japanese (39%–

54%) and Chinese (50%) populations (Levecque et al., 2001). Subsequently, Liu et al. (2002) showed that UCH-L1 exhibits a second, dimerization-dependent, ubiquityl ligase activity. The above polymorphic variant S18Y has reduced ligase activity but comparable hydrolase activity as the wild type (wt) enzyme. Therefore, this study demonstrated that one gene can encode two related enzyme activities, for the case of UCH-L1, a beneficial hydrolase activity and a dimerization-dependent ligase activity that is at least partly pathogenic. The authors speculated that decreasing UCH-L1 expression could have a therapeutic benefit or ideally a PD therapeutic would, like the protective S18Y polymorphism, inhibit the ligase activity and, in addition, promote the hydrolase activity.

More recent findings confirm that UCHL-1 is a susceptibility gene for PD and a potential target for disease-modifying therapies (Maraganore et al., 2004). Finally, UCH-L1 has been implicated in two other neurodegenerative diseases: spinocerebellar ataxia (SCA), in which a UCH mutant is a genetic enhancer of degeneration in SCA transgenic flies (Fernandez-Funez et al., 2000b), and Huntington's disease (HD), in which the UCH-L1 S18Y polymorphism is linked to age at onset (Naze et al., 2002).

1.3.3 New Genes for Parkinson's Disease: *DJ-1* and *PINK1*

1.3.3.1 *DJ-1*

DJ-1 (PARK7) was reported as a new gene associated with recessively inherited PD with a homozygous exon deletion and a homozygous point mutation in two families (Bonifati et al., 2003). Clinically, these two families demonstrate early-onset disease in homozygotes, typical PD symptoms, nigral loss and overall the picture of the disease is very similar to parkin cases, significantly overlapping with idiopathic PD. To date, DJ-1 mutations that have been linked to autosomal recessive PD include missense, truncating and splice-site mutations and large deletions (Abou-Sleiman et al., 2003; Cookson, 2003). The mutations are even less frequent than parkin (PARK2) mutations in early-onset PD (Hedrich et al., 2004; Cookson, 2003).

The DJ-1 gene has seven exons and encodes a 189 amino acid protein. DJ-1 is a protein with several known protein-protein interactions. However, our knowledge is too limited to understand how this gene fits in the PD puzzle, together with the other genes. Before DJ-1 was identified to be linked to inherited PD, it had been shown to interact with c-myc and increase cell transformation (Nagakubo et al., 1997), whereas CAP1, the rat homologue, was associated with infertility (Wagenfeld et al., 1998). DJ-1 was also shown to inhibit the RNA binding activity of a multiprotein complex which is responsive to cyclic AMP and to stabilise mRNA (Hod et al., 1999). Then, (Takahashi et al., 2001b) reported that DJ-1 interacts with PIASxα, which interacts with the androgen receptor, inhibiting gene expression. PIASxα is an E3-like enzyme that sumoylates proteins and interestingly DJ-1 is sumoylated at K130 (Hegde and DiAntonio, 2002). In relation to the study by Takahashi et al. (2001), mentioned above, Niki et al. (2003) later suggested that DJ-1 positively regulates the androgen receptor. They found a novel DJ-1 binding protein (DJBP) that negatively regulates the androgen receptor by recruiting a histone deacetylase complex. Therefore DJ-1 has been shown to have quite important roles in different studies.

After the report by Bonifati et al. (2003), in which DJ-1 mutations were shown to be the cause of recessively inherited PD, further studies reported that wt DJ-1 has cytoprotective roles. Wt DJ-1 protects under oxidative stress, since reactive oxygen species were eliminated by self oxidation of wt DJ-1, but mutant DJ-1, including the PD L166P mutant, lead to cell death (Yokota et al., 2003; Taira et al., 2004). In specific, when Yokota et al. (2003) knocked down the endogenous DJ-1 in a neuronal cell line, there was increased cell death induced by oxidative stress, ER stress, and proteasome inhibition, but not by pro-apoptotic stimulus. In contrast, cell death from H_2O_2 was reduced by wt DJ-1 over-expression, but not by L166P mutant DJ-1. In another study, DJ-1 expression was shown to be induced by oxidative stress, whereas there was a reduction in the protective effect from H_2O_2-induced cell death by over-expression of various DJ-1 mutants (Takahashi-Niki et al., 2004).

In addition, the following studies provided an insight to some of the properties of wt and mutant DJ-1. Miller et al. (2003) found that L166P destabilizes DJ-1 and promotes its degradation through the ubiquitin-proteasome system. Subsequently, (Olzmann et al., 2004) found that the L166P mutation impaired the intrinsic folding propensity of DJ-1 protein, resulting in a spontaneously unfolded structure that was incapable of forming a homodimer with itself or a heterodimer with wt DJ-1. As a result of protein misfolding, the mutant DJ-1 was selectively polyubiquitinated and rapidly degraded by the proteasome. Therefore, as a misfolded and less stable protein, L166P DJ-1 may cause cytotoxicity by overwhelming the cellular protein degradation systems and by undergoing abnormal subcellular localization, for instance in mitochondria (Moore et al., 2003).

Finally, Canet-Aviles et al. (2004) has recently demonstrated that wt DJ-1 protects against neuronal death, and that this is signaled by acidification of the cysteine residue, C106. The acidic isoform accumulates after oxidative stress, suggesting that DJ-1 has a protective effect under these particular conditions. The same study reported that wt or C53A DJ-1 was readily oxidized in cultured cells but an artificial C106A mutant was not. They also observed that oxidation-induced mitochondrial relocalization of DJ-1 and that protection against cell death was lost in C106A but not C53A or C46A mutants. Overall, these data correlate the ability of DJ-1 to oxidize, translocate to mitochondria in response to oxidation and protect against death.

The studies that have been conducted so far, however, have not shed much light into the mechanism *via* which DJ-1 mutations cause PD and therefore further studies are essential before a conclusion can be drawn for the part of DJ-1 in the puzzle of PD.

1.3.3.2 *PINK1*: New Gene for Autosomal Recessive Parkinson's Disease

Valente et al. (2004) reported that a newly identified familial form of PD is caused by a mutation in a putative mitochondrial protein kinase called PINK1 (PTEN-induced kinase 1). PINK1 contains a mitochondrial localization sequence, is in fact localized to the mitochondria and protects cells against loss of mitochondrial membrane potential resulting from incubation of cells with proteasome inhibitors, as found in the same study. Interestingly, the protection is abrogated by the disease mutations. It is however unknown if PINK1 regulates mitochondrial function; also the substrates for its putative kinase activity are unknown. Since PINK1 locates to the mitochondria, it is expected that its substrate(s) include mitochondrial proteins. Further studies of PINK1, especially those utilising *in vivo* systems, and human material, are expected to answer some important questions in the field.

1.3.4 α-Synuclein: An Overview

As mentioned earlier, α-Syn was the first protein to be found mutated in humans and lead to exhibition of the full clinical features of PD. Mutations in α-Syn are inherited in an autosomal dominant manner and are the focus of numerous studies, including the present study. Importantly, α-Syn is a major component of the neuropathological hallmark of the disease, the Lewy body, found in sporadic and in some but not all familial cases of PD. This was thought to be of particular importance for PD research as there might be molecular pathways that are common in the disease process in both sporadic and familial PD. Therefore, understanding the molecular pathogenesis of PD, along with further evaluation of the relative importance of environmental and genetic factors in PD, will help identifying pharmacological targets and develop new effective therapies.

The normal function of α-Syn is not yet very clear. α-Syn is a 140–amino acid protein that contains repetitive imperfect repeats of KTKEGV in the amino-terminal half, a hydrophobic region, and an acidic carboxy-terminal region (Clayton and George, 1998) (Figure 1.1.A). It has been suggested that α-

Syn might have a role in the modulation of synaptic vesicle turnover and synaptic plasticity but its normal role and its role in PD is still not very well understood and this will become apparent in later sections of this thesis.

α-Syn belongs to a family of proteins with at least three different members, α-, β-, and γ-Synuclein, which are expressed from three different genes (reviewed by Clayton and George, 1998). Only α-Syn is present in LBs and the role of β- and γ-Synuclein are still unknown, although there is a report by Hashimoto et al. (2001) showing that β-Syn expression in mice suppresses the PD phenotype that resulted from expression of α-Syn. An additional *in vitro* study also supports a beneficial role by β-Syn in inhibiting α-Syn fibrils and protofibril formation (Park and Lansbury, Jr., 2003). Figure 1.1.B illustrates the role of β-Syn in a proposed model of α-Syn induced toxicity (adapted from Dawson and Dawson, 2003).

Synucleins share structural similarity with apolipoproteins but they are also present in the cytosol and presynaptic terminals. Overall, synucleins are abundant brain proteins whose physiological functions are poorly understood. Interestingly, no synuclein homologues have been identified in *Caenorhabditis elegans* or in *Drosophila*. α-Syn and β-Syn have identical C-terminals and are found in neuronal terminals, whereas γ-Synuclein (a protein that strongly correlates with breast cancer progression named also as persyn) is present throughout the neuron (Clayton and George, 1998). A recent report identified γ-Synuclein as a novel heat shock protein associated chaperone that stimulates estrogen receptor–alpha signaling that leads to mammary tumorigenesis (Jiang et al., 2004). Finally, inactivation of the γ-Synuclein gene in mouse midbrain dopaminergic neurons leads to reduced numbers of dopaminergic neurons. However, no significant motor dysfunction was found, and the same is true for the double α- and γ-Syn null mice (Robertson et al., 2004). Interestingly, resistance to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity was found in all three mutant mice suggesting a possible activation of unknown compensatory mechanisms for the neuronal survival upon MPTP stress (for

MPTP see later; a toxin that inhibits mitochondrial complex I and produces PD in humans and other organisms).

As described earlier in the section on the genetics of PD, mutations in α-Syn were associated with early onset PD after linkage analysis of several Greek families and the Contursi kindred. The point mutation identified by Polymeropoulos et al. (1997) was the A53T substitution. Subsequently, in 1998, α-Syn gene sequencing in a German family with autosomal dominant PD revealed an A30P point mutation (Kruger et al., 1998) and recently a similar approach by Zarranz et al. (2004) has revealed a third E46K mutation in α-Syn in a Spanish family. The E46K mutation substitutes a basic amino acid with an acidic, which is a more profound change for a protein than the two other point mutations. The E46K mutation could change the physicochemical and molecular properties of this protein such as polarity, affecting its lipid interactions (with membrane phospholipids) that could potentially alter vesicular trafficking and transmitter release. Furthermore, a lysine residue could be a site for potential covalent modifications by ubiquitin or by the small ubiquitin-related modifier (SUMO). Therefore, stability, aggregation propensity or other protein interactions of α-Syn may be altered.

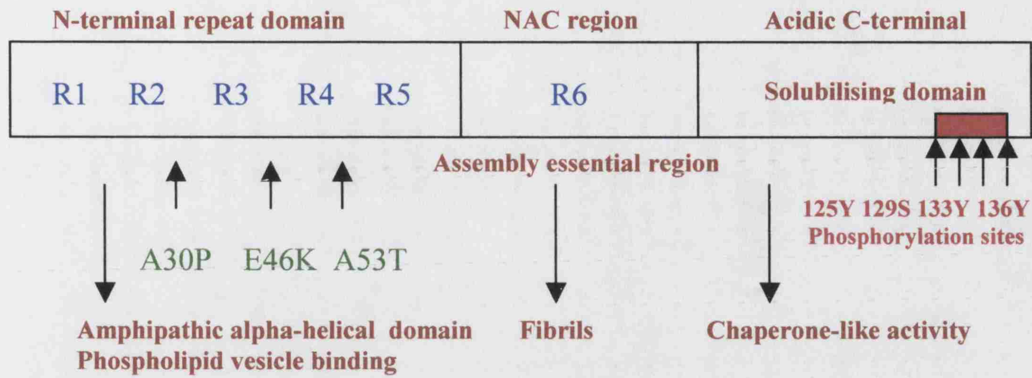
The above three mutations are dominant, suggesting that they cause PD *via* a toxic gain-of-function rather than a loss of function. Each mutation lies in (for A53T, among) the six tightly spaced 11-amino acid imperfect repeats that bear the consensus sequence KTKEGV near the conserved N-terminus of the 140-amino acid α-Syn accounting for reversible lipid binding properties (Bussell, Jr. and Eliezer, 2003). The six 11-amino acid repeats in α-Syn do not assume secondary structure in the dissolved state but they probably form α-helices when bound to lipids (Chandra et al., 2003). When the helices are disrupted by mutations, the protein misfolds, can aggregate and form fibrils (Takeda et al., 1998) (Figure 1.1B). The aggregates are argued that they may impair the function of the proteasome, leading to a potential inability to degrade α-Syn (Tanaka et al., 2001). In addition, Jensen et al. (1998) found that α-Syn with the A30P mutation loses its vesicle-binding activity and proposed that

mutant α-Syn may accumulate, leading to assembly into LBs. It has also been shown that α-Syn is abundant in presynaptic terminals and binds to a number of proteins as well as lipid membranes (Jo et al., 2000). Subsequently, Jo et al. (2002) demonstrated that the A30P mutant α-Syn is defective in binding to phospholipid vesicles in vitro as determined by vesicle ultracentrifugation, circular dichroism spectroscopy, and low-angle X-ray diffraction. Their data also suggest that α-Syn may bind to the lipid vesicles as a dimer, which suggest that this species could have some physiological relevance. The A53T mutant had normal membrane-binding activity that was comparable to this of wt α-Syn. In addition to its altered interaction with lipids that cause fibril and protofibril formation, some other aspects are illustrated in Figure 1.1.B but will be described later.

Surprisingly, an American family of mixed northern European origin with autosomal dominant, young-onset PD was found to have a *α-synuclein* gene triplication - with four fully functional gene copies resulting in two-fold α-Syn over-expression (Singleton et al., 2003). A similar triplication in a Swedish-American family was also reported (Farrer et al., 2004). These findings suggest that excess normal α-Syn can lead to PD. Finally, a polymorphic marker in the promoter region of *α-synuclein* is now associated with sporadic PD, after comparing polymorphic allele frequencies in patients and controls (Holzmann et al., 2003).

Overall, the physiological role of α-Syn is unknown, but there is speculation supported by a significant amount of observations, as will be discussed later. A key point is that mutations in α-Syn or increased gene dosage can cause PD in humans. However, it is still not clear whether α-Syn has a direct causative effect in the most common, sporadic PD or whether it is merely one of the consequences of the pathogenic process (a marker of the pathology). It will be very interesting to find out precisely how those mutations cause PD and also what is the role of normal α-Syn in the neuron.

A



B

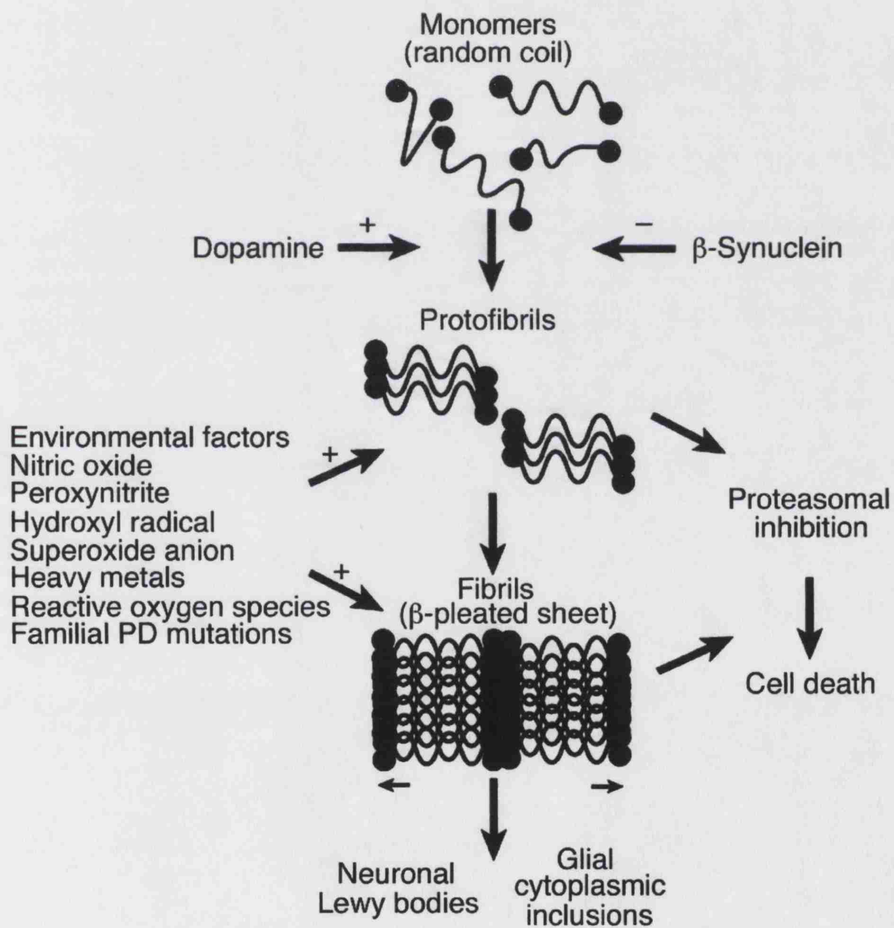


Figure 1.1 Structure of α-Syn and a simplified model of α-Syn aggregation and toxicity.

In the top panel **(A)**, the PD-associated mutations are illustrated as well as some other features of α-Syn, such as the imperfect (KTKEGV) repeats (R) and the PD mutation sites. The bottom panel **(B)** illustrates a model of α–Syn aggregation and toxicity based on experimental evidence and the proposed factors that enhance (+) or inhibit (–) the formation of toxic aggregated forms of α-Syn. Dopamine (DA) enhances the formation of the protofibrillar form of α-Syn and prevents it from aggregating into the fibrillar form (Panel B reproduced from Dawson and Dawson, 2003, with permission of Am. Soc. for Clin. Investigation, *via* Copyright Clearance Center, Inc).

1.3.4.1 α-Syn Knockout and Transgenic Animals

This section presents some important transgenic fly, α-Syn knockout mouse and transgenic wt and PD mutant α-Syn mouse models as well as marmoset α-Syn models. It should be noted that none of the mammalian transgenic models fully recapitulate PD, but they have been useful for the study of α-Syn-induced neurodegeneration and also studying the normal or mutant α-Syn roles in normal and PD brain respectively (Masliah et al., 2000; van der Putten et al., 2000; Matsuoka et al., 2001; Giasson et al., 2002; Lee et al., 2002; Richfield et al., 2002). Overall, none of mouse models has dopaminergic neuronal death in the substantia nigra but abnormal accumulation of detergent-insoluble α-Syn and abnormal proteolytic processing of α-Syn appear to be associated with neurodegeneration in most models.

The *Drosophila* model was developed by Feany and Bender in the year 2000. When normal and mutant forms of human α-Syn are over-expressed in *Drosophila*, the flies develop an adult-onset progressive loss of DA neurons and filamentous interneuronal inclusions that contain α-Syn, despite the fact there is no fly homologue of α-Syn. As will be discussed later, over-expression of heat shock protein hsp70 rescues the motor and neuropathologic features of transgenic flies expressing normal and mutant forms of α-Syn (Auluck et al., 2002), so the fly model has been useful in the investigation of some aspects of the disease pathogenesis.

On the other hand, α-Syn knockout mice are viable, fertile and have normal brain structure and normal complement of dopaminergic cell bodies, fibres and synapses but have reduced dopamine levels on the striatum (Abeliovich et al., 2000). A loss of function of α-Syn is unlikely to cause PD, and mutations in α-Syn that cause PD are likely to be gain-of-function mutations. However, these knockout mice have increased dopamine release following paired stimuli and an attenuation of dopamine-dependent locomotor responses to amphetamine, which suggest that α-Syn may be an essential presynaptic, activity-dependent negative regulator of dopaminergic

neurotransmission (Abeliovich et al., 2000). Cabin et al. (2002) demonstrated that α-Syn knockout mice exhibited significant impairments in synaptic response to repetitive stimulation that depletes docked as well as reserve pool vesicles and they suggested that the normal function of endogenous α-Syn is in regulating synaptic vesicle mobilization at nerve terminals.

Subsequently, Dauer et al. (2002) showed that α-Syn null mice display striking resistance to MPTP-induced degeneration of dopaminergic neurons and dopamine release, and this resistance appears to result from an inability of the toxin to inhibit complex I. A third line generated by Schluter et al. (2003) showed that deletion of α-Syn had no significant effects on brain structure or composition. In particular, the levels of synaptic proteins were not altered, and the concentrations of dopamine, dopamine metabolites, and dopaminergic proteins were unchanged. However, although upon acute MPTP challenge α-Syn knockout mice were partly protected from chronic depletion of nigrostriatal dopamine, mice carrying the spontaneous deletion of the α-Syn gene exhibited no protection.

In addition to models where α-Syn expression is eliminated, there are also transgenics over-expressing human wt or mutant α-Syn using various promoters (reviewed by Fernagut and Chesselet, 2004). For instance, human wt α-Syn expression in mice using the platelet-derived growth factor (PDGF) promoter leads to dopaminergic terminal loss, non-fibrillar α-Syn LB-like inclusion in the substantia nigra, hippocampus and cortex and motor deficits at one year of age (Masliah et al., 2000). In the later report the fact that there are numerous non-fibrillar α-Syn LB-like inclusion support the idea that the α-Syn protofibril (intermediate in the fibrillization process) may be pathogenic.

Interestingly, Manning-Bog et al. (2003) found that in mice over-expressing α-Syn, either the human wt or the A53T mutant, displayed paraquat-induced protein aggregates but were completely protected against neurodegeneration (paraquat is a known toxin to cause PD in various models as seen later). These resistant animals also exhibited increased levels of hsp70. Such observations supporting a role of α-Syn against toxic insults and suggest

that its involvement in human neurodegenerative processes may arise not only from a gain of toxic function, as previously proposed, but also from a loss of defensive properties. Wild type α-Syn expression in other mouse models caused inclusion formation but no dopaminergic neuron loss nor motor deficits, whereas A53T mutant expression lead to motor deficits (Giasson et al., 2002). In another model, the A53T α-Syn mutant causes significantly greater *in vivo* toxicity as compared with other α-Syn variants (Lee et al., 2002).

When comparing the transgenic mouse models (reviewed by Fernagut and Chesselet, 2004) it becomes apparent that expression levels range from 0.5 to 30-fold the endogenous levels and the lines expressing the highest have more severe behavioural and neuropathological abnormalities, independently of the promoter driving transgene expression. High expression of α-Syn in neurons under Thy-1 promoter (van der Putten et al., 2000; Richfield et al., 2002; Rockenstein et al., 2002) or milder expression in glia and neurons under the platelet-derived growth factor beta (PDGF-β) promoter (Masliah et al., 2000) might also explain some differences in behaviour and dopaminergic deficits, which are more severe in the later study and imply a crucial role for glial cells in synucleinopathies. Strain differences in the various studies are also notable but it is more likely that threshold and duration of the transgene expression are more important. Finally, we have to consider that the human α-Syn is expressed in mice co-expressing their own α-Syn and this might account for a less pronounced disease phenotype, based on *in vitro* studies (Rochet et al., 2000).

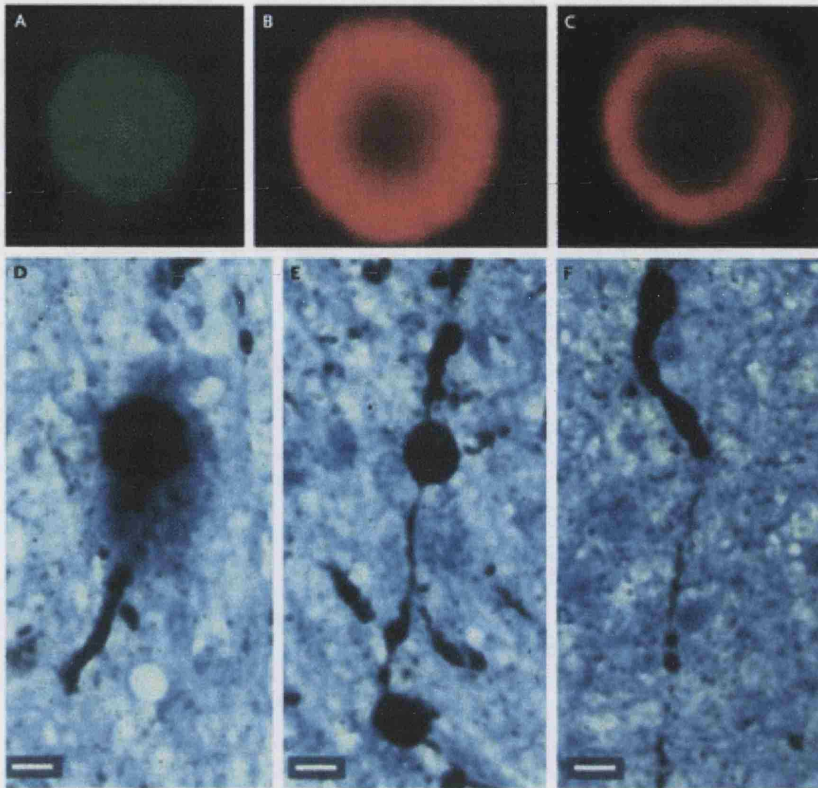
The primate model developed by Kirik et al. (2003) utilised a high-titre recombinant adeno-associated virus vector to express wt or mutant human α-Syn in the substantia nigra of adult marmosets. Over-expression of either wt or mutant α-Syn induces a PD-like neuropathology in the nigrostriatal system, dopamine neuron loss and motor impairment. This model is of particular interest because it develops the disease slowly over time, like human PD, and expresses neuropathological features similar to human PD (α-Syn-positive inclusions and dystrophic neurites).

As a conclusion, the transgenic *Drosophila* and mouse models, along with primate models, have been persuasive in implicating α-Syn in the pathogenesis of PD (reviewed by Dawson et al., 2002 and Beal, 2001).

1.3.4.2 α-Syn in Human Brain, Lewy Bodies and Aggregates

Solano et al. (2000) reported a study of α-Syn, parkin and UCH-L1 mRNA in human brain, where α-Syn mRNA was found in various levels in many parts of the brain showing strong mRNA levels in the substantia nigra. The expression patterns of alpha-synuclein and parkin mRNAs were similar, suggesting that, taken with the rest of the evidence (Shimura et al., 2001), these two proteins are involved in common pathways contributing to the pathophysiology of Parkinson's disease.

α-Syn appears to be the primary component of the LB (Figure 1.2) (Spillantini et al., 1997; Mezey et al., 1998). It has the ability to polymerize into approximately 10-nm fibrils in vitro, and bundles of these fibrils are the major component of LBs and LNs (Figure 1.2). LBs contain numerous other normal and abnormal proteins, many of which are ubiquitylated (Pollanen et al., 1993) and they are thought to be a specialized 'aggresome-related' inclusion (see below for aggresome definition) specific to dopaminergic neurones of the substantia nigra (McNaught et al., 2002a). LBs were discovered about 90 years ago, but the mechanism underlying their formation and their role in the disease are still unknown. Some studies relate them to aggresomes, which are defined as cytoprotective proteinaceous inclusions formed at the centrosome that segregate and facilitate the degradation of excess amounts of unwanted and possibly cytotoxic proteins (Olanow et al., 2004). Cytochrome c was found in LBs (Hashimoto et al., 1998) as well as multiple ubiquitin-proteasome system (UPS) components were detected (Ii et al., 1997; Shimura et al., 1999), with parkin and UCH-L1 among them (Schlossmacher et al., 2002; Lowe et al., 1990). Lewy bodies were also immunopositive for SIAH (Liani et al., 2004), together with various hsps like hsp70, hsp40, hsp27, αB-crystallin, torsinA, hsp90, hsp110 (Sherman and Goldberg, 2001; McNaught et al., 2002b; Schmidt et al., 1996; McLean et al., 2002) and many other proteins.



<u>Figure 1.2</u> Immunohistochemical Analysis of Sections from the Substantia Nigra of a Patient with Sporadic PD.

Panel **A**: Lewy body stained with antibody against ubiquitin (green) (x3000). Panel **B**: The same Lewy body stained with antibody against α-Syn (red) (x3000). Panel **C**: merge of Panel **A** and **B** image, shows that Lewy bodies contain a central core of ubiquinated proteins and α-Syn surrounded by a rim of α-Syn-positive fibrillar material. Panels **D**, **E**, and **F**: Neuronal processes from the substantia nigra of a patient with sporadic PD. Neurites are ballooned, dilated and stain for α-Syn (black stain). Scale bars in Panels **D**, **E**, and **F** indicate 10 μm. (Reprinted with permission from Mezey et al., 1998, copyright 1998, Macmillan Publishers Ltd).

A property that may underlie α-Syn involvement in LB formation and its contribution to the pathogenesis of PD may be because of its unfolded structure. α-Syn is prone to self-aggregation and causes the aggregation of other proteins (Giasson et al., 1999; Conway et al., 1998). More specifically, α-Syn is self-aggregating *in vitro* into fibrils in a time-, temperature-, pH-, and concentration-dependent manner (Giasson et al., 1999). Parameters such as mutations and oxidative stress have also been shown to increase α-Syn aggregation *in vitro* (Hashimoto et al., 1999; El Agnaf et al., 1998). Some environmental triggers to α-Syn aggregation were summarized in Figure 1.1.B previously.

Furthermore, in another study, Giasson et al. (2003) elegantly demonstrated that α-Syn induces fibrillization of tau and that co-incubation of tau and α-Syn synergistically promotes fibrillization of both proteins. They also showed that, *in vivo,* α-Syn and tau filamentous amyloid inclusions co-occur in humans, in single transgenic mice that express A53T human α-Syn in neurons, and in oligodendrocytes of bigenic mice that express wt human α-Syn plus P301L mutant tau. They finally concluded that α-Syn induces the formation of tau fibrils and that both tau and α-Syn synergistically effect the polymerization of each other into fibrillar amyloid lesions.

In addition, Sharon et al. (2003) recently reported that the formation of highly soluble oligomers of α-Syn is regulated by fatty acids and it is enhanced in PD. All the cytosols of mesencephalic neuronal cells, in normal and α-Syn transgenic mouse brains and in normal and PD human brains, contained highly soluble oligomers of α-Syn: polyunsaturated fatty acids increased α-Syn oligomer levels in living mesencephalic neuronal cells, whereas saturated faty acids decreased them, transgenic mice accumulated soluble oligomers with age and PD brains had elevated amounts of the soluble, lipid-dependent oligomers. They concluded that α-Syn interacts with polyunsaturated fatty acids *in vivo* promoting the formation of highly soluble oligomers that precede the insoluble α-Syn aggregates associated with neurodegeneration.

Finally, additional support for the role of protofibrils in PD is provided by the observation that protofibril formation is promoted by catecholamines like dopamine (Conway et al., 2001). In contrast, the failure of transgenic mice that over-express the protofibrillogenic A30P α-Syn to exhibit neurodegeneration provides strong *in vivo* evidence that protofibrillar α-Syn is not the primary toxic form of the protein (Lee et al., 2002). In agreement to this, only when the A30P mutant forms inclusions and fibrils do transgenic flies and mice exhibit neurodegeneration (Feany and Bender, 2000; Kahle et al., 2001; Neumann et al., 2002).

In conclusion, due to the presence of conflicting reports, no definitive answer can be given to the question whether α-Syn inclusion formation *in vitro* and LB formation in the human brain is protective or deleterious and hence it is difficult to conclude on the precise role of α-Syn aggregation in the pathology of PD.

1.3.4.3 α-Syn and Dopamine

So far evidence from animal models implicates α-Syn in PD pathogenesis. It is important to also consider here in this section α-Syn in the context of the dopaminergic environment. A difficult question to answer is how mutations in α-Syn cause selective degeneration of dopaminergic neurons, since α-Syn is a ubiquitously expressed protein in the brain. However, oxidative ligation of dopamine to α-Syn leads to the accumulation of the α-Syn protofibril, which may be the toxic α-Syn moiety (Conway et al., 2001). α-Syn can form adducts with other catecholamines related to dopamine, and it has therefore been suggested that adduct formation provides an explanation for the dopaminergic selectivity of α-Syn-associated neurotoxicity in PD (Conway et al., 2001). Interestingly, α-Syn toxicity in human dopaminergic neurons was reported to require endogenous dopamine production, it is mediated by reactive oxygen species and it does not seem to involve dopamine adduct formation but, instead, is mediated by 54- to 83-kDa soluble protein complexes containing α-Syn and a 14-3-3 protein (14-3-3 family members are dimeric, phosphoserine

binding proteins that regulate signal transduction, apoptotic, and checkpoint control pathways and are elevated selectively in the substantia nigra in PD) (Xu et al., 2002).

The pathogenesis of PD in relation to dopamine, vesicles and α-Syn was extensively reviewed by Lotharius and Brundin (2002), who proposed that defective sequestration of dopamine into vesicles is a key event in the loss of dopaminergic neurons in PD, as it leads to the generation of reactive oxygen species in the cytoplasm. Therefore, a common pathway that underlies both genetic and sporadic forms of PD can be considered: Cytoplasmic dopamine is increased in nigral neurons in the PD brain and this may cause dopamine oxidation and reactive oxygen species generation that can be very damaging or even trigger death in neurons. The above scenario is favoured in the event of impaired vesicular storage of dopamine and indeed evidence (reviewed by Lotharius and Brundin, 2002) shows that mutant α-Syn expression in a human dopaminergic cell line increases cytoplasmic dopamine levels and raises the levels of superoxide radicals in the cytoplasm. In summary, dopamine-induced oxidative stress, impaired synaptic vesicle function and mutated or oxidatively damaged α-Syn aggregation, might be components in a vicious cycle that eventually kills dopaminergic neurons and leads to PD.

1.3.4.4 Key *in Vitro* Studies of α-Syn

In addition to studies with animal models and human material, there is an extensive list of published studies utilising *in vitro* model systems, a lot of which provide useful insights to the role of α-Syn in the context of PD. There are data published from different laboratories, as well as the published findings that have emerged from this thesis (see appendix for published reports) that argue strongly that the presence of PD associated-mutant α-Syn may cause enhanced sensitivity to any type of insult, leading to apoptosis associated with increased oxidative damage. Wt α-Syn expression, however, can be protective or deleterious in different systems or under different stresses.

Lee et al. (2001) found that over-expression of wt α-Syn in neuronal cells delayed cell death induced by serum withdrawal or H_2O_2, but not by 1-methyl-4-phenylpyridinium ion (MPP^+, a product of MPTP that inhibit mitochondrial complex I). By contrast, wt α-Syn transfectants were sensitive to staurosporine, lactacystin or 4-hydroxy-2-trans-nonenal (HNE). Decreases in glutathione levels were attenuated by wt α-Syn after serum removal, but were aggravated following lactacystin or staurosporine treatment. Mutant α-Syn increased the levels of 8-hydroxyguanine, protein carbonyls, lipid peroxidation and 3-nitrotyrosine, and increased the cell death in response to all the insults examined. The decrease in glutathione levels was enhanced in mutant α-Syn expressing cells.

In agreement with the above results on mutant α-Syn effects, immortalized dopaminergic neurons expressing mutant α-Syn are hypersensitive to damage by 6-hydroxydopamine (Kholodilov et al., 1999) and other insults (Saha et al., 2000). This mutant sensitivity has been observed in studies with primary cultures and transgenic mice (da Costa et al., 2000; Ostrerova-Golts et al., 2000; van der Putten et al., 2000; Zhou et al., 2000). On the other hand, wt α-Syn can be either protective (Manning-Bog et al., 2003; Hashimoto et al., 2002) or deleterious (Masliah et al., 2000; Ostrerova-Golts et al. 2000). Interestingly, Seo et al. (2002) showed that the neuroprotective effect of wt α-Syn is dose dependent: the addition of nanomolar doses to PC12 cells protects against various insults, such as oxidative stress, whereas exposure to micromolar concentrations or over-expression is cytotoxic.

Zhou et al. (2002) was the first to over-express wt and A53T α-Syn in cultured, human embryonic mesencephalic neurons using an adenoviral transfer technique. Human dopaminergic neurons expressing A53T α-Syn showed a two-fold increase in apoptotic cell death compared to the effect observed in cells that over-expressed the wt protein. Xu et al. (2002) transfected human embryonic mesencephalic cultures with wt, A53T and A30P and found an increase in apoptosis. α-Syn-induced apoptosis was blocked by either depleting cells of intracellular dopamine using a tyrosine hydroxylase inhibitor or by

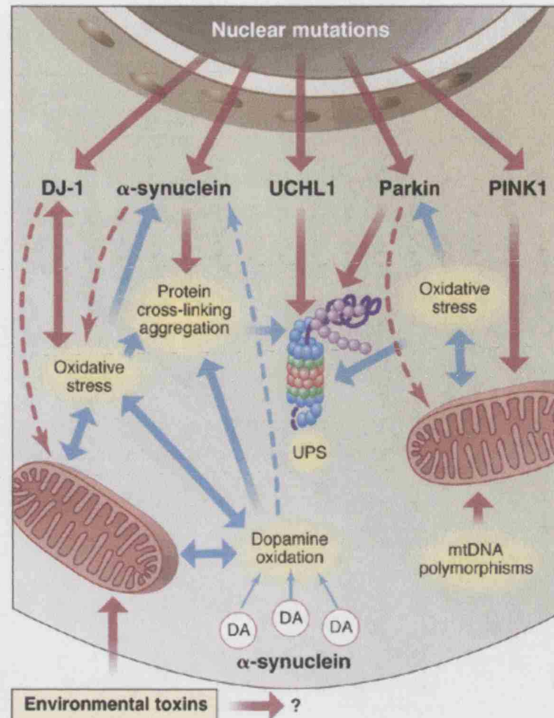
antioxidant treatment, showing that mutant-α-Syn-induced cell death is dependent on dopamine.

In addition, lentiviral-mediated transfer of A53T α-Syn into differentiated MESC2.10 cells (of human origin) decreased the exocytotic dopamine release and increased the amphetamine-induced dopamine overflow, demonstrating a depletion of vesicular dopamine and an accumulation of cytoplasmic neurotransmitter (Lotharius and Brundin, 2002). Cells over-expressing the A53T mutant protein had higher basal levels of cytoplasmic dopamine immunofluorescence and superoxide radicals and reduced levels of VMAT2 (vesicular monoamine transporter 2, it is responsible for dopamine uptake into vesicles). So, there is either a reduced number of vesicles or an abnormal capacity of vesicles to accumulate dopamine.

In conclusion, in terms of protection and toxicity due to α-Syn over-expression, the literature has been conflicting. Wt α-Syn over-expression can be either protective against various insults or deleterious upon stress or by over-expression alone. In the case of either mutant α-Syn expression in vitro or in vivo most studies have reported a toxic effect with or without the presence of enhanced α-Syn aggregation.

Further reported roles of α-Syn and the disease-associated mutants in cell signalling and death/survival pathways will be discussed in the relevant sections of Chapters 3, 4 and 5, presenting the results of the present work and are therefore not mentioned here to avoid repetition. The following figure was adapted from Greenamyre and Hastings (2004) and summarises a lot of the issues raised in the above sections that described the genes implicated in familial PD (Figure 1.3).

The sections that follow include some aspects of cell death in PD, the role of apoptosis and the mitochondria in PD and finally will provide an introduction to the current knowledge of the heat shock proteins and their relevance in PD and neurodegeneration.



<u>Figure 1.3</u> Genes linked to familial PD: Clues to mechanisms leading to PD

The above diagram summarises current mechanisms of PD pathogenesis. They involve mitochondrial and ubiquitin proteasome system (UPS) impairment, oxidative stress, and protein misfolding. α-Syn is oxidatively and nitratively modified and can also form adducts with dopamine quinones (formed in dopaminergic neurones), that are events causing its aggregation. UCHL1 and parkin are components of the UPS that degrades defective proteins. Parkin inactivation, among other roles, also causes mitochondrial impairment and may be inactivated in PD *via S*-nitrosylation. DJ-1 protects against oxidative stress and may become localized in mitochondria upon oxidative stress. PINK1 is a nuclear-encoded, mitochondrial protein kinase. Some toxins or pesticides impair mitochondrial function and cause parkinsonian phenotype. **<u>Key:</u>** Red arrows: putative primary causes of PD, Dashed arrows: secondary effects, Blue arrows: secondary mechanisms of PD that rise after genetic or environmental causes (primary causes), mtDNA, mitochondrial DNA (Illustration by K. Sutliff from Greenamyre and Hastings, 2004, reprinted with permission from AAAS).

1.4 The Ubiquitin-Proteasome System: Relevance to PD

This pathway is overviewed here because it is relevant to PD pathology and because some of the genes that are linked with familial PD are implicated with the Ubiquitin-Proteasome System (UPS): parkin and UCH-L1 are components of the system, α-Syn and DJ-1 are its targets.

The UPS is important for (a) protein-quality control and (b) the timely regulation of the level of short-lived proteins, such as proteins involved in cell-cycle progression, signal transduction, and metabolism. Therefore, proteasomal degradation by the 26S proteasome regulates the expression of many proteins ranging for example from cell cycle regulators, proto-oncogene products, MHC molecules and NF-kB inhibitors (inhibitors of a transcription factor that controls cell growth, inflammatory and stress responses) (Pickart, 2001). Overall, more than 30% of newly synthesized proteins are rapidly degraded (Schubert et al., 2000). Under non pathological conditions, the disposal of damaged or misfolded proteins in a cell can be facilitated by the UPS or by hsps *via* the lysosomal route or the UPS. The UPS is an extralysosomal, energy dependent system and it is present in the nucleus and in the cytoplasm and contributes to cellular defence against various stresses through the elimination of unfolded protein.

The degradation of unwanted proteins begins with their conjugation to a chain of ubiquitin molecules, which is a signal for their recognition and degradation by the proteasome. Ubiquitin monomers are activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent manner. Then, the activated ubiquitin is transferred to a ubiquitin conjugating (carrier) enzyme (E2) and then covalently linked to unwanted proteins *via* an ATP-dependent reaction catalyzed by a ubiquitin protein ligase (E3). These reactions are repeated for the sequential addition of ubiquitin molecules to the selected protein resulting in the formation of a polyubiquitin chain, in which ubiquitin molecules are linked onto the previously ligated ubiquitin (most commonly to the lysine residue at position 48). This chain is the signal recognized by the 26S proteasome multisubunit protein complex localized on the inside of the barrel-

shaped core complex (also called the 20S proteasome). These are large multicatalytic proteases found in the cytoplasm, endoplasmic reticulum, perinuclear region, and nucleus of eukaryotic cells (Voges et al., 1999). The 19S cap, residing in both ends of the 20S complex, recognizes the polyubiquitin chain, which is removed and cleaved into monomers by deubiquitinating enzymes, such as UCH-L1. Then the targeted protein is unfolded and cleaved inside the inner chamber of the 20S core complex. The small peptides resulting from the proteolysis are then released into the cytosol.

In mammalian cells, a single E1 enzyme, more than 20 E2 enzymes and hundreds of E3s, with each mediating the ubiquitination of a single or a very limited number of specific proteins, have been identified to date (Pickart, 2001). Polyubiquitin-protein conjugates and non-ubiquitinated proteins/peptides (e.g. short-lived regulatory proteins) are degraded by 26S and 20S proteasomes respectively. There are also numerous de-ubiquitylating enzymes, but their functional role in the regulation of the system is unclear. They may serve as a proof-reading mechanism by limiting the length of the ubiquitin chain to less than four ubiquitin molecules and therefore preventing selection for proteasomal degradation. Alternatively, they may also hinder protein aggregation that could occur if the polyubiquitin chains became too long. Examples of de-ubiquitylating enzymes include the PD linked UCH-L1 (ubiquitin C-terminal hydrolase-L1, also known as PGP9.5) and ataxin-3 which are both abundant in brain tissue and are essential for normal neuronal function (Wilkinson et al., 1989; Burnett et al., 2003).

It has been found that the UPS is impaired by protein aggregation (Bence et al., 2001) and this is a finding with important implications in PD. Numerous other studies, summarised below, point to the fact that UPS has a special importance in PD. Figure 1.4 pictures some findings that link familial PD with the UPS.

As a summary, mutations in UPS components such as parkin (an E3 ligase) and UCH-L1 (a de-ubiquitinating enzyme) lead to PD and also aggregation or excessive levels of α-Syn (mutated in some PD forms) can

inhibit the UPS. In PD α-Syn is found in LBs and this might reflect the outcome of spontaneous α-Syn self-aggregation and the inability of the UPS to clear aggregated synuclein. Notably, LBs correlate strongly with disease severity (Giasson and Lee, 2003). Conway et al. (2000) found that α-Syn *in vitro* undergoes a concentration depended self-aggregation whereas α-Syn gene triplication leads to increase protein expression and PD in humans (Singleton et al., 2003). In addition, mutant α-Syn can inhibit the proteasome in catecholaminergic neurons in primary midbrain cultures in a manner similar to exposure to proteasome inhibitors, while parkin is capable of rescuing the toxic effects of mutant alpha-synuclein or proteasome inhibition in these cells (Petrucelli et al., 2002). Whether enhanced levels of α-Syn are the prime cause of impairment of the proteasome has to be further studied. Previously Bennett et al. (1999) showed that mutant α-Syn is degraded slower than wt by the proteasome, whereas McNaught et al. (2003) reported reduced proteasome activity in the substantia nigra of PD brain.

Indeed the role of proteasome in PD is important, as indicated by recent findings reviewed by McNaught et al. (2003), demonstrating that defects in the UPS may be a common feature in familial and sporadic PD. Further support to this is argument also comes from a recent study by McNaught et al. (2004), who demonstrated the generation of a rat PD model induced by naturally occurring (epoxomycin) and synthetic (PSI) proteasome inhibitors. Rats developed progressive parkinsonism with bradykinesia, rigidity, tremor, and an abnormal posture with indications of degeneration of the nigrostriatal pathway, striatal dopamine depletion and dopaminergic cell death, and inflammation in the substantia nigra pars compacta. At neurodegenerative sites (substantia nigra pars compacta, the locus coeruleus, dorsal motor nucleus of the vagus, and the nucleus basalis of Meynert, also affected in humans), inclusions resembling LBs were seen in neurons. This study adds evidence to support the hypothesis that proteasome dysfunction causes PD.

In conclusion, despite the current evidence that links PD with the UPS and α-Syn aggregation, it is difficult to say what the precise roles of protein aggregation and the UPS are in PD (deleterious or protective?).The hypothesis

that protein aggregation itself is truly harmful to the neurons could be tested by designing and using pharmacological compounds that could improve the UPS activity in PD. If those compounds could improve protein turnover and reduce protein aggregation this would be strong evidence supporting the above hypothesis i.e. aggregation compromises the UPS and is a prime cause of neurodegeneration *in vivo*.

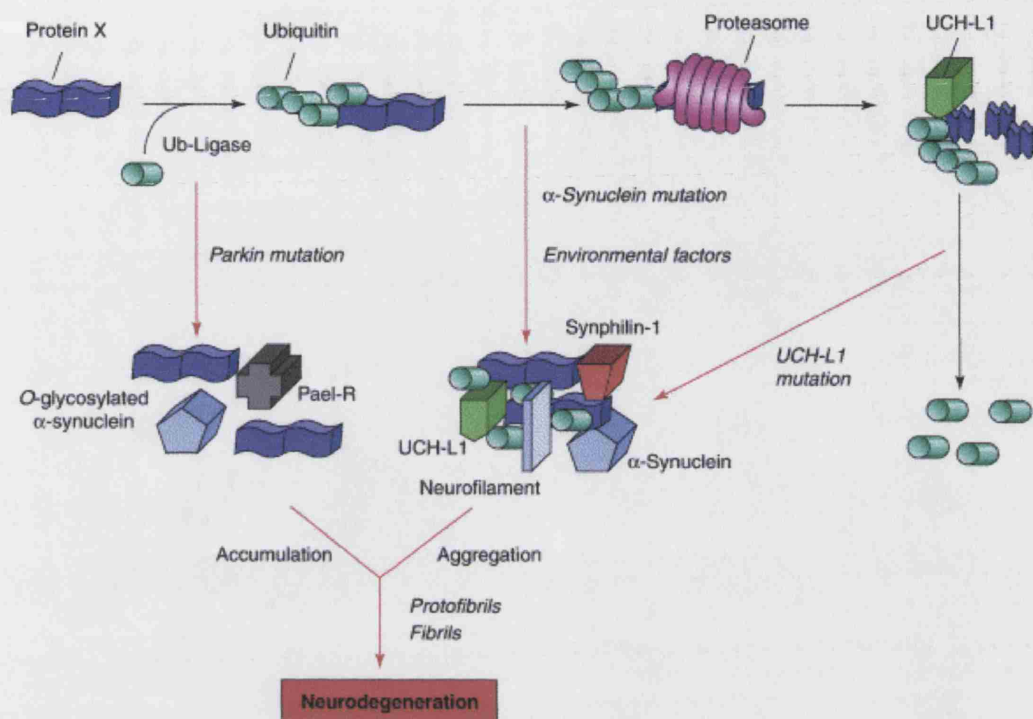


Figure 1.4 The Ubiquitin Proteasome System of Protein Degradation in PD.

Misfolded proteins like α-Syn undergo proteasomal degradation. Missense or triplication mutations in the α-Syn gene lead to an increase spontaneous aggregation. Parkin (E3 ubiquitin ligase) loss-of-function mutations might result in impaired ubiquitination and accumulation of (non-ubiquitinated) protein substrates (e.g. *O*-glycosylated α-Syn, Pael-R) that cannot be degraded. Synphilin-1 is both an α-Syn-interacting protein and a substrate of parkin. Co-expression of synphilin-1 and α-Syn results in the formation of intracytoplasmic aggregates. UCH-L1 allows ubiquitin re-utilization, and mutations in *UCH-L1* might reduce its enzymatic activity (Figure reprinted from Kruger et al. 2002, copyright 2002, with permission from Elsevier).

1.5 Cell Death and Apoptosis in Parkinson's Disease

The work described in this thesis utilises a model cellular system where apoptosis is a key feature and hsp27 has a protective anti-apoptotic effect. Since hsps inhibit apoptosis, it is therefore important that this section briefly overview the molecular pathways of programmed cell death (intrinsic and extrinsic pathways), before proceeding to overview hsps. It will be also highlighted how this physiological process, which acts in all multicellular organisms, can contribute to neurodegeneration and PD in particular. It should be noted here that some researchers, for example Vila and Przedborski, use the term programmed cell death to include all types of cell death that are characterised by active intracellular events, whereas apoptosis is only one morphological form of programmed cell death, characterised by cell shrinkage, nuclear condensation, DNA fragmentation, membrane blebbing (Vila and Przedborski, 2003). In the sections below apoptotic pathways are first described in general before they are discussed in the context of PD.

The proteins involved in programmed cell death include caspases, which are proteases that mediate cleavage of certain substrates at specific aspartic acid residues. They exist in cells as inactive pro-caspases and after activation upon various cellular signals can initiate (pro-caspases 2, 8, 9 and 10) or execute (pro-caspases 3, 6 and 7) programmed cell death. For instance, initiator pro-caspases 8 and 9 are activated through recruitment to complexes including FADD (Fas-associated protein with death domain) and Apaf-1 respectively (apoptotic protease-activating factor 1). The activated initiator caspases subsequently activate by cleavage the effector caspases that start proceeding with events leading to nuclear membrane breakdown, DNA fragmentation and so on. The initiator caspases have long N-terminal pro-domains that contain specific interaction motifs. Activation of pro-caspase 8 and 9 is triggered by dimerisation of the pro-caspase on a dedicated adaptor protein or a scaffold (FADD and Apaf-1, respectively).

The extrinsic pathway (death receptor pathway) activates caspase-8 (and also caspase-10 in humans) *via* FADD. Ligand-induced aggregation of tumor necrosis factor (TNF) receptor family members (Ashkenazi, 2002) attracts FADD, which recruits pro-caspase-8 through death effector domains. Proximity of the pro-caspase leads to dimerisation and subsequent autocatalysis and activation (Boatright and Salvesen, 2003).

On the other hand, the intrinsic, stress pathway (mitochondrial pathway), activates caspase-9. The scaffold protein here is Apaf-1. Apaf-1 interaction with cytochrome c released from the mitochondria, leads to a conformational change that allows it to recruit pro-caspase-9, *via* specialised caspase recruitment domains, and oligomerises into a megadalton complex, the apoptosome. In the apoptosome, pro-caspase-9 is activated by allosteric change and dimerisation (Rodriguez and Lazebnik, 1999; Boatright and Salvesen, 2003). Following these events, caspase-3 and caspase-7 are activated by apoptosome processing and a cascade of proteolysis takes place in the cell that is destined to die.

Mitochondria have a major role in apoptotic cell death following many triggers and their membrane permeability is crucial for the fate of the cell; release of caspase-activating molecules (see above, cytochrome c release and caspase-9 activation) and caspase-independent death effectors, for example, are events closely linked to mitochondrial outer membrane permeabilization. Such mechanisms are not fully understood but seem to be regulated by proteins from the Bcl-2 family, mitochondrial lipids and putative components of the permeability transition pore and have been recently reviewed by Green and Kroemer (2004). For example, members of the Bcl-2 protein family are critical regulators of mitochondrial integrity. An apoptotic stimulus may activate one or more of the "BH3-only" pro-apoptotic members of the Bcl-2 family, which proceed to directly or indirectly activate one or both of the terminal Bcl-2 family death effectors BAX and BAK at the mitochondria (reviewed by Scorrano et al., 2003). The antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-xL, directly antagonize the activity of the BH3-only proteins.

There are four different signalling pathways that lead to apoptotic cell death with features involving DNA fragmentation. Two are caspase-dependent: The cytochrome c, Apaf-1 and pro-caspase-9 system depends on caspases, as well as the second mitochondrion derived activator of caspases (SMAC)/direct IAP binding protein (DIABLO) system. The two caspase-independent systems are the apoptosis initiating factor (AIF) system and the endonucleases G system (see review by Tatton et al, 2003).

Dopaminergic cell death is a prominent feature of PD and this is the reason why intense research has been carried out on pathways of programmed cell death in PD brain and also various PD models. For example, assessment of apoptosis by TUNEL in PD brain was accompanied by many problems and overall conflicting results have been obtained by studies of post mortem PD tissue (Tatton et al., 2003).The problems identified in those studies were mainly associated with the fact that detection of apoptotic cells was difficult since the daily rate of appearance of apoptotic neurons is thought to be low and more importantly, post mortem specimens are usually of patients in late disease stages at which most dopaminergic neurons have already been lost. In addition, the detection of apoptotic cell death in PD brains relied on TUNEL technique (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling). TUNEL helps visualising DNA fragmentation (the broken ends of double-stranded DNA) and its limitations are discussed later in Chapter 4 of this thesis. It should be noted here however that TUNEL is not absolutely specific to apoptosis as DNA damage can occur in other situations such as hypoxia in the brain of patients of which post mortem samples are studied.

Consequently, additional components of the apoptotic pathway have been studied in combination. These components, for instance caspases, have been studied successfully in various models of neurodegeneration and it has been confirmed by many studies that programmed cell death has a role in PD (see review by Vila and Przedborski, 2003), enabling at the same time the identification of drug targets (see also later section). In brief, two pre-mitochondrial signalling pathways of cell death have been shown to participate in neuronal cell death in human PD brain: (a) the p53-GAPDH-BAX pathway

and (b) FAS or TNF-a receptor-FADD-caspase 8-BAX pathway (see review by Tatton et al. 2003, for description of pathways).

In addition to the above, in a number of studies using post mortem tissue from PD brain many components of programmed cell death machinery were shown to have altered expression levels (Hartmann et al., 2000; Hartmann et al., 2001a; Hartmann et al., 2001b; Hartmann et al., 2002; Tatton et al., 2003). However, there are some reports where apoptosis does not seem to occur (Kosel et al., 1997; Banati et al., 1998; Wullner et al., 1999). Nevertheless, strong evidence implicates BAX (Hartmann et al., 2001a; Tatton, 2000), Bcl-x_L and caspase-3 (Hartmann et al., 2000; Hartman et al., 2002) and caspase-8 (Hartmann et al., 2001b; Viswanath et al., 2001) and caspase -9 (Viswanath et al., 2001) in human PD brain. Along with evidence from the human brain studies, there are also many more studies in PD models that reveal a direct role of PCD in this disease and they are mentioned later in the Introduction and Discussion sections of the results Chapters 3, 4 and 5.

Both the extrinsic and intrinsic apoptotic pathways were shown to be involved in PD, however the major mechanism of neuronal death seems to be the mitochondrial apoptotic pathway (intrinsic) in most studies. Nigrostriatal degeneration in the MPTP model of PD can be prevented by a dominant negative Apaf-1 inhibitor. In addition, caspase inhibitors attenuate MPTP toxicity in primary neuronal cells (Ferraro et al., 2003). It has been suggested that apoptosis may not be the only type for neuronal death in PD and that it is possible that multiple interrelated or independent pathways regulate dopaminergic neuron loss. This is supported by the fact that cell death can occur caspase-independently or dependent upon other proteases like cathepsin and calpains (Ferraro et al., 2003). Overall, while the existence of alternative pathways is clear, the evidence for involvement in PD is poor up to date, with the exception of AIF pathway.

Finally, another puzzle in neurodegeneration is the role of cell death at disease onset and progression. In other words, apoptotic cell death in PD brains can be a cause of the disease or it may be a secondary consequence of various

upstream events that cause PD. Intracellular protein aggregates of misfolded proteins accumulation in the cells can lead to UPS inhibition, multiple neuronal dysfunction and eventually death. So, apoptosis could finally eliminate these dysfunctional neurons. Maybe by the time neurons are eliminated by apoptosis, neurons are already so damaged that some researchers believe that blocking it might be an action that would lead to necrosis and inflammation in the tissue.

Therefore, the importance of programmed cell death in PD has become evident while the importance of the mitochondrion in the death pathways has also been clarified. The section that follows aims to overview the links between the mitochondrion and PD.

1.6 The Mitochondrion in Parkinson's Disease

This section provides an overview of the role of mitochondria in both apoptotic cell death and in PD because - as it has already been mentioned in sections above (also see Figure 1.3) - there are some important relations of this organelle with PD pathology. It is therefore worth highlighting the role of such an important organelle in PD because (a) it is a regulatory switch of apoptosis, which is a death mechanism in many neurodegenerative disorders and (b) it is directly involved in PD pathology in other ways i.e. *via* production of reactive oxygen species (ROS) or interactions with PD linked gene products such as parkin, α-Syn, DJ-1 and PINK1. However, some issues and important literature findings relevant to the present study are further addressed in Chapter 5 of this thesis and hence they are not going to be dealt with in this introductory section.

Firstly, the hypothesis that mitochondrial dysfunction may play a key role in PD pathogenesis is supported by postmortem studies that have shown mitochondrial impairment and oxidative damage in PD brains (Beal, 2003; Jenner and Olanow, 1998). Secondly, mitochondrial complex I inhibitors, such as MPTP, rotenone, and paraquat, produce a parkinsonian syndrome in experimental models and humans, as described below (Langston et al., 1983; Betarbet et al., 2000; Dawson and Dawson, 2003; Dauer and Przedborski, 2003;

Song et al., 2004). Complex I deficiency and free-radical damage are very strongly correlated: a complex I defect causes increased release of superoxide ions from the respiratory chain whereas free radicals impair the activity of respiratory chain enzymes. The damaging effect of rotenone in rodents seems to be mediated not by ATP depletion but by generation of free radicals. The rotenone rat model may be an example of the production of α-Syn inclusions owing to mitochondrial inhibition and free radical generation. However, it is difficult to conclude whether mitochondrial dysfunction and dopaminergic neuronal loss is causal or a mere consequence of degeneration caused by other upstream effectors.

The following examples of intoxication PD models provide additional evidence of the importance of mitochondria in the parkinsonian phenotype. Among these, the model produced by the neurotoxin MPTP has a further advantage over all the other toxic models because it causes a syndrome almost identical to PD in humans, nonhuman primates, and other mammalian species, including mice. The MPTP mouse model differs from human PD in some ways but offers a unique means to investigate *in vivo* molecular events underlying dopaminergic neuron death. Interestingly, Jenner and colleagues (2003) have developed a 'partial' lesioned MPTP primate model. By giving low doses of MPTP over three consecutive days they can reproducibly induce 50%–60% nigral cell loss. This model reliably represents the pathological situation seen in early PD, and as in the clinical situation, the animals exhibit only mild parkinsonian symptoms and are being used for the determination of the optimum therapeutic strategy for the long-term control of Parkinson's disease with a minimal risk of dyskinesia.

Finally, there are also other intoxication models. One uses the herbicide 1,1'-dimethyl-4,4'-5 bipyridinium (paraquat) administered with the fungicide manganese ethylenepistithiocarbamate (maneb) and this leads to selective degeneration of dopaminergic neurons in mice (Thiruchelvam et al., 2000). Paraquat is a complex I inhibitor with structural similarity to (methyl-4-phenylpyridinium ion (MPP+), and it leads to up-regulation and aggregation of

α-Syn in mice (Manning-Bog et al., 2002). Rotenone, another complex I inhibitor that is a widely used insecticide and fish poison which results in parkinsonian symptoms in rats after intravenous or subcutaneous administration. Unlike MPTP and paraquat, rotenone is not concentrated in dopaminergic neurons, yet it induces selective dopaminergic cell death, which suggests that those neurons are uniquely sensitive to complex I impairments (Sherer et al., 2003). Interestingly, the chronic nature of rotenone toxicity leads to LB-like inclusions containing α-Syn (Sherer et al., 2003; Betarbet et al., 2000).

Another issue to be discussed is how the genes mutated in familial PD relate/interact with pathways linked to the mitochondrion (Greenamyre and Hastings, 2004). Firstly, there are numerous *in vivo* and *in vitro* studies of α-Syn that implicate mitochondrial pathology or other interactions and those are presented in Chapter 4. As a summary, the following points highlight mitochondrial and α-Syn interactions in PD: (a) mitochondrial are leading generators of reactive oxygen species and their dysfunction through inhibition causes oxidative damage and lead to increased α-Syn expression and/or aggregation *in vitro* and *in vivo* (Kowall et al., 2000; Vila et al., 2000; Betarbet et al., 2000; Sherer et al., 2002; Lee and Lee, 2002) (b) on the contrary, α-Syn over-expression causes mitochondrial dysfunction *in vitro* and *in vivo* (Hsu et al., 2000; Tanaka et al., 2001; Song et al., 2004; Beal, 2004).

Apart from α-Syn, strong experimental evidence link the other genes associated with inherited PD and mitochondrial dysfunction, presented also later in Chapter 5, but some of those finding will be summarized here. Firstly, loss of parkin function in mice causes mitochondrial dysfunction and oxidative damage that supports a causal role of mitochondrial dysfunction in nigral degeneration (Palacino et al., 2004). Changes in the levels of mitochondrial proteins of the electron transport chain and various proteins involved in the protection from oxidative stress were observed in parkin knockout mice ventral midbrain (Palacino et al., 2004). Furthermore, decreases in mitochondrial respiratory capacity and age-dependent increases of oxidative damage were detected. In addition, the flight muscle of *Drosophila* parkin null mutants exhibits

mitochondrial pathology that may be responsible for the observed apoptosis in the flight muscle, which is a heavily energy-dependent tissue (Greene et al., 2003 and Pesah et al., 2004). In addition, over-expression of parkin *in vitro* decreases sensitivity to mitochondria-dependent apoptosis (Darios et al., 2003). It is very unclear, however, if parkin regulates mitochondrial function directly or only indirectly through its E3 ligase activity. Shen and Cookson (2004) suggested that it is possible that mitochondrial protein expression changes could be a downstream effect of parkin on the proteasome function.

Finally, the mitochondrion is further implicated in PD since there is preliminary evidence for relation of the mitochondria with the two new genes associated with familial PD: DJ-1 localizes to the outer mitochondrial membrane and its over-expression protects against mitochondrial damage (Canet-Aviles et al., 2004) and PINK1 is a nuclear-encoded, mitochondrial protein kinase, the substrates of which remain to be defined (Valente et al., 2004) but it is reasonable to hypothesise that they are mitochondrial proteins. New studies are awaited to elucidate the precise relation of those genes in PD pathology and their relation to the mitochondrion and apoptotic cell death pathways.

1.7 Towards the Development of PD Therapies

There is currently no cure for PD. This section briefly overviews what is currently available to patients and what is under development. Gene therapy using viral vectors as gene delivery tools is also discussed, followed by a description of the HSV-1-based vectors utilised here.

1.7.1 Current Treatments and New Targets.

Current therapies for PD provide effective control of symptoms but most patients develop motor complications after long-term treatment. No drug currently alters the rate of the disease progression. Levodopa remains the most effective treatment for PD but is associated with a wide range of adverse effects. Dopamine agonists are increasingly considered as effective early monotherapy in the treatment of PD (Jenner, 2002). They are antioxidants that reduce dopamine turnover and thus the generation of free radicals, and potentially

decrease the excitotoxic input into the substantia nigra. For instance, pramipexole prevents the fall in mitochondrial membrane potential, reduces cytochrome c release, and decreases caspase 3 activation. A study showed that pramipexole can significantly reduce nigral cell death in the MPTP primate model of PD (Jenner, 2002). Other drugs alleviate dyskinesias such as an NMDA receptor antagonist, amantadine (Table 1.2).

Surgery has been an effective approach to advanced PD tremor treatment (by thalamotomy) and reduction of rigidity and dyskinesias (by palidotomy). Deep brain stimulation also proved to be beneficial for advanced PD patients because it greatly reduces rigidity and bradykinesias. It is performed by stereotactic electrode implantation in subcortical structures to achieve their inhibition (reviewed by Olanow, 2004).

Table 1.2 Potential neuroprotective drugs in PD

Bioenergetics	Antioxidants	NMDA antagonists	Anti-inflammatories	Trophic factors	Antiapoptotic
Coenzyme Q10, Creatine	Vitamin E, Coenzyme Q10, Rasagiline, Selegiline, Dopamine agonists	Amantadine, Remacemide	COX I+II inhibitors, Minocycline	GPI 1485, GM-1, ganglioside, GDNF, BDNF	Minocycline, Rasagiline, Selegiline, Dopamine agonists, TCH 346 CEP 1347

However, new neuroprotective therapies that might slow, stop, or reverse disease progression are needed. Experimental findings (reviewed by Schapira, 2004) have suggested various agents (Table 1.2, adapted from the above reference). Many of those agents, however, were effective in animal models but not in human trials or provided inconclusive results. It is essential that some of them are re-examined in clinical trials with better experimental design (Olanow, 2004) and with the use of appropriate markers so we can be confident of either a true neuroprotective effect (Schapira, 2004).

A few other examples of current research aiming for therapeutic agents for PD are as follows. Iravani et al. (2003) reported that 3,4-methylenedioxy-methamphetamine (MDMA or ecstasy) administration suppressed motor activity and exploratory behaviour of common marmosets but in MPTP-treated, L-DOPA-primed common marmosets, MDMA transiently relieved motor disability, but over a period of an hour worsened motor symptoms. Administered together with L-DOPA, however, MDMA markedly decreased chorea and reduced locomotor activity to normal. Furthermore, minocycline, a caspase inhibitor that also inhibits the inducible nitric oxide synthase, both of which are important for apoptotic cell death, might have some neuroprotective benefit (Thomas and Le, 2004).

In addition to drug discovery efforts, neurotransplantation and stem cell research have been around for 15-20 years but are still in their infancy due to legal and bioethical blocks. Human fetal mesencephalic tissue transplanted in PD patients appeared promising but had finally little clinical benefit since many patients developed dyskinesias (reviewed by Olanow, 2004). Many hypotheses exist but the true reason why those efforts did not work is unknown. As for the use of embryonic stem cells there is some encouraging evidence that this approach might be beneficial to humans but has to be further explored. For example, a recent advance is the study by Bjorklund et al. (2002) that restored cerebral function and behaviour in an animal model of PD by transplanting mouse embryonic stem cells into the rat striatum, which resulted in a proliferation of these cells into fully differentiated dopaminergic neurons.

1.7.2 Gene Therapy and HSV-based Viral Vectors

HSV-based vectors were used in this study (Chapters 4 and 5) as a means of highly efficient hsp gene delivery method in cells *in vitro*, but they were originally developed as part of an effort to produce vectors for gene therapy.

1.7.2.1 Viral gene delivery for PD?

In addition to trials and intensive research towards the development of effective PD treatments, described above, gene therapy, utilising viral vectors or

other means, has also been developed in the recent years. There are two types of gene therapy; the first involves the delivery of a functional gene to an individual who has a defective copy of that gene and the second involves the delivery of genes, the protein products of which have a beneficial effect in treating the symptoms of a disease. In PD the second approach is promising.

The strategy for the development of gene therapy procedures for PD aims to deliver factors protective for the dopaminergic neuron or factors with neurorestorative properties in a safe and standardised manner. For example, delivery of the gene encoding tyrosine hydroxylase to boost dopamine production or the delivery of genes encoding neurotrophic factors such as GDNF to promote the survival of dopaminergic neurons (Latchman and Coffin, 2001). HSV-1 has a number of properties which suggest a good candidate in the development of vectors for the delivery of genes to the nervous system and hence development of PD therapies: (a) natural tropism for neurons, (b) large ~150 kb viral genome allowing the insertion of potentially multiple therapeutic genes <20 kb (c) ability to establish asymptomatic life-long latent infections. However, disadvantages include vector toxicity or an inability to maintain long term transgene expression (due to latency-associated problems) and studies to overcome those problems are ongoing.

Although many studies were reported to have delivered various genes in the substantia nigra or the striatum of different animal PD models by utilising different viral vectors, the optimal vector remains uncertain. Examples of successful trials utilising viral gene delivery include the study by During et al. (1994) in which a defective herpes simplex virus type 1 vector expressing human tyrosine hydroxylase was delivered into the partially denervated striatum of 6-hydroxydopamine-lesioned rats, used as a model of Parkinson's disease. More recently, Hurtado-Lorenzo et al. (2004) utilised replication-defective adenoviral vectors that were injected into the brains of rats in order to express the dopaminergic neuron differentiation factor sonic hedgehog, its downstream transcription factor target Gli-1, and the orphan nuclear receptor, Nurr-1, necessary for the induction of the dopaminergic phenotype of nigrostriatal neurons. The toxin 6-hydroxydopamine was used to lesion the nigrostriatal

dopaminergic innervation and significant protection of the dopaminergic nigrostriatal neuronal cell bodies was observed. But perhaps the most significant study to date is this by Luo et al. (2002) in which subthalamic nucleus neurons were transduced with glutamic acid decarboxylase (GAD), the rate limiting enzyme for the synthesis of the inhibitory neurotransmitter gamma-amino butyric acid, utilising an adeno-associated virus vector. A clinical trial has been approved and is aiming to reduce the over stimulation of the globus pallidus interna in patients with PD.

1.7.2.2 Basic HSV-1 biology and rationale in the development of vectors

The optimum HSV viral vector should deliver the transgene efficiently allowing long term expression, have no cytopathic effects, be incompetent of replication, be non-immunogenic and should not disrupt the host cell's normal functions. The virus naturally infects neurons, where it remains latent and episomal. It has evolved the ability of retrograde transport from the site of infection at the periphery to the site of latency in the spinal ganglia. Part of the long repeat region of the viral genome is transcribed during latency, generating RNA species called the latency-associated transcripts (LATs).

As reviewed by Latchman and Coffin (2001), infection of permissive cells by HSV-1 leads to expression of more than 80 viral genes in a well coordinated manner of immediate early (IE), early (E), and late (L) gene products (Lokensgard et al., 1994). Subsequently, the cells are lysed and progeny virions are released. The IE genes are transactivated by the virion protein VP16 that binds to TAATGARAT elements of the IE gene promoters. There are five virally encoded IE genes: ICP0, ICP4, ICP22, ICP27, and ICP47. Of these, ICP4 and ICP27 are absolutely required for viral replication and the deletion of ICP0 and ICP22 significantly impairs virus growth. The ICP0, ICP4, ICP22, and ICP27 proteins regulate the expression of the later replication and structural (E and L) genes. ICP47 is not a regulatory IE protein, but inhibits the transporters of antigen processing in the host cell.

ICP4 is the major viral transactivator protein, and its deletion dramatically reduces the expression of the ~80 HSV-1 genes. However, despite

the fact that the majority of the virus genome is not transcribed, ICP4 deletion mutants are still cytotoxic (Marshall et al., 2000). This toxicity is mainly due to the products of the other regulatory IE genes, ICP0, ICP22, and ICP27 (Marshall et al., 2000; McFarlane et al., 1992; Palella et al., 1988). But when they are inactivated together with ICP4, the toxicity is further eliminated. Therefore, an HSV vector should not express significant amounts of any of the regulatory IE genes.

1.7.2.3 Viral vectors used in this study

The viruses utilised in the present study were made by Dr. Marcus Wagstaff and were shown to be very useful in efficiently over-expressing hsps or HSF-1 in neuronal (Wagstaff et al., 1999; Wagstaff et al., 1998) or non neuronal cells (Brar et al., 1999; Jamshidi et al., 2004). Details of those vectors are provided in Chapters 2 and 4.

As a conclusion, the above introductory section provided an overview of the current therapies for PD and highlighted some aspects of new approaches toward the development of more effective neuroprotective or neurorestorative treatments. However, something that was purposely not discussed above is the idea that molecular chaperones could be potentially neuroprotective and aid neurons to cope with protein misfolding or protein mishandling, various cellular insults and stresses or even inhibit programmed cell death. Recent findings in the field of molecular chaperones and on the molecular pathology of some neurodegenerative diseases have lead researchers to think that chaperones are good candidates to be tested for their potential to confer neuroprotection in dopaminergic neurons. In PD brains there is prominent protein misfolding and aggregation, oxidative damage and other abnormalities that result in dopaminergic neuronal loss. The work presented in this thesis utilises a model of α-Syn toxicity in mammalian neuronal cells to test neuroprotective effects of heat shock proteins. Therefore the following sections provide an overview of the scientific background of heat shock proteins that led the present study to test the hypothesis of them having a neuroprotective effect in a model of α-Syn toxicity.

1.8 Heat Shock Proteins

1.8.1 Overview

The idea that heat shock proteins (hsps) are therapeutic targets for neurodegenerative diseases comes with significant amount of experimental evidence, as will be described later. Before reviewing the links of hsps to neurodegenerative disease and specifically to PD, it is essential to overview some of the hsp families and their roles in the cell.

Heat shock proteins form the molecular machinery of chaperones, which is an ancient defence system in all living organisms. Their induction in response to environmental, chemical or physical stress is able to protect cells from damage, facilitate recovery and modulate pathways of programmed cell death. Ritossa and Vonborstel (1964) reported that the exposure of the larval salivary gland cells of *Drosophila* to heat shock (37°C for 30 min followed by recovery at 25°C) led to the appearance of "puffing" in the chromosomes of the cells and later there was found to be an increase in the expression of 70 and 26 kDa heat shock proteins (Tissieres et al., 1974). The induction of hsps by elevated temperature is well documented but these proteins are also induced by a variety of stimuli that can potentially lead to cell damage and/or death. It has therefore been proposed by many researchers that heat shock proteins are more suitably called "stress proteins" (Latchman, 2001).

Chaperones are highly conserved and ubiquitous proteins. This remarkable conservation of their amino acid sequence throughout evolution highlights their importance in cells. Accumulated evidence suggests that they have indeed critical roles within the cell under both normal conditions and stress. What is definitive for chaperones is their tendency to bind to folding and misfolding proteins, ensuring that the exposed hydrophobic domains of a target protein are shielded from inappropriate nearby protein interaction that could potentially be devastating for the cell. Hence, molecular chaperones assist appropriate protein folding under normal conditions and maintenance of this native folding under stress, but they are not themselves components of the final

structure of the folding protein. They also have many other roles such as facilitating protein translocation across cellular membranes, and transfer of defective proteins to the proteasome for degradation, signal transduction mediating stress responses and anti-apoptotic roles (reviewed by Hartl and Hayer-Hartl, 2002; Kiang and Tsokos, 1998; Concannon et al., 2003; Parcellier et al., 2003a and Beere, 2004). As highlighted by (Uversky, 2003), a newly synthesised polypeptide chain in a cell has the following three potential fates: folding into a functional molecule with a unique three dimensional structure, misfolding or remaining unfolded (for the class of natively unfolded proteins). Many hsps play a role in the first two routes but, in addition to their chaperoning role, they also have other roles within a cell, through interactions with a number of cellular systems.

Hsps are grouped into families according to their molecular weight in kilo Daltons (kDa) but there is some confusion in their nomenclature. A table including the major hsp families is shown below; references are not included in the table but are cited in the text when an hsp is mentioned (Table 1.3). There are multiple co-chaperones acting together with hsps leading to the performance of different roles in the cell. These are discussed mainly in the section of Hsp70, but are not included in this table.

Table 1.3 Heat shock protein families and functions of some of their members

Heat Shock Protein Family	Main Members	Examples of Function
Hsp90	Hsp90α, Hsp90β, Grp94	Major roles in signal transduction, maintains inactive form of steroid receptor, >100 known "clients"
Hsp70	Hsp70(Hsp72), Hsc70(Hsp73), Grp78 (BiP), Grp75(mito-chondria), Hsx70 (primates only)	Molecular chaperone, translocation assembly of multiprotein complexes, protects from many stresses, anti-apoptotic actions, degradation of substrates *via* the proteasome
Hsp60	Hsp60 (mitochondria), GroEL (procaryotic)	Folds "molten globule" proteins or domains, mitochondrial protein folding
Hsp56	Hsp56 (FKBP56)	Protein folding, maintains inactive form of steroid receptor,hsp90 cohort
Hsp40	Hdj1 (Hsp40), Hdj2(Hsdj1), Hsc40, Csp, Tpr2, auxilin, etc	Protein folding, stimulates hsp70 ATP-ase activity, in PD and polyglutamine disease inclusions
Hsp32	Hsp32 (HO1)	Antioxidant
Hsp27	Hsp27, Hsp25, Hsp22,Hsp26, αA-crystallin, αB-crystallin	Actin binding, protein folding, anti-apoptotic functions, certain substrate degradation through the proteasome, protection against stresses
Others	Ubiquitin Hsp10	UPS-Protein degradation (ubiquitin) Hsp60 cohort (Hsp10)

Major hsps are discussed here in individual sections and in detail relevant to the present study. Therefore, in the following sections hsp90, hsp70, hsp56, hsp27 and other important hsps, which are not part of the present study, (large hsps, hsp60, hsp32 and ubiquitin) are discussed, followed by a description of HSF-1 and hsp gene regulation. Finally, their roles in stress, in cell death pathways, in proteolytic degradation by the proteasome and in neurodegeneration, in particular, will be reviewed.

1.8.2 Hsp90

The hsp90 chaperone family includes hsp90 (90 kDa heat shock protein) in the eukaryotic cytosol, named hsp90 α (major isoform) and β (minor isoform) in humans, hsp86 and hsp84 in mice, hsp83 in *Drosophila*, and hsc82 and hsp82 in yeast. Hsp90, is an ATP-dependent chaperone (Prodromou et al., 1997), a very abundant protein with very important roles, since most of its substrates are proteins involved in signal transduction, such as steroid hormone receptors and signaling kinases (Picard et al., 1990; Xu and Lindquist, 1993).

What is characteristic for hsp90 is that it does not generally act in nascent protein folding. So, hsp90 binds to substrate proteins in a near native state, at a late stage of folding (Nathan et al., 1997; Jakob et al., 1995). Hsp90 is part of machinery in the cytosol, including Hsp70 and also peptidyl-prolyl isomerases like hsp56 or other co-chaperones (Bose et al., 1996; Freeman and Morimoto, 1996).The protein-folding mechanism by hsp90 differs from that of either hsp70 or hsp60 in that the substrate (polypeptide) is held by the closed internally dimerized ATP-bound hsp90. The C-terminal 190 residues are responsible for keeping the homodimerised hsp90 together (Nemoto et al., 1995) and the highly conserved 25 kDa N-terminal domain binds ATP. This N-terminal domain also binds geldanamycin, an ansamycin class drug, which inhibits the hsp90 ATPase with nanomolar affinity (Whitesell et al., 1994).

Most studies on hsp90 have been on its role as a protein that binds to the steroid hormone receptor. Steroid receptors, such as the androgen receptor, the

estrogen receptor, the glucocorticoid receptor and so on, are present in the cytosol and contain many protein-binding domains (Pratt and Toft, 1997). The steroid receptor complex is transcriptionally inactive (since it stays in the cytosol) existing as a monomer bound to a dimmer of hsp90, which is in turn bound to its co-factor, hsp56 monomer (Edwards et al., 1992). When the hormone (ligand) binds to the receptor, the hormone bound receptor is activated by dissociation of the hsps from the complex and hence can enter the nucleus and initiate its nuclear function. In this respect the role of hsps is important. For instance, (Picard et al., 1990) showed that the glucocorticoid receptor is non-functional in the absence of hsp90. The assembly of the inactive receptor complex is ATP-dependent, as both hsp70 and hsp90 possess ATPase activity. It has also been suggested that hsp90 and hsp70 binding to the receptor may be important for maintaining its appropriate conformation in order to be able bind to its ligand. Hsp90 was mutated and tested for this, indicating that functional hsp90 was necessary for signal transduction at the hormone binding level and not at the transcriptional level (Bohen and Yamamoto, 1993). Furthermore, the requirements of the various receptors for hsp90 are variable (Pongratz et al., 1992; Nemoto et al., 1992) and the reasons for this are not known.

As reviewed by Young et al. (2001), hsp90 interacts with various co-chaperones in the cytosol, forming large complexes. Those co-factors regulate the function of hsp90 and hsp70 (see section of hsp70). The largest class of co-chaperones binds to hsp90 *via* a domain of hsp90 containing three 34 amino acid tetratricopeptide (TPR) motifs. One of them is the carboxyl terminus of the Hsc70-interacting protein (CHIP) which links hsp90 with the UPS and leads to proteasomal degradation of the substrate (Connell et al., 2001). Finally, hsp90 is associated with tyrosine and serine/threonine kinases, such as pp60/v-src, Wee-1, Cdk4 and Raf (Xu and Lindquist, 1993; Aligue et al., 1994; Stepanova et al., 1996; van der Straten et al., 1997), with nitric oxide synthase and calcineurin (Garcia-Cardena et al., 1998; Imai and Yahara, 2000) and actin, tubulin, hsp56, hsp70 and others.

The high presence of hsp90 in tumors account for the tumor selectivity of hsp90 inhibitors, such as geldanamycin. Hsp90 inhibition and hence HSF-1

activation and hsp expression has very important consequences not only for cancer (Kamal et al., 2004) but also other diseases including PD and HD. In those diseases, that are characterised by protein misfolding and aggregation, hsp90 inhibition and activation of the stress response would lead to increased hsp expression and a possibly neuroprotective effect (Auluck and Bonini, 2002).

1.8.3 Hsp70

1.8.3.1 Overview

The hsp70 family is an important group of proteins that includes chaperones with very important cellular functions. Slater et al. (1981) found that exposure of HeLa cells to a temperature of 45°C for 10 min lead to an increased synthesis of at least three sets of proteins with molecular masses of about 100 kDa 72-74 k Da and 27 kDa. It was subsequently shown that the major 72-74 kDa band consists of seven polypeptides, designated alpha, alpha-prime, beta, gamma, delta, epsilon, and zeta. Cato et al., (1981) cloned the cDNA sequences encoding the beta, gamma, delta, and epsilon heat-shock polypeptides and Hickey et al. (1986b) isolated cDNA clones representing at least five distinct heat-inducible mRNAs in human cells. Harrison et al. (1987) demonstrated that functional genes encoding *HSP70* map to human chromosomes 6, 14 (*HSPA2*), 21 (*HSPA3*), and at least one other chromosome.

The hsp70 family includes highly conserved proteins that have a 60%-78% amino acid identity among eukaryotic cells and 40%-60% identity between eukaryotic and *E.coli* (Lindquist, 1986; Caplan et al., 1993; Zimmermann et al., 1988). Structurally, those ATP-dependent proteins, are characterised by an N-terminus ATP-ase domain (44 kDa), an 18 kDa domain that binds unfolded and folded peptides (Mallouk et al., 1999) and contain two four-stranded anti-parallel β-sheets and one α-helix and a 10 kDa domain in the C-terminus that caries a terminal EEVD sequence. This sequence is found in all eukaryotic hsp70 and is involved in binding TPR containing co-factors. There are several hsp70 members in eukaryotes with various specific functions and localization in

the cell (in the cytoplasm, the mitochondrial lumen, and the endoplasmic reticulum) but they are all molecular chaperones.

The present study involves the inducible form of hsp70. Hsp70 induction is achieved under physiological conditions, such as during tissue development, pathological conditions, such as malignancy, autoimmunity, infection, ischemia or environmental conditions such as exposure to heavy metals, hyperthermia and antibiotics (see review by Kiang and Tsokos, 1998). The nature, strength and duration of the stimulus capable of inducing hsp70, determines the level of hsp70 protein expression, while the levels of these hsps after stress can remain relatively high for days to weeks in various cells.

1.8.3.2 Hsp70 as a molecular chaperone, the role of co-factors and involvement in signal transduction

The major function of hsp70 is that of a molecular chaperone. Hartl and Hayer-Hartl (2002), have reviewed the mechanisms of *de novo* protein folding in the cytosol. Protein translocation by hsp70 across mitochondrial membranes and across membranes of the endoplasmic reticulum (ER) involves the interaction of different binding motifs and the existence of certain translocation signals. An important consideration when looking into the function of hsp70 is its co-factors (or co-chaperones or cohort proteins) as they play an important role in hsp70 function. They can either regulate the ATP-ase activity of the chaperone (and therefore its affinity to substrates) or assist the recruitment of chaperones to protein complexes or cellular compartments.

For instance, the hsp70 ATP-ase activity is stimulated by its co-chaperone hsp40 (Freeman et al., 1995). In *E.coli* DnaJ (hsp40 is the eukaryotic homologue) stimulates the ATP-ase activity of DnaK (the hsp70 homologue) so that substrates bound to can be released from DnaK (Caplan et al., 1993; Silver and Way, 1993). Apparently, the exposed hydrophobic residues of unfolded or partially unfolded proteins are bound onto the C-terminal substrate binding domain and for the subsequent release of this substrate ATP binding to hsp70 is

essential and hsp40 stimulates the ATP-ase activity. Therefore, the co-chaperone can modulate the substrate binding.

In addition, other hsps such as hsp60, responsible for mitochondrial protein translocation, are also involved in the chaperone function of hsp70. Hip is another example of a co-chaperone that binds to the hsp70 ATP-ase domain, stabilizes the ADP state and hence increases its chaperone activity (Hohfeld et al., 1995), whereas Bag-1 inhibits this activity by uncoupling nucleotide hydrolysis and release of the unfolded substrate (Bimston et al., 1998). The opposing actions of the above two co-factors and their importance in hsp70 regulation is not totally understood. It is speculated that they are biologically very important proteins, partly due to their interactions with components of cell death and signal transduction pathways. For instance, Bag-1 and hsp70 mediate a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth (Song et al., 2001). It was suggested by the same researchers that the regulation of Bag-1 by hsp70 might comprise a checkpoint which regulates growth/death in the event of stress-induced raised levels of hsp70 in the cell.

Finally, hsp70 plays important part in signal transduction pathways through its interaction with a variety of other proteins. For instance, two molecules of hsp90, one of hsp70, one of hsp60 and one of hsp56 altogether can stabilize steroid receptors and deprive them of their capacity to enter the nucleus and perform their function. Therefore, hsp70 is a chaperone that has important roles in signal transduction (in collaboration with Bag-1 and interaction with Raf-1 kinase), hormone response (with Bag-1 and growth factor and hormone receptors), stress response (*via* HSF-1), complex assembly (with Hop and hsp40 in chaperone complexes) and even cell death (*via* Bag-1 and Bcl-2) (for review see Nollen and Morimoto, 2002; Hohfeld et al., 2001 and Beere, 2004).

1.8.3.3 Hsp70 and co-factor roles on protein degradation by the proteasome

In addition to protein folding, co-factors were shown to link chaperones with the proteasome system for protein degradation. For instance, hsp70 and hsp90 were shown to participate in protein degradation by the

proteasome (Schneider and Hartl, 1996; Bercovich et al., 1997). Further examples illustrating the role of hsp70 family members in protein degradation by the proteasome involve the following substrates: actin, αB-crystallin, glyceraldehyde-3-phosphate dehydrogenase, β-lactalbumine, histone H2A, where hsc70 is essential for their degradation (Bercovich et al., 1997).

Summarised below, there is an example of a study that point to the fact that CHIP, one of the co-factors that was mentioned before, provides a link between the chaperones and the ubiquitin proteasome system. CHIP binds hsp70 through a TPR domain and also has ubiquitin ligase activity through a U-box domain. CHIP blocks hsp70 ATP-ase cycle and hence inhibits its folding ability and prevents co-operation of hsp90 with other co-factors that would otherwise enable functionality (Ballinger et al., 1999; Connell et al., 2001). As a result CHIP blocks activation of the glucocorticoid hormone receptor (GR) and induces its degradation by the proteasome (Connell et al., 2001) Similarly, Meacham et al. (1999) found that hsp70 and hsp90 interact with the cytoplasmic domains of cystic fibrosis transmembrane conductance regulator (CFTR) and facilitate its folding and that elevated CHIP levels induces ubiquitilation and degradation of immature CFTR in the endoplasmic reticulum (Meacham et al., 1999).

1.8.3.4 Conclusion on the role of co-factors

The concept that cofactors compete between them for the hsp binding sites and hence there is a mechanism that determines whether certain protein substrates will proceed to folding or degradation was introduced by Hohfeld et al. (1995), based on experimental evidence. An example of such decision making is that of hsp70/hsp90 and their co-factors, which can lead to protein folding (with hsp40, Hip, Hop and maybe other co-factors) or degradation by the proteasome (with hsp40, Bag-1, CHIP and possibly other co-factors). Nollen and Morimoto (2002) pointed to the importance of variation in the levels of chaperones and co-factors and suggested that alterations in such protein levels resulting from stress or disease leads to changes in the cellular response.

1.8.3.5 Examples of the protective function of hsp70

Apart form the roles of hsp70 described so far, there is also strong evidence for a cytoprotective role for hsp70 under different stresses (mainly heat and hypoxia and ischemia/reperfusion), as demonstrated by various *in vitro* and *in vivo* studies (reviewed by Kiang and Tsokos, 1998). The present study deals with the inducible hsp70, the over-expression of which is known to protect several systems under stress.

The following studies provide some examples of *in vitro* protection of different cell types resulting from hsp over-expression. Kabakov et al. (2003) reported that hsp70 over-expressed in human endothelial cells during post hypoxic reoxygenation can protect from delayed apoptosis. Brar et al. (1999) also showed that hsp70 over-expression by using HSV-based vector in primary cardiomyocytes protected against apoptosis-inducing stimuli as well as against thermal or hypoxic stress. In addition, studies utilising transient or stable over-expression of hsp70 cardiomyocyte-derived cell lines or primary cells also indicated a strong protective effect of hsp70 under thermal and ischemic stresses (reviewed by Latchman, 2001).

Over-expression of inducible hsp70 protects from ischemia in cell culture models (Papadopoulos et al., 1996; Xu and Giffard, 1997; also reviewed by Kiang and Tsokos, 1998). On the other hand, hsp70 over-expression by HSV-based viral vectors can protect dorsal root ganglion neurons from thermal or ischemic stress but no protective effect against apoptosis was observed (Wagstaff et al., 1999).

Some examples of *in vivo* protection of different cell types include the following. Transgenic mice over-expressing hsp70 demonstrated less infarction than the wt littermates, following transient focal ischemia, transient global ischemia, or kainic acid-induced seizures with evidence that hsp70 reduced the number of apoptotic cells (Tsuchiya et al., 2003). Over-expression of inducible Hsp70 by either viral or transgenic expression has been shown to provide protection from cerebral ischemia in animal models of stroke (Plumier et al.,

1997; Rajdev et al., 2000; Yenari et al., 1999; Yenari et al., 1998), but the mechanisms of such neuroprotection are still not fully understood. Interestingly, hsp70 was recently shown to reduce protein aggregation in a model of global ischemia and its induction with geldanamycin blocked apoptotic astrocyte death induced by glucose deprivation (Giffard et al., 2004).

There are numerous other published reports where *in vitro* and *in vivo* protection by hsp70 was conferred in various disease models (reviewed by Yenari, 2002). In addition hsp70 over-expression in models of polyglutamine diseases and in a mouse and a fly PD model has raised interest in hsp research in the recent years (Bonini, 2002). However, its important role in protecting from neurodegenerative diseases such as PD and various polyglutamine diseases will be described in a separate section.

1.8.3.6 Hsp70 role in suppressing apoptosis

The emerging role on suppressing apoptosis for hsp70 or for hsp70 and hsp90 acting together is considered to be a function which is separate from their function to act as molecular chaperones. Initially, hsp70 was shown to have an anti-apoptotic function by inhibiting late caspase-dependent events downstream of capase-3, such as activation of cytosolic phospholipase A_2 and nuclear morphology alterations (Jaattela et al., 1998). Further studies in a different system reported that hsp70 over-expression inhibited both caspase activation and downstream events upon apoptosis inducing stresses such as heat shock and TNFα but not against ceramide or ionizing radiation (Buzzard et al., 1998). Apaf-1 interacts with the ATP-ase domain of hsp70 and this prevents apoptosome formation by preventing procaspase-9 recruitment (Saleh et al., 2000; Beere et al., 2000; Beere, 2004). However, in addition to hsp70's role in preventing caspase-dependent cell death, it does also prevent caspase-independent cell death; For instance, AIF is inactivated by direct hsp70 binding, although here the ATP-ase domain is not essential for such function (Ravagnan et al., 2001). Gurbuxani et al. (2003) identified the region of AIF that binds hsp70 and inhibits its apoptotic function by preventing its nuclear translocation.

On the other hand, AIF-derived protein that is non-toxic, interacts with hsp70 and induces apoptosis in various human cancer cells due to sequestration of hsp70 whereas this chemosensitizing effect was absent in cells without hsp70 (Schmitt et al., 2003).

Overall, it becomes clear how important and diverse roles hsp70 and members of the hsp70 family have. The principal function of these proteins, however, is the prevention of misfolding of denatured proteins until they are in the appropriate environment to do so. Under stress, increasing the levels of hsp70 may prevent aggregation and misfolding of denatured proteins and assist either their correct folding or their degradation by the proteasome. Its potential role in neurodegeneration will be discussed in a separate section. Further studies on hsp70 are essential due to its clinical importance and the potential it has as a therapeutic target.

1.8.4 Hsp56

Hsp56 has been referred to as p56, p59, FK506-binding protein 52 (FKBP52) and FKBP59 and it is an immunophilin with both peptidyl-prolyl isomerase activity and chaperone activity (Bose et al., 1996). It is known to bind FK506, which is an immunosuppressive drug that inhibits calcineurin binding to FKBP12 (Andreeva et al., 1999). Hsp56 has important roles in the cell as a co-factor of hsp90 (Peattie et al., 1992), which is essential for normal activity of steroid receptors. For instance, Hutchison et al. (1993) reported assembly of the glucocorticoid receptor into a heterocomplex with hsp90, hsp70, and hsp56. This assembly converts the receptor from a form that does not bind steroid to a high affinity steroid-binding conformation.

In addition, signal-transduction pathways due to hsp56 induction in neonatal rat cardiomyocytes have been studied recently and showed that hsp56 is induced by cardiotrophin-1 (Railson at al., 2001) and mediates hypertrophy *in vitro* in neonatal rat cardiomyocytes *via* many different signalling pathways (Jamshidi et al., 2004)). The authors found that hsp56-induced hypertrophy is

attenuated by inhibition of JAK/STAT, MEK and PI3-K/Akt pathways, whereas it is enhanced by p38 MAPK pathway inhibition.

1.8.5 The Small Heat Shock Protein Family

1.8.5.1 Overview of the small heat shock proteins

The small hsp family (also called the hspB family) has members that are present in virtually all organisms. Proteins that have the α-crystallin domain (an approximately ~85 amino acid sequence in the C-terminus of the protein) belong to the small hsp family. In humans the small hsp family consists of ten members: Hsp27 (HspB1), which is involved in the present study, MKBP (HspB2), which is the myotonic dystrophy protein kinase-binding protein, HspL27 (HspB3), αA-crystallin (HspB4) and αB-crystallin (HspB5), Hsp20 (HspB6), cvHsp27 (HspB7), Hsp22 (HspB8), HspB9 and Odfl (HspB10) (Fontaine et al., 2003).

In vitro, small hsps act as molecular chaperones in preventing unfolded proteins from undergoing irreversible aggregation and insolubilization (Haslbeck et al., 1999). Because of their high binding capacity of up to one substrate molecule/hsp subunit, small hsps are more efficient than other chaperones in this respect (Haslbeck et al., 1999; Ehrnsperger et al., 1998). The range of substrates recognized covers peptides as well as oligomeric enzymes (Ehrnsperger et al., 1998). No substrate specificity has been observed for small hsps so far and complex formation with substrates has not been analyzed in detail. As noted by Benndorf and Welsh (2004), small hsps are not a very well studied protein family and it is postulated that their roles in the cell are quite distinct. Evidence for this is the fact that their C-terminus domains are very different but at the same time they are conformationally very flexible. Another line of evidence for their distinct functional roles is the fact that mutations in different small hsps in humans cause related but not the same disease.

1.8.5.2 Hsp27

1.8.5.2.1 Overview of hsp27

(Hickey et al., (1986a) cloned a HeLa cell cDNA encoding hsp27 and by screening a human genomic library with this hsp27 cDNA they isolated the hsp27 genomic sequence, identifying that the hsp27 gene has 3 exons. Hsp27 is expressed at various levels in different cell types and tissues and it is regulated at both the transcriptional and posttranslational levels. Its C-terminus consists of approximately 100 residues and contains the α-crystallin domain, which is highly conserved, followed by a short more flexible C-terminal extension and it is this C-terminal that is responsible for the chaperone function of hsp27 that will be explained next (Muchowski et al., 1997). The N-terminus is more variable and contains the WDPF motif responsible for oligomerisation (Bova et al., 2000; Theriault et al., 2004).

In the sections below, there are representative examples of findings in various *in vitro* and *in vivo* non neuronal and neuronal systems supporting the cytoprotective role of hsp27. Overall, in the findings reported up to date, the observed protective effects of hsp27 seem to be due to its activity as a molecular chaperone, as a regulator of the cytoskeleton, as oxidative stress modulator and as suppressor of apoptotic pathways.

1.8.5.2.2 Chaperone activity and phosphorylation of hsp27

Hsp27 possesses chaperone activity which, in contrast to hsp70 activity, is ATP-independent (Nicholl and Quinlan, 1994; Jakob et al., 1993) and although it has no protein folding activity, it can keep proteins in a folding-competent state (Ehrnsperger et al., 1997). This was shown *in vitro*, where high-molecular-weight complexes of hsp27 bind denatured proteins and prevent their aggregation by keeping them in a renaturation-competent state (Ehrnsperger et al., 1997). A study with an hsp27 relative from yeast suggested that this function involves the initial binding of the denatured peptides on hsp27 dimers, followed by the reassembly of the hsp27-denatured peptide complexes into high-

molecular-weight structures (Haslbeck et al., 1999). This suggested that the phosphorylation-induced dissociation of the mammalian hsp27 multimers into dimers may promote the chaperone function of hsp27.

Phosphorylation causes a major change in the quaternary structure of hsp27, which shifts from large 600- to 800-kDa homotypic multimers to dimers and monomers. Human hsp27 is phosphorylated in serine residues 15, 78 and 82 whereas hsp25, the mouse homologue, has two such sites, serine 15 and 86 (Stokoe et al., 1992; Gaestel et al., 1991). In normal conditions hsp27 is present in non phosphorylated large oligomeric forms (Rouse et al., 1994; Huot et al., 1995; Lambert et al., 1999). Therefore, under stress hsp27 is phosphorylated in the three serine residues by the activated p38 MAP kinase pathway and MAPKAP kinases 2 and 3 (Landry et al., 1992; Ludwig et al., 1996; Freshney et al., 1994; Stokoe et al., 1992) and dissociates into smaller units (Lambert et al., 1999; Van Montfort et al., 2001 ; Rogalla et al., 1999; Lavoie et al., 1995). Hsp27 activity is hence modulated by phosphorylation and one example is the modulation of the actin polymerization by capping by hsp27 (Miron et al., 1991; Benndorf et al., 1994). The role of phosphorylation of hsp27 on its activity is also illustrated in a system where *in vitro* phosphorylation of hsp27 or molecular mimicry of hsp27 phosphorylation causes decrease of its oligomeric size. The above hsp27 phosphorylation patterns seem to reduce the chaperoning properties of hsp27 in suppressing thermal denaturation and facilitating refolding of citrate synthase *in vitro* while the mutants lose their protective properties against oxidative stress (Rogalla et al., 1999).

Furthermore, large non-phosphorylated aggregates are the active form of hsp27 which controls both intracellular reactive oxygen species and glutathione levels and protects against TNFalpha in NIH-3T3-ras cells (Mehlen et al., 1997). On the other hand, phosphorylated dimers are the species of hsp27 that interact with Daxx, preventing its interaction with Ask1 and Fas and blocking Daxx-mediated apoptosis (Charette and Landry, 2000). Theriault et al. (2004) have recently identified a domain in hsp27 that is required for protection, *in vitro* chaperone activity and maintenance of its oligomeric structure.

1.8.5.2.3 General protective properties of hsp27 in various systems *in vitro* and *in vivo*

Hsp27 is induced by stressors in a variety of non-neuronal cell types and protects them from insults such as ischemia, heat shock, oxidative stress, and noxious chemicals (Huot et al., 1991; Landry et al., 1989; Lavoie et al., 1993; Wu and Welsh, 1996). During heat shock, in particular, Lavoie et al. (1995) provided strong evidence that the modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of hsp27. In summary, during stress, phosphorylation-induced conformational changes in the hsp27 oligomers regulate the activity of the protein at the level of microfilament dynamics, resulting in both enhanced stability and accelerated recovery of the filaments. The level of protection by hsp27 during heat shock was suggested to represent the contribution of better maintenance of actin filament integrity to overall cell survival. Apart from stabilizing F-actin microfilaments during stress (Lavoie et al., 1993; Huot et al., 1995; Guay et al., 1997), hsp27 interacts with various intermediate filaments and colocalises with tubulin and microtubules, but those findings need to be extended before a conclusion can be drawn for the role of hsp27 in the maintenance of cytoskeletal integrity during stress or normal conditions.

Hsp27 is also strongly protective against a number of cytotoxic agents such as chemotherapeutic agents and cytokines (Garrido et al., 1997; Huot et al., 1996). Some of these agents may affect protein structure or microfilament integrity, but it is unlikely that only this activity of hsp27 can explain such a spectrum of protective activities. An explanation could come from its capacity to block apoptosis (reviewed by Concannon et al., 2003 and Beere, 2004). In another study, Brar et al. (1999) utilised viral vectors over-expressing hsp27, and other hsps, and reported that hsp27 protected primary cardiomyocytes from various apoptotic stimuli as well as thermal and ischemic stress.

It has so far become clear that hsp27 expression can protect against oxidative stress, apoptotic as well as necrotic cell death in various systems and

cell types (for instance see Mehlen et al., 1995a; Mehlen et al., 1995b; Mehlen et al., 1996a; Mehlen et al., 1996b; Samali and Cotter, 1996; Wyttenbach et al., 2002; reviewed by Beere 2004). The report by Wyttenbach is of particular interest and is described later. The findings of Mehlen et al., (1995a; 1995b; 1996a; 1996b) are also of interest as they demonstrated that hsp27 protects against reactive oxygen species generation and oxidative stress: constitutive expression of human hsp27 and *Drosophila* hsp27 confers resistance to TNF- and oxidative stress-induced cytotoxicity in murine fibroblasts and mediates the increase in the levels of intercellular glutathione and hence protects from reactive oxygen species formation and death.

An example of a recent *in vivo* study of the protective role of hsp27 is this by Efthymiou et al. (2004), in which isolated perfused hearts from mice over-expressing hsp27 were resistant to ischaemia/reperfusion injury (using infarct size as an end point) compared to hearts from non-transgenic littermates. This is the first study that demonstrates that hsp27 over-expression confers protection from lethal ischaemia/reperfusion injury in the intact heart.

Finally, a further important role of hsp27 (underlying multiple possible protective effects) *in vivo* has been very recently demonstrated by Wang et al. (2004) who generated transgenic *Drosophila* lines by using the upstream activating sequence/GAL4 system. Over-expression of either hsp26 or hsp27 extended the mean lifespan of flies by 30% and this was accompanied by increased stress resistance of these flies.

1.8.5.2.4 Protective properties of hsp27 in neuronal systems *in vitro*

Wagstaff et al. (1999) demonstrated for the first time that *in vitro* hsp27 over-expression protects primary sensory neurons and a neuronal cell line against both thermal and ischemic stress and against apoptosis induced by nerve growth factor withdrawal or retinoic acid treatments and serum withdrawal.

More recently, Shimura et al. (2004) found that hsp27 rescued cells from pathological hyperphosphorylated tau-mediated cell death in an *in vitro* study. Hsp27 bound preferentially only the pathological hyperphosphorylated

tau and facilitated its degradation without ubiquitination, which is otherwise not efficiently degraded by the proteasome.

An additional new property of hsp27 was also described by Wyttenbach et al. (2002), who identified that hsp27 *in vitro* can suppress polyQ-mediated cell death in a cellular model of Huntington's disease (HD). Mutant huntingtin caused increased levels of ROS in neuronal and non-neuronal cells, leading to cell death. Hsp27 significantly reduced ROS content in those cells, providing evidence that hsp27 may protect cells against oxidative stress. This protection by hsp27 was regulated by its phosphorylation status and was independent of its ability to bind to cytochrome c. In contrast to hsp40 and hsp70, hsp27 suppressed polyQ death but not polyQ aggregation.

1.8.5.2.5 Protective properties of hsp27 in neuronal systems *in vivo*

Another example of the neuroprotective role of hsp27 is the study by Lewis et al. (1999), which showed that in sensory neurons hsp27 plays a role in promoting neuronal survival after axotomy *in vivo* and after NGF withdrawal *in vitro*. Their findings suggest that hsp27 contributes significantly to the survival of sensory neurons under these conditions and is likely to be an important factor for survival of adult sensory neurons as well. An important study by Benn et al. (2002), extending the above findings, showed that motor and sensory neurons in adult rats up-regulate hsp27 in its active phosphorylated form following injury to a peripheral nerve. In neonatal rats, however, the expression of hsp27 is much lower following such injury, and only a minority of those cells expressing the protein survives. There also seemed to be an inverse relationship between the expression of hsp27 and the expression of activated apoptotic factors and other signs of cell death. Delivery of hsp27 into developing cells could mimic the resistance of adult cells: after infecting neurons of neonatal rats with an adenovirus expressing human hsp27, more of the neurons survived when the sciatic nerve was damaged. By using a herpes simplex virus encoding antisense hsp27 they found that adult neurons began to die after nerve injury. Finally, hsp27 was shown to interact with cytochrome c and prevented down stream events.

Further roles of hsp27 in *in vivo* neuronal systems, which are relevant to the present study are those described by Kalwy et al. (2003) and Akbar et al. (2003). Kalwy et al. (2003) utilised HSV-based viral vectors to exogenously express hsp27 in the rat hippocampus resulting in a significant increase in survival following kainic acid administration. This was the first report demonstrating protection by exogenously expressed hsp27 in an *in vivo* model of neuronal cell death. Subsequently, Akbar et al. (2003) developed transgenic mice, expressing human hsp27 at high levels in the brain, spinal cord and other tissues and tested the hypothesis of hsp27 conferring *in vivo* neuroprotection against kainite induced neuronal cell death. Indeed, hsp27 expression in the mouse reduces kainate-induced toxicity and mortality by at least 50% in two mouse lines, reduces neuronal cell death in the CA3 region of hippocampus and attenuates caspase-3 induction.

1.8.5.2.6 Novel role of hsp27 in proteasome-mediated protein degradation

Hsp27 has also a newly discovered and important role in protein degradation of certain substrates *via* the proteasome. Parcellier et al. (2003b) reported that hsp27 over-expression increased proteasome activation after cytokine stimulation (TNF-α and interleukin 1β) or the cytotoxic drug etoposide. Under those stresses hsp27 increased NF-κB activity. The authors reported a direct association of hsp27 with the 26S proteasome, with ubiquitin chains, and with phosphorylated I-κBα (main inhibitor of NF-κB). An increase in NF-κB activity (due to degradation of its inhibitor) leads to suppression of apoptosis, as it is an important function of NF-κB. Therefore, hsp27 facilitates phosphorylated I-κBα degradation by the proteasome, which could explain the protective effect of hsp27 under stressful situations.

1.8.5.2.7 Hsp27 roles in human disease

In affected members of a Russian family with Charcot-Marie-Tooth disease type 2F reported previously (Ismailov et al., 2001), Evgrafov et al. (2004) has recently identified a Ser135-to-Phe (S135F) substitution which

occurs in a highly conserved α-crystallin domain of the hsp27 protein. The same mutation was also found in affected members of an unrelated family from the United Kingdom with distal hereditary motor neuropathy. The S135F mutant protein expression *in vitro* led to reduced survival of neuronal cells and impaired the assembly of neurofilaments. In addition, in a Belgian family with distal hereditary motor neuropathy the same group identified an Arg127-to-Trp (R127W) substitution in hsp27 and in a Croatian family a Thr151-to-Ile (T151I) substitution in hsp27 and a Pro182-to-Leu (P182L) substitution in affected members of an Austrian family with the same condition. These mutations occur again in the highly conserved α-crystallin domain. The authors suggested that these deficits may be responsible for premature axonal degeneration, which underlies both Charcot-Marie-Tooth disease and distal hereditary motor neuropathy.

1.8.5.2.8 Hsp27 roles in suppressing apoptosis

Finally, the role of hsp27 and other hsps in suppressing apoptosis initiated by various stresses is becoming well documented in the last few years and is also discussed extensively later in this thesis, in view of experimental findings. Some of this evidence is also summarised separately later and is reviewed by Beere (2004), Parcellier et al. (2003a) and Conncanon et al. (2003).

Briefly, hsp27 was found to bind to cytochrome c after its release from the mitochondria and to procaspase-3 and hence preventing apoptosome formation and events downstream the mitochondrion (Bruey et al., 2000; Pandey et al., 2000; Paul et al., 2002). By binding to cytochrome c, hsp27 negatively regulated procaspase-9 activation and apoptosome formation (Bruey et al., 2000). Inhibition of caspase-independent apoptosis by hsp27 has also been shown (Charette et al., 2000). In this instance hsp27 prevents Daxx translocation to the membrane and its interaction with Fas receptor and Ask 1 (so, Fas-mediated apoptosis is blocked).

Moreover, hsp27 regulates apoptosis through interaction with protein kinase B (Akt) in a variety of systems. For instance, Konishi et al. (1997) found

that under stress the activation of Akt is *via* association with hsp27 and it is possible that suppression of apoptosis by Akt activation is modulated by an hsp27 interaction. Interestingly, Rane et al. (2003) showed that hsp27 has a novel role as an Akt substrate, which dissociates from it after phosphorylation. The authors reported that the interaction between hsp27 and Akt impaired Akt activation and increased neutrophil apoptosis. Therefore, control of neutrophil apoptosis is through regulation of Akt activity by hsp27.

1.8.6 Other Important Hsps: Large hsps, Hsp60, Hsp32 and Ubiquitin

The large hsp105/110 family represents a diverged subgroup of the hsp70 family but it is not very well studied. Hsp110 family members are localized in both the nucleus and cytoplasm and are regulated by hyperthermia (see review by Easton et al., 2000). Over-expression of hsp110 in cultured mammalian cells confers thermotolerance. Importantly, Hsp110 is found in conjunction with Hsp70 in the cytoplasm and nucleus of every eukaryotic cell examined, from yeast to humans. All hsp110s loss of function or deletion mutants listed in the *Drosophila* deletion project database are lethal (Easton et al., 2000).

Hsp105 was shown to suppress protein aggregation in stressed cells in which ATP is greatly decreased (ADP is present), in contrast to hsp70/hsp40 proteins that need ATP to perform such function (Yamagishi et al., 2003). The same group recently showed that hsp105alpha is able to regulate the substrate binding cycle of Hsp70/Hsc70 by inhibiting the ATPase activity of Hsp70/Hsc70, hence it was proposed to be a negative regulator of Hsp70/Hsc70 system (Yamagishi et al., 2004).

On the other hand, hsp104p is considered to be protective against reactive oxygen species formation and, interestingly, it was reported to regulate mutant α-Syn fibril formation (Kong et al., 2003). A homologue to yeast hsp104 (which is capable of disaggregating prions according to Kushnirov et al., 2000) is torsinA. TorsinA was found to be in LBs in PD (Sharma et al., 2001) and its mRNA levels are highest in the dopaminergic neurons of the substantia nigra (Augood et al., 1999). TorsinA is also mutated in dominant cases of early onset

torsion dystonia (Ozelius et al., 1997). In addition, McLean et al. (2002) co-expressed α-Syn and synphilin-1 in a neuroglioma cellular model that forms intracytoplasmic inclusions, and found that torsinA co-localises in the inclusions with α-Syn, hsp70, hsp40 and hsp60 but not hsp27, hsp90 or hsp110. When wt TorsinA, but not a mutant form, was co-transfected with α-Syn and synphilin-1, the number of intracytoplasmic inclusions was reduced by 46%, suggesting that TorsinA might have a role in suppressing aggregate formation.

Previously, Glover and Lindquist (1998) reported an elegant study where yeast hsp104 was found to reactivate already denatured proteins and such remodelling activity relied on hsp70 and hsp40 interactions with hsp104. It should be noted here that such remodelling activity of already denatured proteins is not reported so far for other hsps. Furthermore, this yeast chaperone hsp104 and its bacterial homolog, ClpB (structurally unrelated to hsp70 family) together with Hsp70/Hsp40 can solubilize protein aggregates of expanded polyQ protein (Satyal et al., 2000; Carmichael et al., 2000).

Another family worth mentioning is the hsp60 family (chaperonins). Hsp60, GroEL in *E.coli*, locates in the mitochondria and chloroplasts of eukaryotes and in the cytosol of bacteria. Hsp60 needs mt-hsp70 and ATP to fold newly imported proteins. Bacterial GroEL-like heat shock protein 60 protects epithelial cells from UV radiation-mediated epithelial cell death through activation of ERK1/2, which inhibits caspase-3 activation (Zhang et al., 2004). Another example of the protective role of hsp60 is provided by the study by Shan et al. (2003), in which over-expression of hsp60 and its co-factor hsp10 in cardiomyocytes suppressed doxorubicin-induced apoptosis, increased the abundance of the anti-apoptotic Bcl-xl and Bcl-2, reduced the protein content of the pro-apoptotic Bax and stabilized mitochondrial transmembrane potential and inhibited caspase-3.

Hsp32 is an interesting hsp called haem oxygenase 1 (HO1) and it is an enzyme that degrades heme (haem and haem proteins are potential pro-oxidants) into iron, carbon monoxide, and biliverdin which is rapidly converted into bilirubin (Stocker et al., 1990). Both biliverdin and bilirubin have been

demonstrated to have anti-oxidant function (Stocker et al., 1987). Hsp32 is induced by heat shock and oxidative stress (Dwyer et al., 1992) and it has been shown that increased levels of hsp32 localise in neurofibrilary tangles in AD (Smith et al., 1994), raising the possibility of an involvement in this disease.

Finally, ubiquitin is a conserved, small 7-8 kD protein that is very important for all eukaryotic cells because it targets proteins for degradation by the 26S proteasome. As discussed earlier, it acts by being attached to the protein to be degraded by ubiquitin-conjugating enzymes in an ATP-dependent manner. It binds protein at lysine residues *via* its C-terminal glycine and forms a poly-ubiquitin chain that signals for UPS degradation (Pickart, 1997). UBB+1 (an aberrant ubiquitin form found in intracellular protein inclusions in Alzheimer's disease and progressive supranuclear palsy) is the product of molecular misreading with an extra 20 aminoacids at its C-terminal (van Leeuwen et al., 1998) and hence it cannot ubiquitinate target proteins, whereas its over-expression strongly inhibits the 26S proteasome and leads to hsp induction that protects from oxidative stress (Hope et al., 2003). Histones H2A and H2B are ubiquitin conjugated. Therefore, ubiquitination can alter chromatin structure and hence gene expression. For instance, a recent study demonstrated that ubiquitylation and de-ubiquitylation are both required for gene activation, suggesting that ubiquitylation of histones has an important role among histone modifications and can potentially regulate chromatin alterations (Wyce et al., 2004).

1.8.7 Heat Shock Protein Regulation and HSF

Heat shock gene transcription is induced by the transcription factors called heat shock factors (HSFs) under normal or stressful conditions. They are highly conserved in structure from yeast to humans, homotrimerise and bind to *cis*-acting DNA promoter elements, the also highly conserved heat shock elements (HSEs). The repression or activation of HSF activity depends on various post-translational modifications and protein interactions (Wu, 1995). In mammals there are three genes that encode HSF-1, HSF-2 and HSF-4. Identified by Nakai et al. (1997), HSF-4 is only found in humans, preferentially

in the heart, brain, pancreas and skeletal muscle and interestingly it does not have transactivation properties. HSF-3 is an extra form found in chickens (Nakai and Morimoto, 1993).

Hsps are expressed in response to heat shock and other stresses *via* HSF-1 and in turn, HSF-1 is stress activated. In summary, such activation involves the formation of the HSF trimer that is ready to bind to DNA, followed by the exposure of the transcriptional activation domain, which enables its transcriptional activating ability (Morimoto, 1993). Deletion of the second leucine zipper from HSF-1 allows trimerisation and exposure of the activating domain without the need of previous stress induction (Zuo et al., 1995). This particular mutant was also used in experiments presented in this thesis (see Chapter 4).

HSF-1 has four leucine zipper motifs and the first activating step of HSF-1 is the disruption of the leucine zipper interactions upon stress. Three uncoiled HSF-1 monomers interact *via* their N-terminus leucine zipper motif and trimerise (Rabindran et al., 1993). Then the trimer translocates to the nucleus and bind HSEs (Rabindran et al., 1993; Westwood et al., 1991) to initiate transcription, after it has been phosphorylated by MAPK members in a *ras*-dependent manner (Knauf et al., 1996; Kim et al., 1997).

HSF-1 regulation is by phosphorylation and partly by down-regulation by hsp70 (Morimoto et al., 1996), hsp90 and the nuclear heat shock binding protein 1 (Wu, 1995). In summary, a proposed model of HSF-1 regulation is as follows, although details in such mechanism are not fully described. In unstressed cells the monomeric HSF-1 remains inactive without DNA binding activity due to its interaction with hsp70, but after stress, such as heat shock, it trimerises and binds to HSE in the heat shock gene promoters and is phosphorylated. Hsp70 expression levels increase, hsp70 interacts with HSF-1 and subsequently dissociates giving rise to the three inactive monomers.

Hsp90 has also an important role as a negative regulator of HSF-1. It has been suggested that an hsp90/HSF-1 heterocomplex may keep HSF-1 in a

repressed state. Disruption of this heterocomplex by stress would allow HSF-1 to form trimers and become transcriptionally active. As a result of disruption of these heterocomplexes during stress there is accumulation of denatured polypeptides and hsp90 binds such denatured polypeptides (Bharadwaj et al., 1999; Zou et al., 1998; Guo et al., 2001). During recovery from stress, HSF-1 trimers have been found in separate complexes with hsp70 and in hsp90/hsp56 complexes (Zou et al., 1998). Heat shock factor-binding protein 1, which interacts with HSF-1 trimers together with hsp70, has also been isolated (Satyal et al., 1998), and this interaction appears to also be a negative regulator of HSF-1 transcriptional activity.

Interestingly, Morley and Morimoto (2004) confirmed previous studies by suggesting that the transcriptional activator HSF-1 regulates stress resistance genes that extend longevity, independently of DAF-16 in *C. elegans*. To date only small hsps, hsp70 and hsp90 have been shown to be expressed after HSF activation and it was only recently attempted to identify the full range of HSF targets in yeast (Hahn et al., 2004). Targets representing 3% of the genome were identified (including loci encoding proteins functioning in protein folding, degradation, metabolism, cell signaling, transcription, molecule trafficking), suggestive of a broad role for HSF.

1.8.8 Heat Shock Protein Involvement in Neurodegeneration

The involvement of hsps in neurodegeneration and their therapeutic importance have only recently become evident. AD, PD and HD are distinct neurodegenerative disorders that have something in common: brain lesions that consist of proteins with the propensity to form amyloid, an ordered fibrilar structure (see reviews by Selkoe, 2001; Goedert, 2001; and Rochet and Lansbury, Jr., 2000). These brain lesions are characterised by the presence of molecular chaperones and components of the ubiquitin-proteasome system (reviewed by Sherman and Goldberg, 2001). This section provides a short account on the aspects of heat shock protein roles in various neurodegenerative disorders, as shown in many studies utilising cellular *in vitro* models or *in vivo* models of the diseases and human post mortem material.

1.8.8.1 Polyglutamine diseases

Huntington's like Parkinson's disease is usually a late-onset, progressive neurodegenerative disease associated with selective neuronal loss and abnormal protein accumulations. HD belongs to polyglutamine (polyQ) repeat diseases. This class also includes dentatorubropallidoluysian atrophy (DRPLA), spinal and bulbar muscular atrophy (SBMA) and spinocerebellar ataxias type 1, 2, 3 (also known as Machado–Joseph disease, MJD), 6, 7, and 17. PolyQ diseases are characterized by the expansion of glutamine within the ORF of the respective proteins. The expanded polyQ domain is toxic and leads to neuronal dysfunction and degeneration. Abnormal protein accumulation as nuclear inclusions that contain the disease protein and many other components, led to multiple *in vitro* and *in vivo* studies where molecular chaperones are tested for their ability to protect from abnormal aggregate formation and toxicity, as seen next.

1.8.8.1.1 Huntington's Disease

Muchowski et al. (2000) demonstrated that hsp70 and hsp40 *in vitro* in yeast can interact with mutant huntingtin and inhibit the formation of detergent insoluble fibrillar aggregates. There are several other reports that demonstrate that increased expression of the hsp70/hsp40 chaperone system can suppress polyQ-induced neurotoxicity in fly models (Kazemi-Esfarjani and Benzer, 2000; Warrick et al., 1999; Chan et al., 2000; Fernandez-Funez et al., 2000) and a mouse model of polyQ disease (Cummings et al., 2001).

Some other examples include the study by Dedeoglu et al. (2002) where homozygous and heterozygous hsp70 over-expressing mice and wt controls received 3-nitropropionic acid or malonate and the striatal lesion sizes were evaluated. Mice over-expressing hsp70 show increased resistance to malonate and 3-nitropropionic acid. Malonate and 3-nitropropionic acid are well-characterized animal models of HD and they inhibit succinate dehydrogenase, inducing mitochondrial dysfunction, which triggers the generation of superoxide radicals, secondary excitotoxicity and apoptosis.

However, Hansson et al. (2003) studying a mouse model of HD found that hsp70 has only modest effects on disease progression. They crossed R6/2 mice, expressing exon 1 of the HD gene with an expanded CAG repeat, with mice over-expressing hsp70. The resulting R6/2-hsp70 transgenics exhibited 5- to 15-fold increases in hsp70 expression in neocortical, hippocampal and basal ganglia regions which correlated with a delayed loss of body weight compared to R6/2 mice. However, the number or size of nuclear inclusions, the loss of brain weight, reduction of striatal volume, and reduction in size of striatal projection neurons, development of paw clasping phenotype and early death of the mice were not affected by hsp70 over-expression. It was also notable that in older R6/2-hsp70 mice a large proportion of the Hsp70 protein was accumulated in nuclear inclusions.

Hay et al. (2004) also recently reported a progressive decrease in Hdj1, Hdj2, hsp70, alphaSGT and betaSGT brain levels in the R6/2 mouse model of HD. These proteins were all found to co-localise with nuclear but not with extranuclear aggregates. At the mRNA level, Hdj1 and alphaSGT showed that these do not change so maybe the decrease in protein levels is a result of their sequestration into aggregates, or an increase in protein turnover, possibly as a consequence of their relocation to the nucleus. By crossing the R6/2 mouse to hsp70 transgenic mouse, hsp70 over-expression delays aggregate formation by one week, has no effect on the detergent-solubility of aggregates and does not alter the course of the neurological phenotype. However, by utilising organotypic slice culture assay and pharmacolocical agents such as radicicol and geldanamycin (see below also), it was shown that chaperone induction was maintained by both agents for at least three weeks, and altered the detergent soluble properties of polyQ aggregates.

The first demonstration that huntingtin protein aggregation in cells can be suppressed by chemical compounds activating a specific heat shock response utilised geldanamycin (Sittler et al., 2001). Geldanamycin is a benzoquinone ansamycin that binds and inhibits heat shock protein hsp90 and activates a heat shock response in mammalian cells. Treatment of mammalian cells with geldanamycin at nanomolar concentrations induces the expression of Hsp40,

Hsp70 and Hsp90 and inhibits HD exon 1 protein aggregation in a dose-dependent manner. As mentioned in the previous paragraph, Hay et al. (2004) used geldanamycin and radicicol to induce various hsps in organotypic slice cultures from transgenic HD mouse and this altered the solubility properties of polyQ protein aggregates in this model.

Finally, Wyttenbach et al. (2002) reported an interesting role of hsp27 *in vitro.* Hsp27 suppressed polyQ-induced ROS formation in cells and conferred protection to neuronal and non-neuronal cells against poly-Q-mediated toxicity without reducing polyQ-protein aggregation. These findings demonstrate that oxidative stress caused by polyQ expansion contributes to cell death and suggest an important new role of hsp27 in preventing toxicity associated with polyQ expansions.

1.8.8.1.2 Hsps and other polyQ diseases

More recently, Adachi et al. (2003) cross-bred spinal and bulbar muscular atrophy (SBMA) transgenic mice with mice over-expressing human hsp70 and they showed amelioration of the disease phenotype in addition to the finding that the nuclear localisation of the mutant androgen receptor (AR) as well as of monomeric mutant AR was reduced, which suggest a possible hsp70-mediated AR degradation. Earlier to this study, in an *in vitro* cellular system hsp70 and hsp40 overexpression led to enhanced mutant AR solubility and proteasomal degradation and a decrease in the mutant AR half-life (Bailey et al., 2002).

In spinocerebellar ataxia type 1 (SCA1), which is characterized by loss of motor coordination due to the degeneration of cerebellar Purkinje cells and brain stem neurons, the expanded protein aggregates into nuclear inclusions that contain chaperones, ubiquitin, and proteasomal subunits (components of the protein refolding and degradation machinery). Cummings et al. (2001) crossbred SCA1 mice with inducible hsp70 over-expressing mice in order to determine whether enhancing chaperone activity could mitigate the phenotype in a mouse model by reducing protein aggregation,. Although the amount of nuclear inclusions in Purkinje cells remained the same, further analysis revealed

that high levels of hsp70 protect against neurodegeneration and preserved dendritic arborization in the cerebellum.

Interestingly, Hsp104 and its bacterial homolog, ClpB, can solubilize small protein aggregates in concert with Hsp70/Hsp40modulators of polyQ aggregation. Direct interaction of the prokaryotic ClpB (Hsp104) and DnaK (Hsp70) in the ATP-dependent resolubilization of aggregated proteins was reported and the chaperone complex described seems that it might facilitate transfer of intermediates between ClpB and DnaK during refolding of substrates from aggregates (Schlee et al., 2004).

1.8.8.2 Hsps and Parkinson's Disease

As reviewed by Bonini (2002), molecular chaperones were the first modifiers defined to interfere with the progression of neurodegeneration in *Drosophila*. Hsp70 was identified as a potent suppressor of disease phenotype in HD and PD in flies. In specific, hsp70 prevented dopaminergic neuronal loss associated with α-Syn expression in *Drosophila*. In addition, interference with the endogenous chaperone activity in flies accelerated α-Syn toxicity (Auluck et al. 2002). Interestingly, although increased expression of hsp70 suppressed the toxicity induced by α-Syn, it did not alter the microscopic appearance of the neuronal inclusions formed.

In the same report, hsp70 and hsp40 were shown to be present in LBs and LNs of post mortem PD brain. As mentioned in section 1.3.4.2 previously, LBs, which are the neuropathological hallmarks of the disease, were shown to contain many other hsps among which hsp27, αB-crystallin, torsinA, hsp90, hsp110 and ubiquitin. The presence of hsps in LBs provides an additional link between hsps and the disease; however their role in the process of neurodegeneration is still unclear.

Overall, the various *Drosophila* studies have established an important role of hsps in neurodegeneration, i.e. chaperones are potent modulators of polyQ toxicity (see studies described above) and α-Syn toxicity in polyQ disease and PD models respectively. These studies have also proved that

interference with the endogenous chaperone activity accelerates pathogenesis. The later finding comprises strong evidence that chaperone activity is central to the disease progression that is being modulated by over-expression or interference. It is notable that *Drosophila* models proved to be valuable for the identification of modifiers that interfere with the disease progression.

The report by Auluck et al. (2002) on α-Syn suppression of toxicity by hsp70 was followed by a recent study by Klucken and colleagues (2004), in which α-Syn and hsp70 transgenic mice were crossed. Hsp70 had an effect on reducing high molecular weight and detergent insoluble α-Syn species in this model but did not change the total amount of soluble monomeric α-Syn. The same study reported protection from α-Syn toxicity by hsp70 over-expression in an *in vitro* cellular model of α-Syn aggregation (human H4 neuroglioma cells) as well as reduction of detergent insoluble α-Syn species. Auluck et al. (2002) had suggested that hsp70 protection from α-Syn toxicity can be dissociated from prevention of α-Syn aggregation. It would therefore be interesting to see whether hsp70 over-expression in the double transgenic mouse, apart from reducing aggregation, could also ameliorate dopaminergic cell death and improve the disease phenotype of the model described by Klucken et al. (2004).

1.8.8.3 Alzheimer's Disease and Amyotrophic Lateral Sclerosis

In Alzheimer's disease (AD), which is a progressive amnestic dementia, post-translational hyperphosphorylation, enzymatic cleavage, and conformational alterations of the microtubule-associated protein tau are evident. A recent study showed that CHIP and hsp70 regulate tau ubiquitination, degradation and aggregation (Petrucelli et al., 2004). CHIP was found to directly interact with the microtubule-binding domain of tau, induce tau ubiquitination, increase the levels of insoluble aggregated tau and interestingly CHIP was present in tau lesions in human post mortem brain tissue. In the same study, the levels of tau were reduced in mice over-expressing hsp70 and also hsp70 induction by geldanamycin or HSF-1 led to a decrease in steady-state tau levels and in detergent insoluble and hyperphosphorylated tau. Previously, Shimura et al. (2004) had reported tau binding to Hsc70, and the requirement for

phosphorylation prior to ubiquitination by CHIP. CHIP rescued from phosphorylated tau-induced toxicity, although the study by Petrucelli et al. (2004) further explores the opposing action of hsp70 and CHIP on tau ubiquitination and aggregation. Imai et al. (2002) showed that CHIP, hsp70, parkin, and Pael-R formed a complex *in vitro* and *in vivo*. The amount of CHIP in the complex increased during ER stress. CHIP promoted the dissociation of hsp70 from parkin and Pael-R, thus facilitating parkin-mediated Pael-R ubiquitination. Finally, Dou et al. (2003) found that in transgenic mice and in AD brains tau aggregation is inversely related to hsp70 and hsp90 levels and importantly their elevated levels enhance tau solubility and its binding to microtubules.

In familial cases of amyotrophic lateral sclerosis (ALS), which is a fatal neurodegenerative disorder characterised by death of motorneurons of the spinal cord and the motor cortex, patients carry mutations in the Cu / Zn superoxide dismutase gene (*SOD-1*). In a model of this disease, which is a transgenic mouse over-expressing human SOD-1, acrimoclomol treatment resulted in delay in the disease progression, by inducing hsps (Kieran et al., 2004). This drug is a hydroxylamine derivative that acts as a co-inducer of hsp expression and successfully led to increase in HSF-1 activation and an increase in the protein levels of hsp70, hsp90 and, at a lesser extend, of hsp27 in the spinal cord of treated mice. Importantly, treated mice exhibited significant improvement in motorneuron survival and hind limb muscle function and had increased lifespan by 22%.

In other studies, mutant SOD-1 toxicity was delayed by gene transfer of hsp70 (Bruening et al., 1999) and, interestingly, αB-crystallin and hsp27 were upregulated in the spinal cord of mutant SOD-1 mice compared to mice over-expressing wt SOD-1, while hsp70 levels were normal (Vleminckx et al., 2002). Finally, Batulan et al. (2003) showed that there is high threshold for induction of the stress response in motor neurons which is due to impaired ability to activate HSF-1. The above examples implicate hsps in ALS and raise the possibility that hsps might be important therapeutic targets for this neurodegenerative condition.

1.8.9 Heat Shock Proteins: Therapeutic Targets for Parkinson's Disease?

In addition to the various previous reports on the role of hsps in neurodegeneration in general, there are a few important recent studies related to hsps in various PD models and in post mortem PD brains. These studies have been described in previous sections of the Introduction of this thesis (Klucken et al., 2004; Shulman et al., 2003; Manning-Bog et al., 2003; McLean et al., 2002; Bonini, 2002; Auluck et al., 2002; Braak et al., 2001). These studies highlight the potential of hsps as therapeutic targets for this disease, but further work is necessary for a better understanding of the role of hsps in PD pathology. Evidence from the aforementioned reported studies in the context of PD and in models of other neurodegenerative conditions, suggests that agents that pharmacologically induce the expression of molecular chaperones in neurons could be a fruitful strategy for the treatment of PD.

Muchowski (2000) discussed the roles of protofibrils in neurotoxicity in a number of neurodegenerative conditions and by evaluating experimental evidence he suggested that maybe chaperones protect in various *in vivo* models by acting at the protofibril level. In PD the precise role of LBs is not yet defined and more studies are necessary before we have a clear understanding of which exactly is the toxic species in the aggregate formation pathway.

In view of the multiple functions of hsps, in addition to suppressing protein misfolding and aggregation, it becomes clear that manipulation of the hsp expression in animal models could become a starting point to develop neuroprotective strategies for PD and other diseases. As a summary, PD is characterised by not only aggregation of protein in the brain but also mitochondrial dysfunction, reactive oxygen species formation, oxidative stress and cell death. Hsps have been implicated in all the above pathways, as seen in the introduction of this thesis, therefore hsps comprise eligible candidates for neuroprotection in PD. Pharmacological chaperone induction should be further evaluated pre-clinically in mammalian *in vivo* models of PD, as it has been done for HD.

Neurodegenerative diseases like PD, AD, ALS, and the prion diseases all involve the assembly of structurally unrelated proteins into intracellular or extracellular amyloid fibrils. Therefore, since all the intermediates that are formed early in these diseases can be highly cytotoxic for the neurons (Bucciantini et al., 2002; Walsh et al., 2002), it is possible that there might be common mechanisms in amyloid formation and toxicity leading to the development of common strategies for neuroprotection.

1.9 Aims and Objectives

The aims of the project of which results are presented in this thesis were as follows:

- To establish, characterize and utilize an *in vitro* α-Syn toxicity cellular model system in order to test various hypotheses with regards to the role of wt and PD-mutant α-Syn in neuronal cells.
- To test the protective role of hsps (in particular hsp27 and hsp70) against α-Syn-associated toxicity under various conditions relevant to PD in the above *in vitro* system.
- To subsequently investigate the underlying mechanisms of the protective effects of hsps in this model: preliminary studies of apoptotic pathways to be performed and the role of the mitochondrion is also investigated.

The following three Chapters describe:

- The establishment and characterization of this model system and discuss its limitations and advantages in relation to the literature as well as suggest further experiments (Chapter 3).
- The utilization of this model system and HSV-based vectors to study the neuroprotective properties of hsps (Chapter 4).
- Preliminary investigations into the mechanism of the protective action of hsp27 (Chapter 5).

CHAPTER 2

Materials and Methods

2.1 Laboratory Reagents

2.1.1 General Suppliers

General laboratory chemicals were of analytical grade or molecular biology and were purchased from the following companies: Sigma Chemical Company Ltd., Poole Dorset, UK; BDH Merck Ltd., Lutterworth, Leicestershire, UK; Boehringer Mannheim, Lewes, East Sussex, UK. Phosphate buffered saline (PBS) was made using PBS tablets (1 tablet in 500ml ddH_2O) from Invitrogen Ltd, Paisley, UK. Millipore water was used for solutions and was autoclaved where necessary. General laboratory plasticware was purchased from BDH Merck Ltd. and Ependorff, Cambridge, UK.

2.1.2 Bacterial Reagents

Bacto®-Yeast extract, Bacto®-Tryptone and Bacto® Micro-agar bacterial growth medium were from Duchefa, Harlem, Nehterlands. XL1-blue *Escherichia coli* (*E. coli*) cells were from Stratagene, La Jolla, California, USA.

2.1.3 Molecular Reagents and Plasmids

All the restriction endonucleases and DNA modifying enzymes and their respective buffers were supplied by Promega Southampton, UK, apart form the enzyme *Bam*H1 that was purchased from New England Biolabs, UK. DNA molecular weight marker (1 kb) was from Invitrogen Ltd, Paisley, UK. QIAquick® gel extraction kit, QIAprep Mini and Midi DNA prep kits were from Qiagen Ltd, Crawley, UK. DNA sequencing was performed by MWG-Biotech AG (Ebserberg, Germany). The plasmid vectors used were: pMAMneo from BD Biosciences, Clontech, Palo Alto, CA, USA (GenBank Accession # UO2432) which has a dexamethasone responsive promoter, under which wild type (wt) and mutant A30P and A53T α-Syn cDNA was subcloned in sense orientation after excision from their original vectors as detailed in the Methods.

Those plasmids with the wt and mutant α-Syn cDNA were kinldy provided by Dr. Ross Jakes (Cambridge, UK).

2.1.4 SDS-PAGE and Western Blotting Reagents

Acrylamide/bisacrylamide (30% w/v) solution for polyacrylamide gels was obtained from Amresco Ltd., Ohio, USA. Protein molecular weigh Rainbow^{TM} marker, Hybond^{TM}-C nitrocellulose membranes, Enhanced Chemiluminescence system (ECL) and Kodak X-OMAT imaging photographic film were purchased from Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK. Photographic developing and fixing chemicals were obtained from X-OGRAPH Ltd., Tetbury, UK.

Hsp27 antibody, hsp56 antibody (both goat polyclonal, diluted 1:1000) and β-actin antibodies (goat polyclonal, diluted 1:3000) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Hsp70 antibody (mouse monoclonal, diluted 1:2000) and the HSF-1 (rabbit polyclonal antibody used 1:2000) was from Stressgen Biotechnologies Inc., San Diego, CA, USA, and α-Synuclein antibody (mouse monoclonal, diluted 1:500) was from BD Transduction Laboratories, Lexington, KY, USA. The hsp40 antibody was used 1:3000 and was a goat polyclonal antibody, specific for human hsp40 (the virus expresses human hsp40) and was a kind gift by Dr Rohan de Silva, UCL, UK. Secondary horseradish peroxidase-linked antibodies used for Western immunoblotting were all from DAKO Ltd., Glostrup, Denmark.

2.1.5 Tissue Culture Reagents

Tissue culture media and reagents and plasticware were purchased from Invitrogen Ltd, Paisley, UK. Disposable sterile 0.2 μM filters were obtained from Millipore, Watford, UK. Dexamethasone was dissolved in methanol and kept in -20°C (Sigma). *All trans*-Retinoic acid was from Sigma and was resuspended in dimethylsulfoxide (DMSO) and kept in -70 °C in stocks

of 3 mg/ml, always minimizing exposure to light. Geneticin® (G418-sulphate) and Zeocin™ were purchased from Invitrogen Ltd, Paisley, UK. All caspase-inhibitors were from Calbiochem® Z-VAD-FMK (inhibits caspase -1-like proteases), Z-IETD-FMK (irreversible caspase-8 inhibitor), Z-LEHD-FMK (irreversible caspase-9 inhibitor) and were all solubilised in DMSO (as suggested by the supplier at 50 mM, 10 mM and 20 mM stock solutions , respectively, which were kept in aliquots at -20°C) and used at a final concentration of 10μM. Hexamethylene bisacetamide (HMBA), from Sigma, was used at a final concentration of 3 mM HMBA. It was solubilised in double distilled water, filtered sterilized and kept in -4°C.

2.1.6 Cell Death Detection and Quantification

TUNEL (terminal dUTP nick end labeling) reagents and the annexin-V-FLUOS staining kit, including propidium iodide solution were both purchased from Roche Molecular Biochemicals, GmbH (Mannheim, Germany).

2.1.7 Determination of Mitochondrial Membrane Potential

Green fluorescent MitoTracker Green FM (M-7514) (MTG) and tetramethylrhodamine, methyl ester, perchlorate (T-668) (TMRM) were from Molecular Probes, Eugene, USA. MTG was solubilised in DMSO (stock concentration 1 mM, kept in -20°C) and TMRM was solubilised in methanol (200 mM stock concentration, kept in -20°C). They were used at the optimum concentrations 50 nM for MTG and 400 nm for TMRM, as shown in Chapter 5. The mitochondrial uncoupler carbonyl cyanide chlorophenylhydrazone (CCCP) was dissolved in DMSO and kept at -20°C. It was added to cell culture media prior to FACS analysis at a concentration of 1 μM).

2.1.8 Equipment

Trans-Blot™ cell transfer tanks, Bio-Rad Laboratories Ltd., Hertfordshire, UK.
Bio-Rad GS-800 densitometer, Bio-Rad Laboratories Ltd., Hertfordshire, UK.

Labsystems Multiskan RC Plate reader, Finland.
Zeiss microscope, Carl Zeiss MicoImaging Inc., New York, USA.
Beckman Coulter EPICS XL FACS machine, Beckman Coulter Inc., California, USA.

2.2 Cell Culture

Media and reagents were all sterile on purchase or sterilised by autoclaving or filtering with 0.2 μM filters. All cell culture work was carried out under sterile conditions in a laminar flow cabinet. Cells were discarded after passage number 25. The number of different clones per cell line used in each experiment is indicated in Chapters 3, 4 and 5.

2.2.1 ND7 Cells Stable Transfection

ND7 cells were originally generated as a fusion of primary sensory DRG neurons and neuroblastoma cells (Wood et al., 1990). ND7 cells were grown in Leibovitz's L-15 medium with 10% fetal bovine serum, 0.375% (w/v) sodium bicarbonate, 0.35% (w/v) D (+)-glucose, 2 mM L-glutamine x100, 100 units/ml penicillin and 100 units/ml streptomycin. They were routinely split 1:10 every 3 days and they were not used for more than 25 passages. Cell passage was by dislodging the cells form the culture flask surface and diluting appropriately this cell suspension with fresh media before aliquoting them in new culture flasks or plates.

ND7 cells were grown in 6-well plates at low density (approximately 10,000 cells per well) and 5 μg per well of the above α-Syn expression vectors as well as a control, empty pMAMneo vector were transfected using the calcium phosphate method (Gorman, 1986). G418-sulphate was added to the media 48h post transfection at final concentration of 800μg/ml, as determined previously by Smith et al. (1997) and Reeves et al. (1999) for ND7 cells. After 5-10 days approximately 80-100 individual G418-resistant colonies per construct were isolated, with the use of a micro pipette, under the x40 objective of a light

microscope that had been previously cleaned and placed in the tissue culture hood. G418-sulphate containing media were replaced every 2-3 days. Clones expressing either wt or mutant α-Syn were identified by means of Western blot, following the induction of α-Syn expression by the addition of 1 μM dexamethasone (Sigma) to the culture medium, unless otherwise stated. The mammalian expression vector pMAMneo contains a neomycin cassette for antibiotic selection of the transfected/resistant colonies (using G418-sulphate) and also the murine mammary tumor virus (MMTV) promoter which is hormone responsive and therefore the transgene (α-Syn) expression is induced by addition of dexamethasone in the culture medium.

2.2.2 Antibiotic Dose Response Curve

The aim of the antibiotic killing curve is to determine the minimum concentration of antibiotic such as G418-sulphate, required to cause complete cell death after 5 days of treatment. Although transfected ND7 cells were selected in previous studies with 800 μg/ml (Reeves et al., 1999), it was confirmed again that for the stock of ND7 cell used for this study the same concentration was the optimum. The selective antibiotic dose response curve was performed according to the manufacturer's instructions. Briefly, cells were plated into 6-well plates so that they would be less than 20% confluent. A range of 100 to 1200 μg/ml of G418-sulphate in 100 μg increments was tested. At 10 days from the addition of G418-sulphate in the replicate wells, cells were washed with PBS and then incubated with 0.5% methylene blue and 50% methanol for 20 min. The plates were then scored by calculating percentage of surviving colonies in the presence or absence of various G418-sulphate concentrations. The dose response curve was constructed and the concentration 800μg/ml was selected as optimal for the ND7 cells.

2.2.3 Preparation of Cells for Liquid Nitrogen Storage

Adherent cells were removed from their tissue culture flask (a 100% confluent 175cm^2 flask were be frozen down in 14 ampoules) and transferred to an appropriate sterile tube, pelleted by centrifugation at 1500 rpm, 10 min, 4°C,

the medium was removed and the pellet was re-suspended in a sterile solution composed by 90% fetal calf serum and 10% DMSO. This cell suspension was very quickly aliquoted into cryovials and stored wrapped in tissue paper in a polystyrene box in -80°C overnight, in order to freeze slowly, prior to liquid nitrogen storage.

The cells were recovered from liquid nitrogen storage by quickly defrosting them at 37°C to minimize the exposure time to DMSO. Before the ampoule of cells is removed from liquid nitrogen, 5-10ml full growth media were aliquoted into a 15ml falcon tube. The ampoule was then transferred from liquid nitrogen into a 37°C water bath to thaw. As soon as it is thawed the cell suspension was quickly transferred in the prepared media and pelleted by centrifugation at 1500-2000 rpm for 10 min at 20°C. The supernatant was carefully removed and the cell pellet was re-suspended in 5ml full growth media supplemented with the appropriate selective agent if necessary, such as G418-sulphate, and transferred in a 25 cm^2 flask and incubated as normal. The day after the medium was changed, if there was significant level of cell death, so that resistant colonies will propagate.

2.2.4 Cellular Stresses

For each experiment, three different clones stably expressing wt and mutant α-Syn were used, as well as two different control clones, unless otherwise stated. The clones used had similar protein expression levels. The use of more than one clone per cell line is an extra control measure which ensures that the response of a cell line to a death inducing stimulus is due to the expression of the gene of interest (wt or mutant α-Syn) and not due to the site of integration of the plasmid DNA into the ND7 cells genomic DNA.

Serum Removal and Retinoic Acid Addition

To induce the cells to cease dividing and undergo morphological differentiation or apoptosis, they were transferred for the indicated length of

time to serum-free medium consisting of a 1:1 mix of Dulbecco's modified Eagle's medium and nutrient mix Ham's F12 supplemented with human transferrin (5 μg/ml), bovine insulin (250 ng/ml), and sodium selenite (30 nM). *All-trans-* retinoic acid (RA) dissolved in DMSO was added to a final concentration of 1 μM to increase the proportion of cells undergoing apoptosis. This procedure has previously been shown to induce cell death by apoptosis in these cells (Howard et al., 1993). Cell death was immediately quantified by the trypan blue exclusion assay.

Dopamine Treatment

Cells were incubated for 24 h in full growth media containing 62 μM dopamine or 125 μM (3,4-dihydroxyphenethylamine; dopamine, Sigma). Cell death was immediately quantified by the trypan blue exclusion assay.

Simulated Ischaemia Followed by Re-oxygenation

Ischaemia can be simulated *in vitro* by incubating the cells in a physiological buffer containing raised level of lactic acid, high potassium, and decreased pH and inhibitors of electron transport and glycolysis. This method is based on one devised for simulating ischaemia upon cardiomyocytes (Esumi et al., 1991) and has been further adapted by Dr. Jing Zhao (The Rayne Institute for Cardiovascular Studies, United Medical and Dental Schools, London, UK).

Control Buffer:	**Ischaemic Buffer:** Control Buffer containing:
118mM NaCl	20mM sodium lactate
24mM $NaHCO_3$	12mM KCL
4mM KCL	**pH 6.2**
1mM NaH_2PO_4	
2.5mM $CaCl_2$	
1.2mM $MgCl_2$	
0.5mM EDTA	
2mM Sodium pyruvate	
10mM D-glucose	
pH 7.4	

ND7 cells were cultured in 6-well plates and induced with dexamethasone 24 h prior to ischaemia. The cells were incubated for 3 h or as otherwise indicated at 37 °C, 95% argon, 5% CO_2 with 1ml per well ischemic buffer in the hypoxic chamber (Esumi et al., 1991). Then the ischemic buffer was replaced by fresh media and the cells were incubated as normal for 24 h at 37 °C, 5% CO_2. ND7 cells were harvested and cell death was assessed after insult.

Staurosporine Treatment

Cells were incubated for 3 h in full growth media containing 1 μM staurosporine in DMSO (Sigma), then gently harvested by centrifugation and resuspended in PBS. Death was immediately assessed.

Heat Shock

Full growth media were pre-warmed to 48°C and 1ml was added per well to ND7 cells. The plates were wrapped in Parafilm and incubated in a water bath at 48°C for 20 min (lethal heat shock) (Fink et al., 1997) or 46 °C (milder heat shock). The plates were then transferred to 37°C / 5% CO_2 incubator for a recovery period of 1h. The cells were then harvested and transferred into a 1.5 ml microcentrifuge tubes and pelleted at 2,000 rpm for 10 min. The pellet was gently re-suspended in 100 μl PBS and the percentage of cell death was assessed by the trypan blue exclusion assay.

2.2.5 B130/2 BHK and M49 Cell Culture and Viral Infection

Cells were cultured in DMEM (Dulbecco's Modified Eagles Medium) with 10% foetal calf serum and 100 units/ml penicillin and streptomycin. The cells were washed with Hanks Balanced Salt Solution (HBSS), trypsinised for 2 min with 2 ml per 80cm^2 flask of 10% (v/v) trypsin in versene at 37°C, and subsequently trypsin was inactivated by addition of full growth media. The cells were then vortexed to avoid clumping and were aliquotted 1:10 in fresh growth

media. Cells were split routinely 1:10 every three days to produce stocks. M49 cells were stably transfected to express ICP4 and ICP27 proteins so that they complement the replication of deficient recombinant HSV vectors used in this study (see Chapter 4), whereas B130/2 BHK cells were complementing the virus only for ICP27. Therefore, the stocks of M49 were always grown in the presence of G418-sulphate (800 μg/ml) and Zeocin (175 μg/ml) and B130/2 BHK cells in the presence of G418-sulphate only (800 μg/ml). However, when the cells were plated for a viral infection none of the above selection agents were used. In order to propagate the virus and produce a sufficient amount to concentrate in high titre stocks for experimental use, the viral infections were carried out by using a crude viral stock to infect cells for 1 h in serum free media and then replace this with fresh media and return to the incubator. This crude stock is a cell culture of a 6-well plate frozen at -80°C and then thawed. Most of the cells were rounded up but not detached from the dish or lysed, which was an indication of optimum viral propagation in the complementing cell line. The 2 ml contents of such well (medium and cells) can be used to infect a medium (85 cm^2) flask of M49 cells. This 2 ml of media are mixed with 8 ml DMEM and 3 mM HMBA and were left in the incubator for 2 days for viral growth to occur. Below there is a full description of the virus propagation and preparation of high titre viral stocks.

2.3 HSV-based Viral Vectors

2.3.1 Propagation

Dr Marcus Wagstaff constructed and characterised the viruses used in this study. Hsp or green fluorescent protein (GFP) or LacZ cDNAs under the cytomegalovirus immediate early (CMV-IE) promoter were introduced into a disabled HSV vector [see also Wagstaff et al. (1999)]. Hsp cDNAs were Chinese hamster hsp27, human hsp40 (the hsp40 and the respective control GFP/LacZ expressing viruses were a kind gift by Dr David Hay, KCL, UK), rabbit hsp56, inducible human hsp70. The vector viruses lack the gene encoding the essential HSV immediate early protein ICP27 and were grown in

complementing B130/2 BHK cells that had been stably transfected to express ICP27 (Howard et al., 1998), allowing for lytic growth of the virus and therefore allowing us to prepare high titre viral stocks. The hsp40 virus was more disabled and details are found in Chapter 4.

As described in section 2.2.5, when the cells in a 175 cm^2 flask looked all infected by the virus, round but still attached on the dish, with minimum number of cells floating dead or having been lysed in the orange coloured medium, the contents of the flask would be frozen at -80°C and then thawed once to release virus from cells and used to subsequently infect two 175cm^2 flasks of 90% confluent M49 cells. This procedure was followed until there is a volume of crude stock sufficient to infect ten to twelve 247cm^2 plates. So, ten 175cm^2 flasks were grown and split into ten to twelve 247cm^2 plates. The plates would be 80-90% confluent the day after and they should then be infected by removing the old media and replacing with 50ml fresh full growth medium and 3mM HMBA containing the virus (i.e. some of the crude stock).The cells were infected at an m.o.i of 0.01 and 0.05. This is approximately 3 x 10^6 pfu / plate. The infection was allowed to proceed at 32°C or 37°C for four or two days respectively. HMBA was added in the culture media during infection in order to induce the immediate early genes in the absence of virion proten VP16 (VMW65), a coat protein required for infection. The recombinant viruses used in this study were grown using the respective complementing cell line, concentrated in high titre stocks as described in the section below.

2.3.2 Preparation of High Titre Stocks

After freezing and thawing once the contents of the twelve 245 cm^2 plates, they were spun at 3500rpm for 45 min in a bench top centrifuge at 4°C. The supernatant was carefully transferred into a clean receptacle. The cell pellets were re-suspended in media and were frozen at -80°C. This cell associated virus is reasonably a high titre stock which can be used for subsequent stock growing.

The supernatant was filtered once through a 0.45 μM. Filtering is necessary to minimize debris carry-over. This filtered supernatant was then poured into autoclaved pots and spun at 12,000 rpm 2 h at 4°C. The supernatant was then discarded carefully and the pots left inverted in the tissue culture hood for a few minutes. The pellet was gently re-suspended in as small volume of serum free medium as possible (100-150 μl per 250 ml pot of a filtered stock).

All virus solution was then transferred in an autoclaved sonicating vial and held in a sonicating water bath until the solution was completely homogeneous (approximately 5 x 5 seconds, briefly chilling on ice between times) and subsequently stored in 5-20 μl aliquots in -80°C overnight and thereafter transferred in liquid nitrogen. All viral vector stocks were titrated (see below) and checked for appropriate over-expression of the gene delivered in ND7 cells.

2.3.3 Titration of Virus on Complementing Cells

Cells B130/2 BHK were used for the titration of all hsp expressing viruses and GFP and LacZ viruses because they are the complementing cells for ICP27 mutant viruses, whereas M49 cells were used for hsp40 and its respective GFP/LacZ expressing control virus, both of which are more disabled viruses as they are ICP4 and ICP27 deficient, so they need to be grown and titred in the complementing double transfected stable cell line M49, expressing both ICP4 and ICP27.

Cells were plated in 6-well plates and infected when they were 80% confluent. Virus was added in the wells in a 1:10 serial dilution of from 10-2 to 10-7 ml of virus stock in 500μl serum free DMEM and the plate was incubate as normal for 1 h at 37°C in 5% CO_2. The media were subsequently replaced carefully with 2 ml fresh full growth media and then returned to the incubator for 48 h, when viral plaques were counted under the microscope.

Viruses like the hsp expressing recombinants had no reporter gene for visualisation of the virus in virally infected cells, however the titration of those

viruses was performed as described for the other viruses containing reporter genes, apart from the fact that the infected cells were observed under light microscopy and "white" plaques were counted in the cell monolayer on the culture dish. A viral plaque can be distinguished clearly as a patch of cells with distinct morphology, i.e. within a viral plaque the infected cells are rounded up but most still alive and attached on the plate. Virus expressing green fluorescent protein (GFP) as a reporter gene was detected by simply viewing the plate of infected cells under a microscope with UV light, so GFP positive cells were green fluorescent. Virus expressing the LacZ reporter gene was detected as follows: Cells were washed with PBS once and then incubated for 10-15 min with 0.05% glutaraldehyde in PBS at room temperature, followed by two washes with PBS. X-gal (4-Cl,5-bromo,3-indolyl-β-galactosidase) solution (0.2% v/v in DMSO) was dissolved in prewarmed at 37°C X-gal buffer (10mM sodium phosphate, 1mM $MgCl_2$, 150mM NaCl, 3.3 mM $K_4Fe(CN)_6$ and 3.3 mM $K_3Fe(CN)_6$ in PBS) and 2 ml of this solution was added in each well of 6-well plate. The plate was returned to the incubator for 2-12 h and then blue viral plaques (indicative of β-galactosidase activity and hence LacZ gene expression) were counted under a light microscope. The buffer was then replaced with 1ml 70% glycerol for storage.

2.3.4 Viral Infection of Cells

Prior to *in vitro* assays, ND7 cells and B130/2 BHK or M49 cells were infected at a multiplicity of infection (m.o.i.) of 10 plaque forming units (pfu) per cell for ND7 cells and 1 pfu per cell for the complementing cells. The virus stock was added in 500μl medium per well of 6-well plate or 250μl per well of 24-well plate in the respective serum free media and the plate was incubated for 1 h at 37°C / 5% CO_2. The virus-containing media were then replaced by full growth media and incubated as usual for 16-24 h prior to exposure to stress or cell harvesting for Western blotting. The transduction efficiency of the cells by these viruses was very high (90-100% of cells express the GFP/LacZ marker or hsp). It should be noted that the infection titre of the virus on cells was defined

by the m.o.i, which is the number o pfu per cell and it was estimated by titration of the viral stock.

2.3.5 Treatment of Cells after Viral Infection

For each experiment involving viral gene delivery (experiments of Chapters 4 and 5), at least two different clones stably expressing wt and mutant α-Syn were used, as well as two different control clones, unless otherwise stated. Dexamethasone was used to induce α-Syn expression at 2μM. The clones used had similar protein expression levels.

Serum Removal

Media in the cells were replaced by serum-free media (Howard et al., 1993) at 16 h post-viral infection with hsp or GFP/LacZ expressing virus. Cell death was quantified after 12 h, 24 h or 48 h from serum withdrawal as indicated in the respective experiment.

Dopamine Induced Cell Death

Virally infected cells, induced to express α-Syn with dexamethasone were incubated for 12 h, 24 h and 36 h in full growth media containing 62 μM dopamine. Cell death was immediately quantified.

Simulated Ischaemia Followed by Re-oxygenation

ND7 cells were cultured in 6-well plates in the presence of dexamethasone and virally infected 16h prior to 2 h, 3 h, or 4 h ischaemia followed by 24 h re-oxygenation (ischemia was simulated as described earlier on). Cell death was assessed immediately.

Staurosporine Treatment

Cells were virally infected 24 h prior to incubation for 1 h, 2 h or 4 h in full growth media containing 1 μM staurosporine, and cell death was immediately assessed.

Heat shock

Cells were virally infected 24 h prior to heat shock. Subsequently, they were incubated at 48°C for 20 min, followed by 1 h recovery at 37°C and cell death was immediately assessed.

Caspase Inhibitors

All caspase inhibitors were added in the media of the cells 1 h prior to treatment and thereafter during the treatment period at 10 μM. Since they were dissolved in DMSO, DMSO alone was used in the non-caspase inhibitor treated cells at all times.

2.4 Propagation, Purification and Manipulation of Plasmid DNA

All manipulations of bacterial cultures were carried out under sterile conditions, over a Bunsen burner flame to prevent contamination. All media and glassware were autoclaved before use at 120°C 10 psi for 20 min and also sterile, disposable plastic ware was used.

2.4.1 Bacterial Transformation

The *E.coli* strain XL 1-blue was used for the propagation of plasmid DNA. XL-1 blue cells were streaked on a plate of Luria Bertani (or Luria Broth) Agar [1%(w/v) Bacto®-tryptone, 1% (w/v) NaCl, 0.5% (w/v) Bacto®-yeast extract and 2% (w/v) Bacto ®-Micro agar]. A single bacterial colony was picked

from a plate 24 hours later and used to inoculate 5 ml of Luria Broth [1% (w/v) Bacto®-tryptone, 1% (w/v) NaCl, 0.5% (w/v) Bacto®-yeast extract]. This started culture was grown overnight in an orbital shaker at 37°C, 200 rpm.

To allow transformation, bacteria were made competent to allow entry of plasmid DNA.100µl of this starter culture was used to inoculate 100 ml Luria Broth and cultured in the orbital shaker until the culture had an optical density of 600nm of 0.4-0.5 units (between 4-6 hours). The cultures were then pelleted in sterile 50 ml tubes by centrifugation at 2500g at 4°C for 10min. The supernatant was carefully discarded and the bacterial pellets re-suspended in 5 ml ice-cold 100 mM $CaCl_2$ for 1 h prior to use.

Bacterial transformation was carried out by aliquoting 100µl of competent cells into chilled 1.5 ml sterile microcentrifuge tubes, containing 1 µg of plasmid DNA and incubated on ice for 30 min. The cells were then heat shocked at 42°C for 90 seconds and then immediately put on ice for 5 min. 800 µl Luria Broth was added and the cells incubated in an orbital shaker for 1h at 37°C. The cells were then pelleted at 145g for 1 min at 20°C, most of the supernatant was discarded and the bacterial cells re-suspended in the remaining supernatant of approximately 100 µl. This cell suspension was then spread onto Luria Broth agar plates containing the appropriate selection marker. Ampicillin was used at a concentration of 50 µg/ml (ampicillin stocks were made using double distilled water, filtered sterilised and stored in -20°C in light-tight containers). Plates were incubated at 37°C overnight and then stored for up to one month at 4°C.

2.4.2 Small Scale Plasmid DNA Extraction from *E. coli*

DNA was prepared using the QIAprep Mini DNA prep kit according to manufacturer's instructions from 10ml bacterial cultures (also see below for principles).

2.4.3 Large Scale Plasmid DNA Extraction from *E. coli*

Starter cultures of *E.coli* were grown in 5 ml of Luria Broth in an orbital shaker overnight at 37°C at 200 rpm and a volume of this culture (1:10,000 v/v) was used to inoculate large volumes of Luria Broth to culture overnight for plasmid preparation. Routinely, 400 ml Luria Broth with the appropriate antibiotic selection was used and the overnight bacterial culture was used for large scale purification of plasmid DNA with the Qiagen-tip 100 midi-prep kit. The purification protocol is based on a modified alkaline lysis procedure of the bacteria plasmid separation on a Qiagen resin, plasmid DNA wash, elution and isopropanol precipitation. The DNA was washed with 70% ethanol, dried briefly at room temperature and resuspended in double distilled autoclaved water to a concentration of approximately 1 $\mu g\ \mu l^{-1}$.

2.4.4 Restriction Digestion of plasmid DNA

Small scale (10µl) restriction endonuclease digestions were performed in order to characterise a plasmid or large scale digests (up to 100 µl) were performed in order to isolate a DNA fragment and subsequently ligate it in an appropriate vector. For all the enzymes used, the manufacturer's instructions were followed for incubation times, incubation temperatures, enzyme units and the type of incubation buffer.

Plasmid DNA digests were set up as follows:

DNA	0.5-1µg
Enzyme	10 units
Buffer x10	1µl
ddH2O	up to 10µl

2.4.5 Isolation and Purification of DNA Fragments from Agarose Gels

Gel electrophoresis of DNA fragments/plasmids

1 x TAE Electrophoresis buffer

	40 mM	Tris-acetate
	1 mM	EDTA pH 8.0
	0.004%	Ethidium bromide (40 ng/ml)

10x TAE loading dye

	50% (v/v)	Glycerol
	50 mM	EDTA pH 8.0
	0.25%	Xylenol orange
	20% (v/v)	50x TAE buffer

Agarose (Boehringer Mannheim, Lewes, East Sussex, UK) was added to TAE buffer (0.8-1g / 100ml) and the solution was heated in order to dissolve the agarose, then left to cool to hand temperature and 2 μl of 10 mg / ml ethidium bromide solution was added. The gel was left to solidify on a gel tray, placed in the gel tank containing TAE buffer supplemented with 200ng/ml ethidium bromide and the samples were loaded (10-40 μl samples containing 1-4 μl loading buffer). The gel was then subjected to a voltage of 100-120 Volts for 45-90 min. The gel was then exposed under ultraviolet light and photographed. The band sizes were estimated by comparing those produced by a 1 kb molecular weight marker loaded into another well of the gel, the band of interest was excised with a scalpel blade and the DNA fragment purified.

The purification of DNA fragments after DNA digestion and electrophoresis on an agarose gel or after blunt ending reaction was carried out by using QIAquick® gel extraction kit according to manufacturer's instructions.

2.4.6 α-Syn subcloning to mammalian expression vectors

Plasmid vectors with the cDNAs of human wt and A30P and A53T mutant α-Syn were kindly provided by Dr R. Jakes (Cambridge, UK). The

cDNAs were excised from those plasmids by double enzymatic digest using Hind III and Nde I restriction endonucleases to completion. Each digestion reaction was electrophoresed on an agarose gel to confirm production of the appropriate sized fragments, which were then excised and subsequently isolated and purified using the QIAquick® gel extraction kit.

The pMAMneo vector (Clontech) was digested to completion with Sal I at 37°C for 8 h (Promega buffer C was used instead of the optimum buffer D for Sal I, because buffer C is better for the subsequent blunt-end reaction) at the multiple cloning site (MCS) and was linearised. A small amount of the reaction mix was electrophoresed to confirm complete digestion and then the remaining reaction mix as well as the α-Syn cDNA fragments were blunt ended as follows: 40 μl of above digestion reaction, 1 μl 25mM dNTPs mixture (dATP, dTTP, dCTP, dGTP), buffer C, 15 units T4 DNA polymerase and ddH2O up to 50 μl were incubated at 37°C for 40 min. This reaction mix was then electrophoresed on a 0.8% agarose gel until visible, clearly separated fragments of the appropriate size were seen on the gel. The bands were excised from the gel using clean scalpels and kept in sterile microcentrifuge tubes at 4°C until purification with QIAquick® gel extraction kit, according to the manufacturer's instructions. The 5' ends of the vector were then dephosphorylated using calf intestine alkaline phosphatase CIAP (Promega) as follows: 50 μl of vector after elution from QIAquick® kit, 39 μl nuclease free ddH$_2$O, 10 μl x 10 CIAP buffer and 1 μl CIAP. The reaction was incubated at 37°C for 45 min.

The vector was again purified using QIAquick® kit, so that the buffer contents of the dephosphorylation were removed so as not to interfere with the ligation reaction. Before setting up the ligation reactions, a small volume of the DNA fragment after purification was run on an agarose gel for quantification purposes, i.e. to determine the yield of cut, blunt-ended and dephoshorylated vector and the yield of blunt-ended α-Syn cDNA fragments. The ligation reactions (giving approximately molar reactions of 9:1 fragment to vector) were assembled for each α-Syn type as follows: 9 μl α-Syn fragment, 1 μl linearised, blunt-end vector pMAMneo, 2 μl T4 DNA ligase buffer (Promega), 6 μl H_2O

and 2 μl ligase (Promega). A control reaction was also assembled by omitting the α-Syn fragment and using linearised, blunt-ended and dephosphorylated vector alone to confirm that the vector ends did not re-ligate with each other due to a possibly unsuccessful dephosphorylation reaction. The ligation reactions were incubated as suggested by the ligase manufacturer (Promega).

Competent XL1-blue *E.coli* cells were prepared by the calcium phosphate method as described in section 2.4.1. The total 20 μl volume of each ligation reaction was used in order to transform the competent cells. The following day, several individual colonies were picked from each ligation plate and were grown in Luria Broth containing 50 μg/ml ampicilin. Small scale plasmid DNA preparation was carried out as described in section 2.4.2 and approximately 500 ng of the plasmid DNA obtained were digested with the BamH I enzyme (Promega buffer E, 37°C for 2 h), in order to identify constructs with inserted α-Syn cDNA and also to identify the orientation of the insert at the MCS (see Chapter 3). The constructs were sequenced by MWG-Biotech AG, (Ebserberg, Germany) and this analysis confirmed that the plasmids contained cDNA of either wt or A30P or A53T mutant α-Syn.

2.5 Analysis of Protein Levels

2.5.1 SDS-polyacrylamide gel electrophoresis and Immunoblotting

SDS-PAGE

To resolve proteins of less than 50 kDa 12% acrylamide gels were used. Gel composition and buffers were adapted from Sambrook et al., (1989).

6 x SDS gel loading buffer:

300 mM	TrisHCl (pH6.8)
600 mM	DTT
10%	SDS
30%	glycerol
0.1%	bromophenol blue

5 x Tris-glycine electrophoresis buffer:

25 mM	Tris.HCl
250 mM	Glycine
0.1%	SDS

Composition in ml of 12% polyacrylamide resolving gels and stacking gel (total volume 10 ml, adjust accordingly for number of large gels):

	Resolving 12%	Stacking gel
H_20	3.3	6.8
30% Acrylamide	4.0	1.7
1.5 M Tris.HCl (pH 8.8)	2.5	-
1.0 M Tris.HCl (pH 6.8)	-	1.25
10% SDS	0.1	0.1
10% Ammonium persulfate	0.1	0.1
TEMED	0.004	0.01

For immunoblotting of cell lysates, 20-50 μg total protein was loaded on gels in 10-20 μl total loading volume. Equal loading of protein samples was confirmed by performing a commercially available protein assay (see below). The appropriate volume of 6 x SDS loading buffer was added and the mixture adjusted to total loading volume by addition of cell lysis buffer. Samples were denatured by heating to 95°C for 5 min prior to loading. Gel plates were cleaned with dH_20 and 70% ethanol prior to use. The resolving gel of appropriate percentage acrylamide was prepared, poured into the gel assembly and over-layered with water saturated isopropanol to create a level interface. After the resolving gel was polymerised the isopropanol was discarded and the top of the gel washed with d H_20 before the stacking gel was poured on top of the resolving gel. Samples and high range molecular weight protein standards (Rainbow Markers, Amersham International Plc, Little Chalfont, Bukcs, U.K.) were loaded on the gels. Large gels were electrophoresed in the appropriate polyacrylamide gel electrophoresis apparatus with Tris-Glycine electrophoresis

running buffer at 180Volts, 40mA per gel, for 5-6 hours or until the dye front had reached the base of the gel.

Transfer of protein

Western transfer buffer (pH 8.3):

25 mM	Tris.HCl
192 mM	Glycine
20% (v/v)	Methanol

The proteins that had been separated on the gel were then transferred to nitrocellulose filters. Nitrocellulose membrane and 3MM (Whatman) paper was cut to the same size as the SDS-page gel. A stack was formed inside the minicell consisting of three pieces of 3MM paper, the SDS-page gel, the nitrocellulose filter and another three pieces of 3MM all of which were presoaked in transfer buffer, with the nitrocellulose membrane closer to the anode. Transfer was performed at 200mA for 6h at room temperature or 100mA, overnight at 4°C in a Bio-Rad Trans-blot^{TM} Cell protein transfer apparatus.

Immunodetection

Following transfer nitrocellulose membranes were rinsed twice in 1x PBS. Non-specific binding of antibody to the membrane was blocked by incubating the membrane in 1x PBS with 4% milk (Marvel) at room temperature for 1 h or at 4°C overnight. The nitrocellulose membrane was incubated with primary antibody diluted in PBS and 4% Marvel for 2 h at room temperature with shaking or 4°C overnight. The membrane was then washed four times with PBS including 0.2% Tween 20 (Polyoxyethyenesorbitanmonolaurate) for 10 min with shaking. Secondary antibody incubation was carried out in PBS with 4% Marvel for 1 h at 4°C. Horseradish peroxidase conjugated secondary antibody dilutions were: anti-rabbit (1:3000), anti-mouse (1:2000), anti-goat (1:3000) (Chemicon). Washing was as previously described for the primary antibody. The membrane was processed using the enhanced chemiluminescence kit (ECL, Amersham International Plc, Little Chalfont, Bucks, U.K) according

to the manufacturer's specifications. Membranes were exposed on X-ray film from 3 sec to 45min, depending on the strength of the signal on the membrane and the film was then processed in a developer. Membranes were stripped by submerging stripping buffer and incubate at 50°C for one hour with occasional agitation, prior to re-blocking and re-probing.

Western blot antibody stripping buffer

100 mM	β-mercaptoethanol
2%	SDS
62.5 mM	Tris.HCl (pH 6.7)

2.5.2 Protein Assay

The amount of protein in the samples was quantified by using a commercially available kit, the bicinchoninic (BCA) Protein Assay Reagent Kit according to the manufacturer's instructions. In brief, bovine serum albumin (BSA) standards were prepared and cell lysates diluted using 10 µl lysate and 40µl loading buffer without bromophenol blue or DTT. 50 µl standards and cell lysates were added to a 96 well microplate, 200 µl BCA working reagent in each well and the plate was incubated for 1h at 37°C. The absorbance was measured at 560 nm on a plate reader (Labsystems Multiskan RC plate reader) using Labsystems Genesis Communication software, and the unknown protein concentration was calculated from the standard curve.

2.6 Cell Death Assessment Methods

2.6.1 Trypan Blue Exclusion Assay

The extent of cell death was quantified by measuring the percentage of viable cells able to exclude trypan blue by adding an equal volume of 0.4% trypan blue in phosphate-buffered saline to an aliquot of the cell suspension. Mixtures were incubated at room temperature for 5 min, and the proportion of cells able to exclude trypan blue was assessed by counting using a

haemocytometer at x 40 magnification (Weber Scientific International Ltd., UK).

2.6.2 TUNEL

DNA fragmentation was evaluated with the TUNEL (terminal dUTP nick end labelling) method. Labelling of 3'-hydroxyl ends of DNA fragments was performed using terminal deoxynucleotidyl transferase (TdT) and rhodamine conjugated nucleotides. The media of the cells were removed carefully, the cells were washed one time with PBS and fixed with 4% paraformaldehyde (dissolved in PBS by heating at 60°C and adjusting the pH to 8.0 by using 10M NaOH) for 10min at room temperature, then washed carefully three times with PBS. Then the cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min, washed two times 5 min each with PBS and incubated with the reaction mix (100µl reaction mix was used per well of a 24-well plate) for 1h in the dark at 37°C. Subsequently, the reaction mix was removed, the reaction was stopped by adding PBS with 1mM EDTA (also containing the Hoechst stain at 2 µg/ml final concentration) for 5 min at room temperature and the cells were washed two times 5 min each with PBS and stored in PBS in the dark at 4°C until microscopy.

Volumes for TUNEL labeling for one well of 24-well plate:

5x Reaction Buffer	20 µl
25mM $CoCl_2$	10 µl
25mM labeled dUTP	1 µl
20 U/ µl Td Transferase	1 µl
Double distilled H_2O	68 µl
	100 µl

All TUNEL–positive cell counts were performed in a blind fashion. A minimum of 300 cells on 3-5 random fields (of the well in which the cells had been plated) were counted. Duplicate wells were counted per independent

experiment. All the above reagents were supplied by Roche Molecular Biochemicals, GmbH (Mannheim, Germany).

2.6.3 Annexin V / Propidium Iodide Staining and FACS Analysis

In the early stages of apoptosis, changes occur at the cell surface (Creutz, 1992). One such change is the translocation of phosphatidylserine from the inner part to the outer part of the plasma membrane, so that phosphatidylserine (PS) becomes exposed at the external surface of the cell (Vermes et al., 1995). The assay used in this study is Annexin-V-FLUOS and propidium iodide (PI) (Roche Molecular Biochemicals GmbH, Mannheim, Germany). The assay utilises annexin V, a Ca^{+2}-dependent phospholipid binding protein with high affinity for PS and hence can be used as a sensitive probe for PS exposure on apoptotic cells' outer membrane. Since necrotic cells also expose PS according to the loss of their membrane integrity, apoptotic cells are differentiated from necrotic by using a DNA stain, propidium iodide (stock solution 50 μg/ml). PI will intercalate into the DNA of cells which have lost membrane integrity. Hence live cells are annexin negative and PI negative, apoptotic cells are annexin positive and PI negative and necrotic cells are annexin positive and PI positive.

One million cells were harvested in their culture media, centrifuged at 200 x g for 5 min, washed in PBS and re-centrifuged. The cell pellet was then re-suspended in 100 μl Annexin-V-FLUOS labelling solution (predilute 20 μl annexin-V-FLUOS and 20 μl propidium iodide solution in 1 ml incubation buffer) and incubated in the dark for 10-15 min at a temperature between 15-25°C. Analysis by flow cytometry (using a Beckman Coulter Epics XL flow cytometer) was carried out within 1 h from the addition of the labelling solution in the cells. At the end of the incubation time, 300μl incubation buffer (containing 10mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) was added in the 100 μl cell suspension. About 20,000 events (cells) were aquired by the flow cytometer. Cells were excited with an argon laser at 488nm. Fluorescein was detected using a 525nm band-pass filter and PI was detected

using a 675nm band pass filter. Single positive controls were used to define electronic compensation of the instrument to exclude overlapping of the two emission spectra. The events were analysed using EXPO 32 software. Histograms of apoptotic versus non-apoptotic and necrotic cells were plotted and, as seen in Chapter 4, the percentage of apoptotic cells was used in order to plot graphs summarising the experiments.

2.7 Measurement of Mitochondrial Membrane Potential Changes

Green fluorescent MTG was added into full growth media at final concentration of 50 μM and red fluorescent TMRM at 400 nM (for determination of optimum incubation time and concentration of the fluorescent probes, see Chapter 5). Cells were incubated with this medium as normal at 37°C/5%CO_2 for 1h. The media were then removed, the cells were washed with PBS, harvested in PBS in an eppendorf, transferred to polypropylene tubes and kept on ice in the dark for no longer than 1h prior to flow cytometric analysis. The protonophore (H^+ionophore) and uncoupler of oxidative phosphorylation in mitochondria CCCP (1 μM) was added to untreated, labelled cells and incubated for 15 min at room temperature prior to flow cytometric analysis as a positive control for membrane depolarisation.

At least 20,000 events were acquired using a flow cytometer. Cells were gated based on their forward activated light scatter and side scatter characteristics. A 488 nm argon laser was used to excite the fluorochromes and the emission was collected at 525 nm bandpass filter (MTG) and 575nm bandpass filter (TMRM). Data were analysed using EXPO 32 software and logarithmic scales. The mean fluorescent intensity (MFI) of each fluorochrome was used for the analysis of data. This allows quantitative measurement of the change in fluorescence of the cells which directly correlates with depolarization of the mitochondria. Information on the two fluorescent probes is provided in Chapter 5.

2.8 Statistical Analysis

Data collected from 3-5 experiments, performed using various cell clones, were pooled and analyzed by one-way analysis of variance (ANOVA) test, to identify differences between treatments (for example different hsp over-expression). One-way ANOVA is a parametric test that makes the following assumptions: the observations are independent, the sample datum has a normal distribution and the scores in different groups have homogenous variances. One-way ANOVA was performed first in order to identify if there are any significant differences between groups. Multiple comparisons were performed with *post hoc* Bonferroni *t*-test, using SPSS PC software. The significance level was set at $p<0.05$ unless otherwise indicated. *t*-Tests are not recommended for multiple comparisons because of the increased risk of a type I error, as more tests are performed. The Bonferroni test is a modified *t*-test in which the P value is multiplied by the number of tests performed on the same data. In other words, the chance of obtaining significance is less likely, so the chance of obtaining a false positive is eliminated.

CHAPTER 3

The Effects of Wild Type and Parkinson's Disease-associated Mutant α-Synuclein Over-expression in the Neuronal ND7 Cell Line.

3.1 Introduction

The alteration in the expression of a protein is a powerful tool for understanding its biological roles. Therefore, cell lines that stably overexpress particular genes of interest are widely used to study the role of proteins in a particular cellular context. This chapter describes the establishment of an *in vitro* cellular system that allowed the study of wt α-Syn and the disease associated mutants, A30P and A53T, which are stably over-expressed in the neuronal ND7 cell line. The advantages and some limitations of the system are also discussed.

The ND7 cell line was prepared by fusing post-mitotic rat dorsal root ganglion neurons and the N18Tg2 mouse neuroblastoma cell line, selecting for the drug resistance of the ganglion cells (Wood et al., 1990). This is a very useful cell line because, while proliferating indefinitely in culture, it also retains many of the characteristics of the neuronal cells and can be differentiated under certain conditions, leading to growth arrest and outgrowth of neuronal processes. It is therefore possible to establish stable neuronal cell lines that proliferate indefinitely, over-expressing the gene of interest and allowing multiple and reproducible experiments to be conducted in large cell populations. There are also well known stresses that induce ND7 cells to undergo apoptotic and/or non-apoptotic cell death (both of which occur in the brains of PD patients) and for this reason these cells provide a suitable model for the assessment of neuroprotection and for cellular toxicity studies.

As discussed in Chapter 1 (Introduction), genetic studies have revealed two mutations in α-Syn (A30P and A53T) that cause PD in a number of pedigrees with autosomal dominant inheritance (Polymeropoulos et al., 1997; Kruger et al., 1998). The A30P and A53T mutations enhance the propensity of α-Syn to aggregate *in vitro* (Narhi et al., 1999; Li et al., 2001). More recently

another dominant mutation E46K was reported in α-Syn (Zarranz et al., 2004) and also a triplication of the α-Syn gene in an American family of mixed northern European origin (Singleton et al., 2004) and in a Swedish-American family (Farrer et al., 2004). A number of studies have demonstrated that over-expression of either of the mutant A30P or A53T forms of α-Syn in mammalian cells enhances cell death in response to death-inducing stimuli whereas the wt form of the protein either has no effect (Tabrizi et al., 2000; Ko et al., 2000) or produces a protective effect against the death-inducing stimulus (da Costa et al., 2000; Lee et al., 2001).

In contrast, other *in vitro* studies have shown that over-expression of wt α-Syn in neuronal cells can increase cell death in response to certain stressful stimuli (e.g. the neurotoxin 6-hydroxydopamine, addition of the MAPK inhibitor U0126, reduced serum in medium) as observed with the mutant forms of the protein (Zhou et al., 2000; Iwata et al., 2001). Similarly, over-expression of wt α-Syn or either of the mutants in *Drosophila* results in the death of dopaminergic cells (Feany and Bender, 2000) whilst over-expression of wt α-Syn in transgenic mice similarly results in dopaminergic neuronal loss, although the mutant forms were not tested in this system (Masliah et al., 2000).

Therefore, from what was known about α-Syn at the time of the establishment of the model system presented here, it was suggested that α-Syn can enhance, reduce or have no effect on the cell death triggered by various stimuli. The work described here adds to the literature and attempts to resolve this discrepancy by demonstrating that wt α-Syn can be neuroprotective upon exposure to serum removal, presence of dopamine or heat shock at 46°C or can be neurotoxic upon exposure to ischaemic stress, incubation with staurosporine or more severe heat shock at 48°C. Therefore, depending on the stress applied to induce cell death in this neuronal cell model, wt α-Syn can be either protective or deleterious, whereas, in contrast, the two PD-mutant forms of the protein A30P and A53T are shown to consistently enhance cell death under each of the stresses that were mentioned above. These findings suggest that the

disease-associated mutations appear to convert α-Syn from a protein which modulates cell death differently under different conditions to forms which have a universal damaging effect (Zourlidou et al., 2003) and this, along with limitations of the system, is discussed in this chapter.

3.2 Establishment of Stably Expressing α-Syn Cell Lines

This section describes the establishment and characterisation of the model system. The mammalian expression vector pMAMneo (BD Biosciences, Clontech, Palo Alto, CA, USA) was chosen on the basis that it has been successfully used in the past in different studies using ND7 cells (Reeves et al., 1999). In ND7 cells also similar plasmid vectors with the dexamethasone-inducible murine mammary tumor virus (MMTV) promoter have been used to establish stable, inducible cell lines over-expressing the Brn-3a transcription factor (Smith et al., 1997). This vector allowed inducible, high protein expression in mammalian cells and simultaneous selection for cells with the inserted gene using G418-sulphate, since the vector also contains a neomycin resistance cassette.

3.2.1 Molecular Subcloning

As seen in Figure 3.1.a the α-Syn cDNAs were excised from their original vectors (a kind gift by R. Jakes, Cambridge, UK) and subcloned in pMAMneo vector (for description see Chapter 2). Figure 3.1.b shows constructs with both orientations of α-Syn fragments which were obtained. For sense orientation, the bands seen on an agarose gel are 917 bp, 2688 bp and 5146 bp long. For antisense orientation, bands of 1205 bp, 2688 bp and 4858 bp were obtained. In addition, the enzymes Nhe I and Xho I were used subsequently to excise the inserts out of the vector (Figure 3.1.c). The constructs were commercially sequenced at both orientations, using appropriate primers in order to verify that the right mutations exist in the DNA sequences.

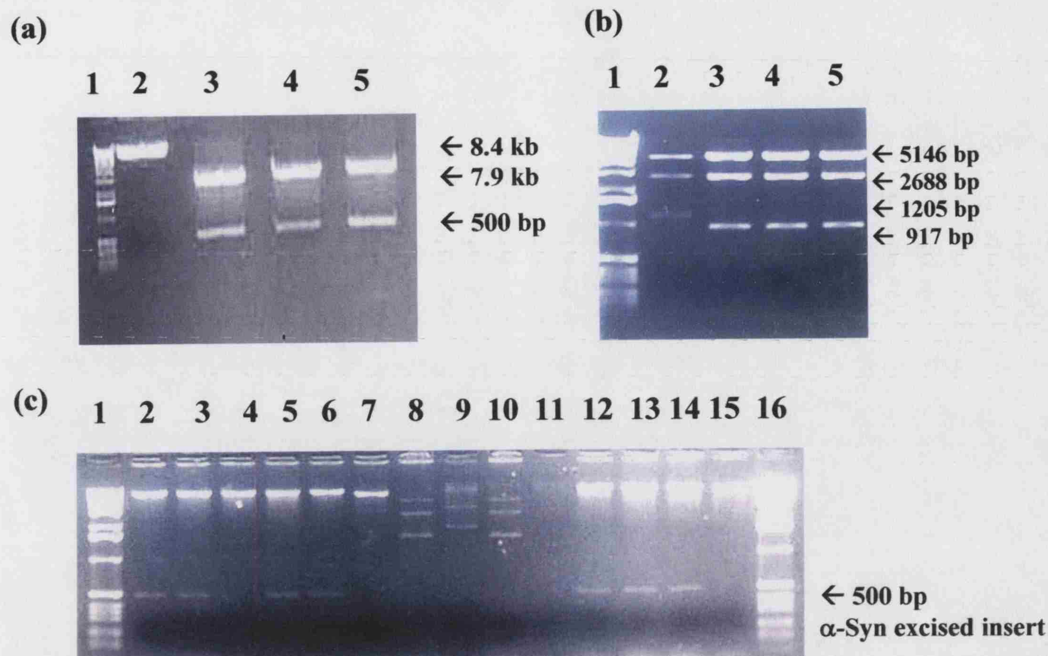


Figure 3.1 Sub-cloning of α-Syn cDNA under the dexamethasone responsive MMTV promoter of the pMAMneo vector for gene expression in mammalian cells.

(a) Digested plasmids run on 1% agarose gel in order to isolate and purify the appropriate DNA fragments. Lane 1: 1 kb DNA ladder. Lane 2: The linearised 8.4 kb pMAMneo vector, resulted from enzymatic digestion with Sal I. Lanes 3 to 5: The original vectors (provided by Dr R. Jakes) digested with Hind III and Nde I in order to excise the 500 bp wt, A30P and A53T α-Syn cDNA, respectively.

(b) BamH I digestions of pMAMneo vectors with the inserted α-Syn at the Sal I site, in order to differentiate between sense and antisense orientation of the inserts.

Lane 1: 1 kb DNA ladder. Lane 2: Antisense α-Syn clone (fragment sizes: 4858 bp, 2688 bp, 1205 bp). Lanes 3-5: Clones with sense orientation of the inserted wt and mutant α-Syn cDNA (fragment sizes: 5146 bp, 2688 bp, 917 bp)

(c) Identification of positive clones and reconfirmation of insertion of α-Syn cDNA in the pMAMneo vector by excision of the inserts by Nhe I and Xho I double enzymatic digestion. Lanes 2, 3, 5, 6, 12, 13 and 14 contain clones with the 500 bp α-Syn insert (the linearised vector can be seen as the 8.4 kb fragment).

3.2.2 Transfection of Plasmid DNA Constructs to Establish Stable Cell Lines.

ND7 cells were grown in cell culture dishes and the above α-Syn expression vectors as well as a control, empty pMAMneo vector were transfected using the calcium phosphate method. G418-sulphate was added to the media 48h post transfection and after 5-10 days approximately 80-100 individual G418 resistant colonies per construct were isolated (for method details see Chapter 2).

3.2.3 Clonal Selection and Growth.

Approximately ten to twenty different colonies were screened from each construct for α-Syn expression, by means of Western blot, following the induction of α-Syn expression by the addition of 1μM dexamethasone to the culture medium for 24 h, as will be described in detail in the next section (3.3.1). Higher concentrations of dexamethasone were also used to check the inducibility and the expression levels of α-Syn but the concentration of 1 μM was routinely used unless otherwise stated. This concentration resulted in sufficient α-Syn expression without the potential effects that higher doses might have and it has also been used in the past in ND7 cells in a different study (Reeves et al., 1999). Finally, only three out of the various screened colonies were chosen for further analysis, based on their similarity of α-Syn expression levels and their normal growth properties which are also described in the next section (3.3.2).

Morphological examination of the colonies that were selected for each construct revealed that in the majority of colonies there were no significant changes in the shape or size of cells. These cells, which also proliferate normally, were subsequently grown to produce large stocks. However, a small number of the colonies that had been transfected with the A30P and A53T α-Syn constructs ceased dividing and extended long or shorter neuronal processes,

indicative of differentiation to a mature neuronal-like phenotype, whereas the control vector and wt α-Syn expressing cells were morphologically similar to non-transfected ND7 cells (data not shown). This phenomenon may be due to various reasons. It can be related to possibly high levels of overexpression of a mutant - potentially toxic - protein that leads to cell death or simply to cellular stress that leads to growth arrest and differentiation. Alternatively, in the case where this phenomenon is not related to stresses imposed by the overexpression of a toxic protein, it can be due to the insertion of the DNA sequence, which was transfected, into a region of the cellular genome that could interfere with the normal growth / proliferation of the cell. Nevertheless, the above explanations remain speculative as to why relatively less A30P and A53T α-Syn clones survived after colony selection as compared to control-empty vector-cells.

3.3 Identification and Characterisation of Over-expressing Clones

3.3.1 Western Blotting

Following colony selection, the clones that were growing normally were screened for expression of α-Syn by means of Western blotting as described in section 2.5.2. As seen in Figure 3.2, clear overexpression of the 19 kDa α-Syn was confirmed in various clones that were incubated for 24 h with 1 μM dexamethasone. The concentration of each of the protein samples was determined by performing a protein assay, which is described in section 2.5.2, and equal protein amounts were loaded in each lane.

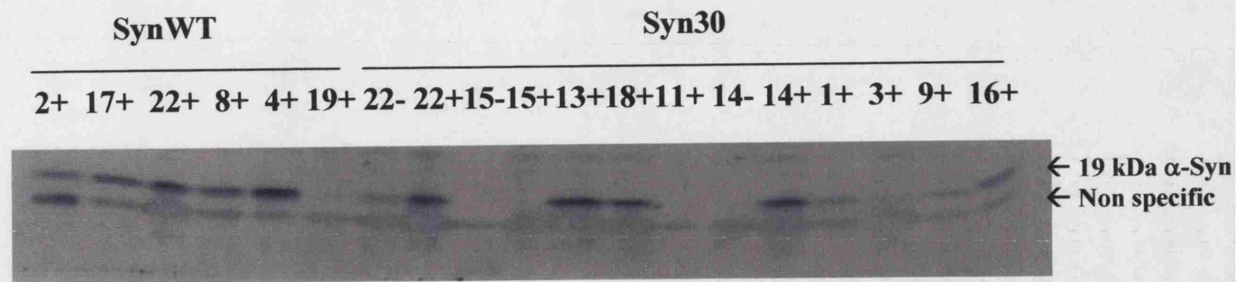
Figures 3.2.a and 3.2.b show the levels of α-Syn expression of some of the clones selected as compared to the control (empty vector) cells. In Figure 3.2.a in particular, clones Syn30.15 and Syn30.22 are two examples of the inducibility of the cell lines by dexamethasone (0 μM is vehicle treatment and + is 1 μM dexamethasone). The rest of the clones in Figure 3.2.a and 3.2.b are

dexamethasone treated and exhibit variable levels of α-Syn expression. Therefore, clones WT.2, WT.17, WT.22, WT8, WT.4, WT.7 and WT.12 express wt α-Syn, whereas WT.19, WT.1, WT.3, WT.10 do not express at all and WT.5 does not express significant amounts. From the A30P mutant α-Syn-transfected clones 30.22, 30.13, 30.18 and 30.14 express significant amounts of A30P α-Syn, whereas 30.16, 30.1 and 30.9 clones have lower expression levels and 30.3, 30.15 and 30.11 do not express at all under the same treatment conditions with dexamethasone. The A53T mutant α-Syn clones 53.3, 53.5 and 53.8 also express α-Syn whereas clones 53.7 and 53.2 do not.

It was also shown that the expression of α-Syn depends on the dexamethasone concentration used; hence no transgene expression was observed in the absence of dexamethasone whereas α-Syn expression was induced after incubation with dexamethasone (Figure 3.2.c). This illustrates the inducibility of the MMTV promoter of the vector used. Clones were selected on the basis of showing good inducible expression (Figure 3.2.c and d).

Figure 3.2

(a)



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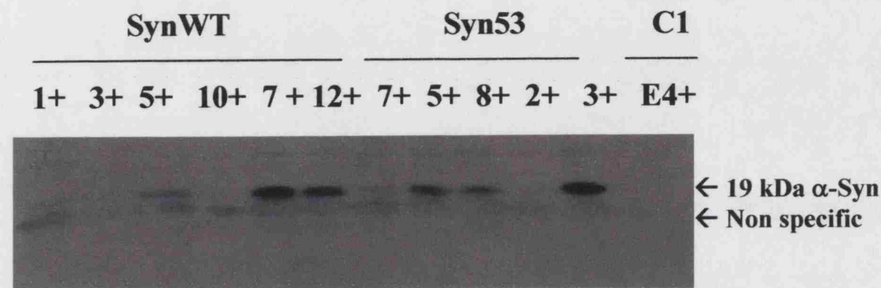
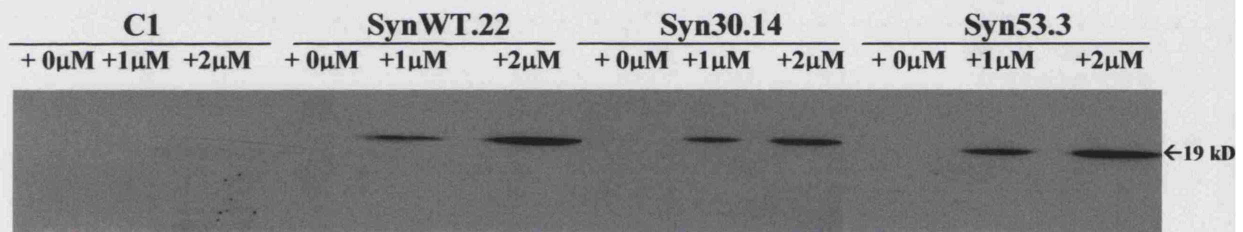
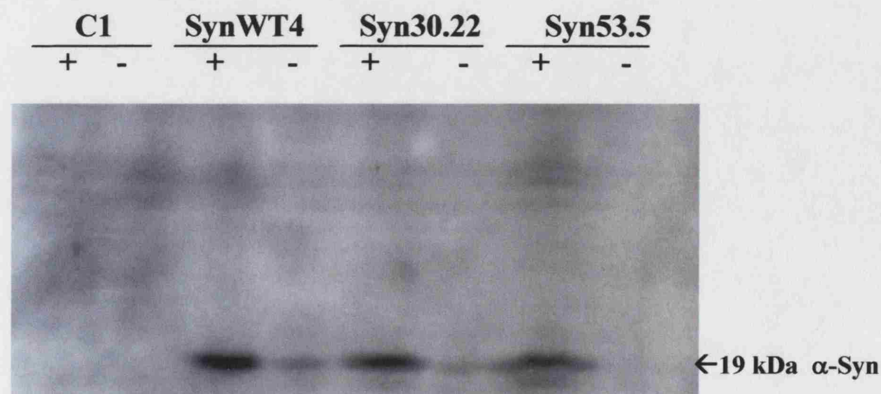


(c)



(d)



<u>Figure 3.2</u> Western blot analysis of various stably transfected clones with wt or mutant α-Syn constructs.

Cells were treated for 24h with dexamethasone in order to induce α-Syn expression, then harvested and electrophoresed on a 12% SDS-polyacrylamide gel, followed by western blot analysis as described in section 2.5.1 and by using a mouse monoclonal anti-α-Syn-antibody. There is a non-specific (NS) band appearing on the blot just below the 19 kDa band of α-Syn. The numbers that label each lane of the blots below are the individual clone numbers.

(a) Clones expressing wt (2, 17, 22, 8, 4, 19) or A30P mutant (22 , 15 , 13, 18, 11, 14, 1, 3, 9, 16) α-Syn. All clones prior to electrophoresis were treated for 24h with 1 μM dexamethasone unless they are labelled as – dex and are vehicle-only treated.

(b) Clones expressing wt (1, 3, 5, 10, 7, 12) or A53T (7, 5, 8, 2, 3) α-Syn or empty vector (E4), all treated for 24 h with 1 μM dexamethasone.

(c) Clones which were used subsequently for all experiments are treated here with vehicle-only (0 μM dex) or 1 μM or 2 μM dexamethasone for 24 h in order to assess their degree of inducibility. However, cells were routinely incubated with 1 μM dexamethasone for all subsequent experiments, unless otherwise stated. C1 is transfected with empty vector.

(d) Some other clones that were incubated with 1 μM dexamethasone (+) or vehicle treated (-). The 19 kDa band indicates α-Syn expression, however in this blot it is clear that there is some leaky expression (see - lanes) of α-Syn by the MMTV promoter of the pMAMneo vector which was introduced into the cells.

3.3.2 Growth Curves

Having established inducible cell lines expressing wt, A30P and A53T α-Syn, and before performing further experiments, it was thought to be important to choose representative clones from each type exhibiting normal growth properties in the absence of dexamethasone (the inducer of gene expression). The alteration of the levels of expression of a gene in cell lines, among other effects, can also have a significant effect on the growth properties of the cell line, i.e. the clonally selected lines can have different growth rates and this effect might be due to over-expression of this particular gene or might be the result of non-specific effects caused by the genomic insertion.

Therefore, three inducible colonies of each α-Syn construct, that had been identified to over-express similar amounts of α-Syn, were selected and their growth curves were constructed. Cells were plated out at 1 x 10^4 cells per 9.6 cm^2 culture dish and were grown for up to 72h in full growth media without dexamethasone in the presence of G418-sulphate. At each time point (12h, 24h, 48h, 72h) the viable cells of duplicate wells were quantified by the trypan blue assay and three independent experiments were performed.

The growth curves of clones C1, SynWT1, Syn30.1 and Syn53.1 are shown in Figure 3.3 and they are shown here as representative examples of the growth properties of the different cell lines. Although statistical analysis was not performed to assess the significance of the small differences between different cell lines, it is still notable that all cell lines are growing at very similar rates and hence they are suitable for further analysis.

Three clones per construct were therefore selected on the basis of their similarity of α-Syn over-expression, normal appearance of cellular morphology and normal growth properties. Two but not three control clones (stably transfected with the neomycin resistance pMAMneo vector alone) were used for subsequent experiments because of accidental loss. The responses of these

representative clones to a variety of stressful conditions are described in the following section.

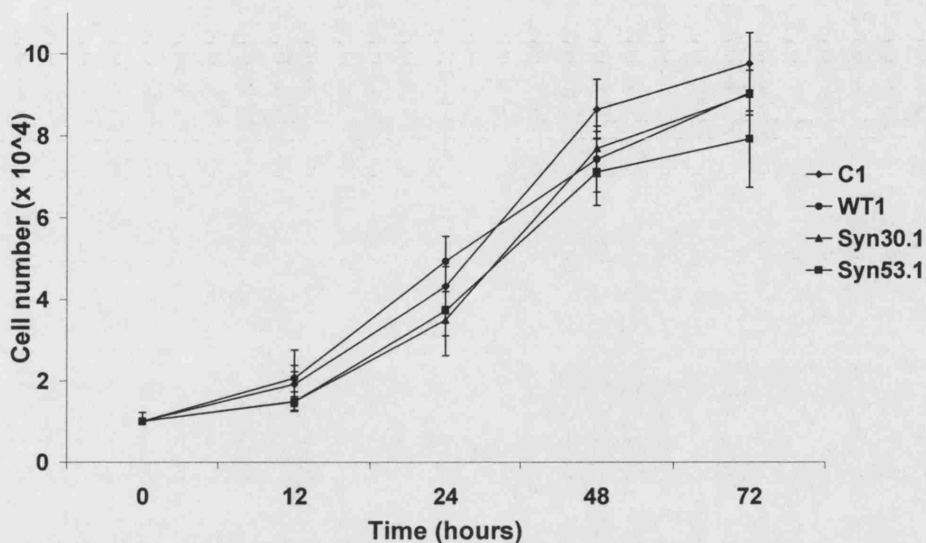


Figure 3.3 Cellular growth curves of clones C1 (vector only), SynWT1, Syn30.1 and Syn53.1 in the absence of dexamethasone.

Cells (1 x 10^4) were plated on 9.6 cm^2 wells in full growth medium supplemented with 800 μg/ml G418-sulphate for up to 72h. Cell viability was assessed by the trypan blue assay at 12 h, 24 h, 48 h and 72 h after seeding, counting duplicate wells each time. Values are the means of three independent experiments ± SD.

3.4 Responses of Cell Lines to Several Death-inducing Stimuli

The following sections describe the responses of multiple clones of control, wt α-Syn or either of the PD-mutants that have been subjected to various death inducing cellular stresses, such as serum removal, dopamine, simulated ischaemia, staurosporine and heat shock (for conditions see section 2.2.4 of Chapter 2).Various studies have utilised stable cell lines expressing toxic or other genes of interest and by using different agents tested their responses to stresses, as seen later in the Discussion. The relevance of the stresses used here to α-Syn and Parkinson's disease is explained in each section below. Overall, three different clones from each wt or mutant α-Syn were used and two different control vector only clones, as explained in section 3.3.3. Cell death was quantified by the trypan blue exclusion assay as described in section 2.6.1. Unless otherwise indicated, C1 is clone Empty vector 4 (E4), C2 is Empty vector 6 (E6), SynWT1 is clone WT.4, SynWT2 is clone WT.22, SynWT3 is WT.8, Syn30.1 is clone 30.14, Syn30.2 is clone 30.22, Syn30.3 is clone 30.13, Syn53.1 is 53.5, Syn53.2 is 53.11 and Syn53.3 is 53.3.

3.4.1 Serum Removal

Serum is essential for maintenance and growth of cell lines and its withdrawal from cell culture media is known to lead to cell death and/or differentiation (for example see Poser et al., 2003). In our system, serum removal is used as a known apoptotic and differentiating stimulus for ND7 cells (Howard et al., 1993). It can be extrapolated and potentially compared to the deprivation of neurones from various survival signals in the brain, a situation which is linked to neurodegeneration. It is therefore used as a potentially relevant stress to the disease.

To investigate whether the over-expression of both wt and mutant α-Syn in neuronal cells has any effect on a stimulus that induces apoptosis, cells were incubated for various lengths of time with appropriate medium without foetal calf serum and in the presence or absence of all-*trans* retinoic acid (RA)

(vehicle was DMSO and was used as a control in the absence of RA). The response of ND7 cells to serum free conditions have been well characterised in the past. ND7 cells undergo either morphological differentiation into a mature neuronal phenotype with dendrite-like processes or programmed cell death (Howard et al., 1993).

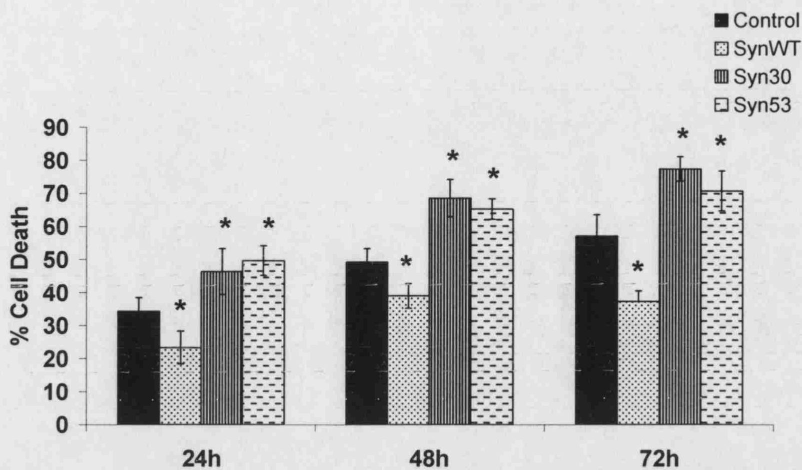
As seen in Figure 3.4 three time points were initially chosen in order to assess the extend of cell death of the cell lines at 24 h, 48 h and 72 h from serum withdrawal in three independent experiments utilising one clone per cell line. The response of the various cell lines was profound at 24 h or 48 h from serum removal but overall the cell death was lower compared to 72 h and the protective effect by wt α-Syn was marginal, although the differences in cell death were statistically significant ($p<0.05$), especially at 24 h (Figure 3.4). Overall, α-Syn cells have lower levels of cell death compared to control cells but both mutant forms exhibit higher levels of cell death and these differences are statistically significant ($p<0.05$) In contrast, a more prolonged stress of 72 h serum withdrawal leads to higher levels of cell death and hence the protective effect of wt α-Syn becomes more profound. Retinoic acid was always used under serum removal conditions as it increases the percentage of cells that undergo apoptosis. Finally, due to the higher differences in cell death the time point of 72 h from serum withdrawal was chosen and was subsequently tested in the various cell lines in order to confirm the above findings in multiple clones.

Figures 3.5 and 3.6 compare the percentage of total cell death of control cells with wt and mutant α-Syn expressing cells. Figure 3.6 summarises the pooled data obtained by two control and three wt or mutant clones in five independent experiments, whereas in Figure 3.5 the responses of the individual clones and the small variation in response to stress between the clonally selected lines are illustrated.

In a smaller number of experiments the cell lines were incubated for the same time period in the presence of normal growth medium and there was

no marked difference between cell death in the various cell lines, instead the cells proliferated normally (described in section, 3.3.3).

The data in Figure 3.6, which summarise the data in Figure 3.5, clearly show that both the mutant forms of synuclein produce enhanced cell death in response to serum removal and this is statistically significant ($p<0.05$). This effect is observed both in the presence or absence of retinoic acid but is particularly marked in the presence of retinoic acid. Interestingly however, the cells over-expressing wt α-Syn showed greatly reduced cell death compared to the cells over-expressing the mutant forms. Moreover, they also showed significantly reduced cell death compared to the control cells which was particularly marked in the presence of retinoic acid ($p<0.05$). These effects were also observed when individual cell lines of each type were compared (Figure 3.5).



<u>Figure 3.4</u> **Effect of different periods of serum withdrawal on cell death in control cells or cells expressing wild type or mutant forms of α-Syn.**

Levels of total cell death in one clone of either control vector, wt, mutant A30P or A53T α-Syn expressing cells that were exposed to serum deprivation for 24 h, 48 h or 72 h in the presence of 1 μM retinoic acid. Dexamethasone (1 μM) was added in the medium of all cell lines to induce α-Syn expression 24 h prior to treatment and thereafter until cell death assessment by the trypan blue assay. Values are the means ± SD of three independent experiments (n=3) and significant differences in means were calculated after one-way ANOVA and *post hoc* analysis using a Bonferroni test. Asterisk (*) indicates significant difference in death compared with control cells receiving the same treatment ($p<0.05$). Control cells are treated, vector only transfected ND7 cells (black bars).

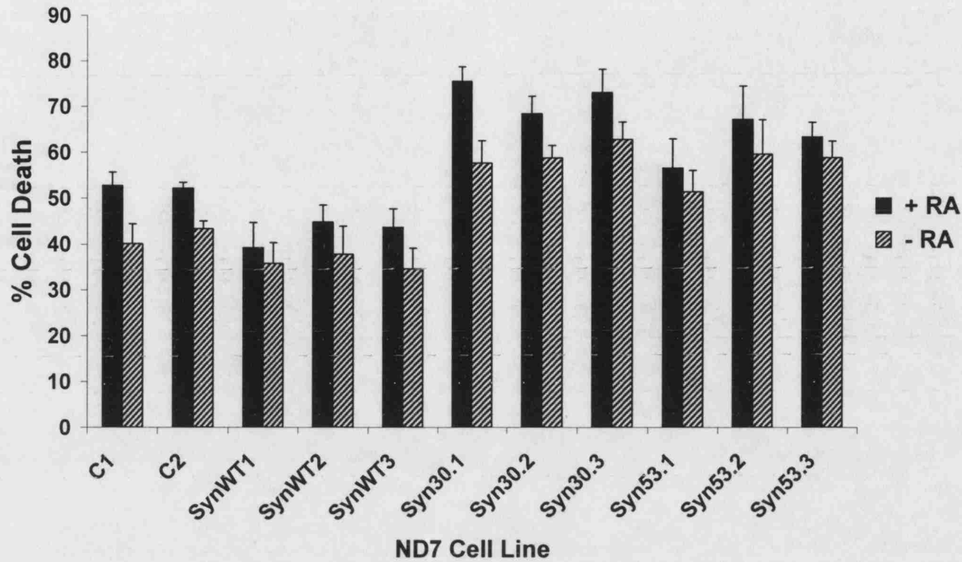


Figure 3.5 Effect of serum withdrawal induced cell death in various clones expressing wild type or mutant α-Syn.

Levels of total cell death in multiple control, wt and mutant A30P or A53T α-Syn expressing ND7 clones that were exposed to serum deprivation for 72 h in the presence (black bars) or absence (striped bars) of 1 μM retinoic acid. Values are the means ± SD of five independent experiments. Statistical analysis was not performed for the individual clones, but it was performed for the pooled results as shown in Figure 3.6.

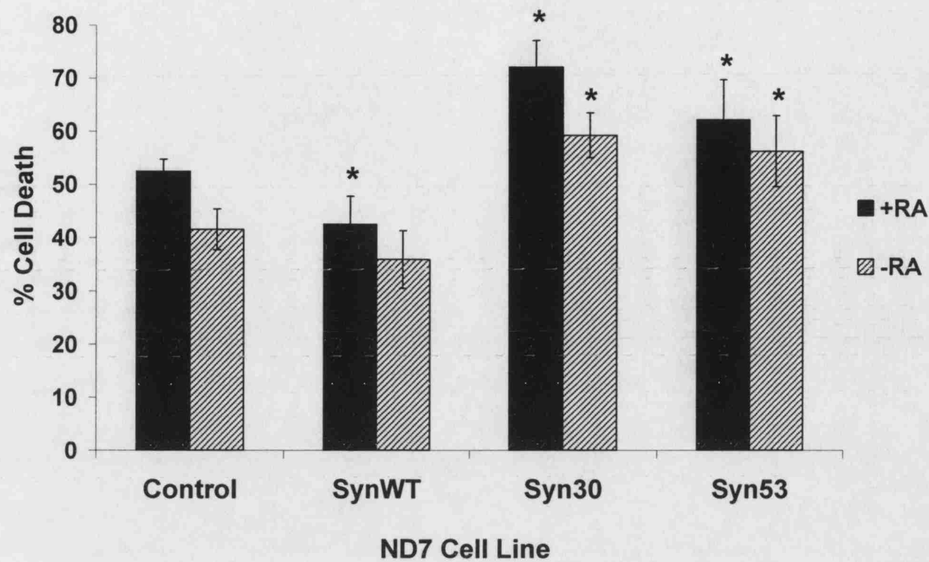


Figure 3.6 Effect of serum withdrawal induced cell death in ND7 cells expressing wild type or mutant α-Syn.

Pooled data from different clones showing the levels of cell death measured by trypan blue exclusion assay in control, wt and mutant A30P or A53T α-Syn expressing ND7 cells exposed to serum deprivation for 72 h in the presence (black bars) or absence (striped bars) of 1 μM retinoic acid. Values are the means ± SD of five independent experiments using three different clones from each wt or mutant α-Syn expressing cell lines (n=15) and two different clones from the control cell lines (n=10). Asterisk (*) indicates significant difference in death compared with control cells ($p<0.05$). Control cells are treated, vector only transfected ND7 cells.

3.4.2 Dopamine

Dopamine (DA) induced neurotoxicity is thought to be involved in the pathogenesis of PD (see review in Chapter 1). It is produced in the substantia nigra and passes messages between the striatum and the substantia nigra. In the case of PD, when the cells of the substantia nigra deteriorate, there is a corresponding decrease in dopamine which is produced by these cells and less dopamine causes striatal neuronal dysfunction, preventing the patient to be able to direct motor function.

At the molecular level, reactive oxygen or nitrogen species are generated in the enzymatic oxidation by monoamine oxidase or auto-oxidation of an excess amount of DA inducing neuronal damage and/or apoptotic or non-apoptotic cell death which can be prevented by various intrinsic and extrinsic antioxidants (see review by Lotharius and Brundin, 2002). DA and its metabolites, containing two hydroxyl residues, are cytotoxic in dopaminergic neurons mainly due to the generation of highly reactive DA and DOPA quinones. These may irreversibly alter protein function through the formation of 5-cysteinyl-catechols on the proteins. Formation of DA quinone-alpha-synuclein consequently increases cytotoxic protofibrils and the covalent modification of tyrosine hydroxylase by DA quinones. The melanin-synthetic enzyme tyrosinase in the brain may rapidly oxidize excess amounts of cytosolic DA, hence preventing slowly progressive cell damage by auto-oxidation of DA and maintaining DA levels.

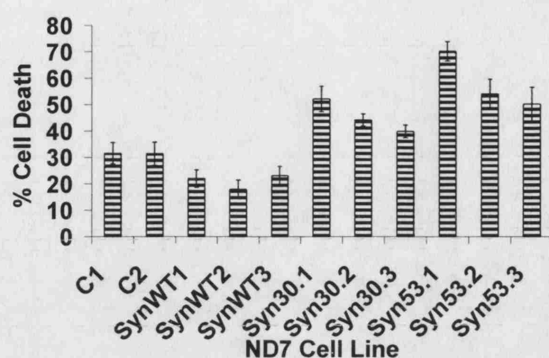
Here, dopamine was used as relevant to the disease stress, in order to assess the response of the various cell lines to oxidative stress, and then compare it with the findings from the use of other stresses.

After 24 h incubation with 62 μM or 125 μM dopamine, the results obtained were similar to those observed following serum removal. They are illustrated in Figure 3.7.a, in individual clones, and also summarised in Figure 3.7.b. Again, wt α-Syn showed significantly reduced cell death compared to

control or cells over-expressing the mutant forms ($p<0.05$). However, both mutant α-Syn expressing cells are more susceptible to dopamine induced cytotoxicity (Figure 3.7.b).

A higher concentration of dopamine (125 μM) at 24 h was also tested in one clone per cell line. At this concentration, dopamine seemed to be more toxic and the same trend is observed, i.e. wt α-Syn reduces cell death in contrast to the disease-associated mutants that enhance it (Figure 3.8).

(a)



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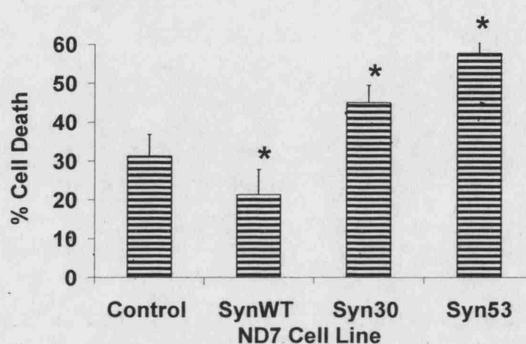


<u>Figure 3.7</u> Effect of dopamine induced neurotoxicity in various clones expressing wild type or mutant α-Syn.

(a) Levels of total cell death in multiple control, wt and mutant A30P or A53T α-Syn expressing ND7 clones. Cells were exposed to 62 μM dopamine for 24 h. Values are the means ± SD of three independent experiments. Statistical analysis was not performed for the individual clones but it was performed for the pooled results as shown in Figure 3.4.2.b. **(b)** Pooled data from different clones showing the levels of cell death measured by trypan blue exclusion assay in control, wt and mutant A30P or A53T α-Syn expressing ND7 cells exposed to 62 μM dopamine for 24 h. Values are the means + SD of three independent experiments using three different clones from each wt or mutant α-Syn expressing cell lines (n=9) and two different clones from the control cell lines (n=6). Asterisk (*) indicates significant difference in death compared with control cells ($p<0.05$). Control cells are treated, vector only transfected ND7 cells.

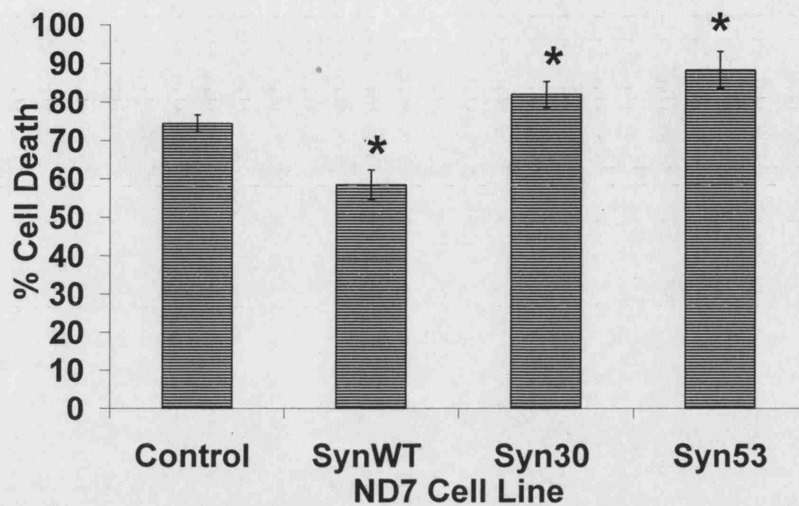


Figure 3.8 Effect of higher dopamine concentration on cell death of ND7 cells expressing wild type or mutant α-Syn.

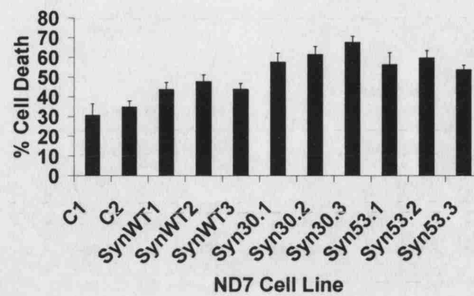
Levels of total cell death in clones C1 (control vector only), SynWT1, mutant Syn30.1 and Syn53.3. Cells were exposed to 125 μM dopamine for 24 h and death was assessed with the trypan blue assay. Values are the means ± SD of three independent experiments. Asterisk (*) indicates significant difference in death compared with control cells ($p<0.05$).

3.4.3 Cell - line responses to Simulated Ischaemia.

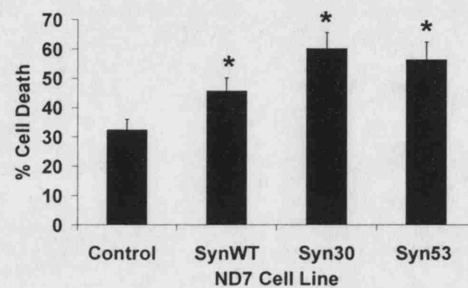
The next cellular stress that was used was simulated ischaemia, as it had not been used in any PD-relevant model system before and it is a stress that was used in the past to induce ND7 cell death and demonstrate the neuroprotective role of hsps (Wagstaff et al., 1999). Briefly, the cells were plated in 6-well plates, induced to over-express synuclein by dexamethasone treatment for 24 h, and thereafter during the treatment period, which was 2 h, 3 h or 4 h of ischaemia followed by a 24 h reoxygenation period. From all the above time points tested one only was selected as representative, for practical reasons, in order to perform multiple experiments with the various clones, and this was the 3 h ischaemia with 24 h reoxygenation which induced cellular stress that lead to significant differences in cell death in ND7 cells. The control buffer (pH 7.4) was used at all times and the cell death observed was always between 5-10% in the various cell lines, but those values were not plotted and therefore those data are not presented in the figures below. It is clear, however, that the cytotoxic effect of the ischaemic buffer is due to its additional components and the decreased pH.

As seen in Figure 3.9 the cells over-expressing either of the mutant forms of α-Syn were again more sensitive to 3 h simulated ischaemia followed by 24 h reoxygenation when compared to control cells and this effect was observed both for the three individual clones of each type (Figure 3.9.a) and for the pooled data ($p<0.05$) (Figure 3.9.b). Most interestingly however, enhanced cell death was also observed in all three cell clones over-expressing wt α-Syn compared to the control clones containing only the empty expression vector ($p<0.05$) (Figure 3.9.b). In addition to the data on all the cell lines with one period of ischemia, Figure 3.10 illustrates the results of three experiments that were performed utilizing one clone per cell line, in order to investigate the response of varied periods of ischaemia, followed by the same reoxygenation period. These data suggest that there is the same trend between cell lines - in terms of increased cell death in wt α-Syn cells and further increase in the mutants compared to control.

(a)



(b)



<u>Figure 3.9</u> **Effect of simulated ischaemia on cell death in various clones expressing wild type or mutant α-Syn.**

(a) Levels of total cell death in multiple control, wt and mutant A30P or A53T α-Syn expressing ND7 clones. Cells were exposed to 3 h ischaemia followed by a 24 h reoxygenation period and cell death was assessed by the trypan blue exclusion assay. Values are the means + SD of three independent experiments. Statistical analysis was not performed for the individual clones but it was performed for the pooled results as shown in panel b. The error bars represent standard deviations of the means calculated from the means of three counts for each sample n (n=3). **(b)** Pooled data from different clones showing the levels of cell death measured by trypan blue exclusion assay in control, wt and mutant A30P or A53T α-Syn expressing ND7 cells exposed to 3 h ischaemia and 24 h reoxygenation. Values are the means + SD of three independent experiments using three different clones from each wt or mutant α-Syn expressing cell lines (n=9) and two different clones from the control cell lines (n=6). Asterisk (*) indicates significant difference in death compared with control cells ($p<0.05$). Control cells are treated, vector only transfected ND7 cells.

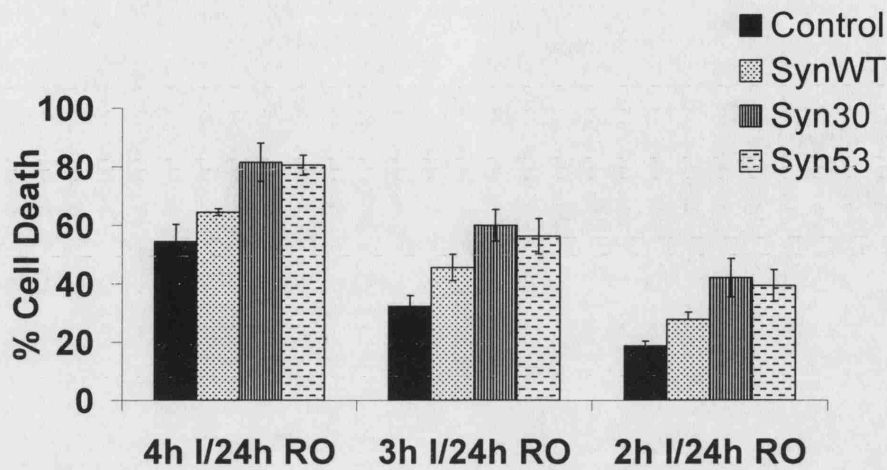


Figure 3.10 **Effect of varied periods of simulated ischaemia on cell death of ND7 cells expressing wild type or mutant α-Syn.**

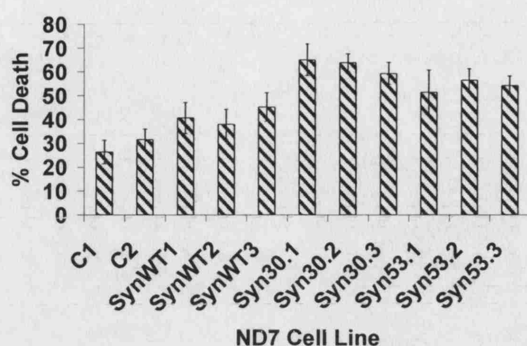
Control, wt and mutant α-Syn cell lines were exposed to 2 h, 3 h or 4 h of ischaemia followed by 24 h of reoxygenation and cell death was immediately assessed by the trypan blue assay. Again, wt α-Syn as well as both mutants enhance cell death with different efficiencies at all time points, although statistical analysis was not performed in order to assess the significance of these differences. One clone per construct was used in three independent experiments and values are the means ± SD.

3.4.4 Staurosporine treatment

Staurosporine is one of the most potent cell permeable general inhibitors of protein kinases, including protein kinase C, the isoforms of which are serine/threonine kinases involved in signal transduction pathways that govern a wide range of physiological processes. Staurosporine shows a strong cytotoxic effect on the growth of various mammalian cells and has been extensively used as an apoptosis inducing agent in various studies, including those relevant to the model system described here (da Costa et al., 2000; Lee et al., 2001; Alves et al., 2002; Marx et al., 2003).

Total cell death was assessed following exposure of the various clones to 1 μM staurosporine (higher concentrations were highly toxic) which also induces apoptotic cell death in a variety of different cell types (see references above and also (Porcelli et al., 2003; Darios et al., 2003). Cells were plated in 6-well plates, treated with 1 μM dexamethasone for 24 h prior to stress and then incubated for three hours in full growth media containing 1 μM staurosporine and 1 μM dexamethasone. The trypan blue exclusion assay was performed immediately after. In this case, the cell death was significantly enhanced in the cells expressing wt α-Syn (41.3%) compared to control cells (28.8%) and further enhanced in the cells expressing the two mutants (62.9% and 54% for A30P and A53T respectively) (Figure 3.11.a and b, for individual clones and for the pooled data respectively) ($p<0.05$ versus control in all cases).

(a)



(b)

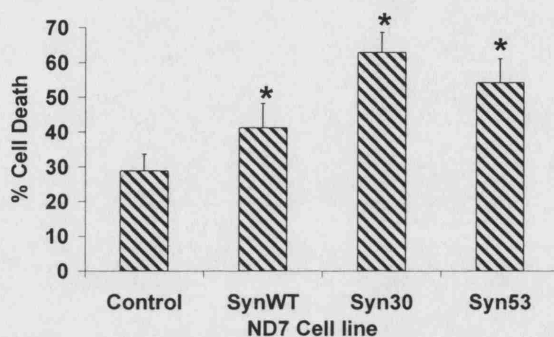


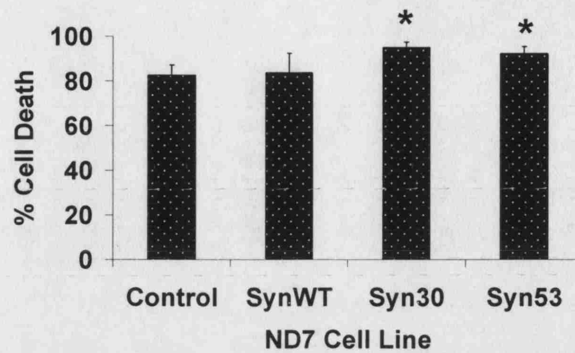
Figure 3.11 Effect of the apoptosis inducing agent staurosporine on cell death in various clones expressing wild type or mutant α-Syn.

(a) Levels of total cell death in multiple control, wt and mutant A30P or A53T α-Syn expressing ND7 clones. Cells were incubated for 3 h with 1 μM staurosporine and total cell death was assessed by the trypan blue exclusion assay. Values are the means ± SD of three independent experiments. Statistical analysis was not performed for the individual clones, but it was performed for the pooled results as shown in panel b. **(b)** Pooled data showing the levels of cell death in control, wild type and mutant A30P or A53T α-synuclein expressing ND7 cells exposed to 1 μM staurosporine for 3 h. Values are the means + SD of three independent experiments using three different clones from each wt or mutant α-Syn expressing cell lines (n=9) and two different clones from the control cell lines (n=6). Asterisk (*) indicates significant difference in death compared with control cells ($p<0.05$). Control cells are treated, vector only transfected ND7 cells.

3.4.5 Heat Shock

The effect of this particular stress on ND7 cells has been previously studied and is known to induce cell death whereas exogenous hsp over-expression can confer neuroprotection (Wagstaff et al., 1999). We therefore extended this study to investigate whether α-Syn overexpression had any effect during this stress. One clone of each cell line was therefore exposed to 20 min heat shock at 48°C (severe heat shock), followed by 1 h recovery at 37°C following which the cell death was assessed by the trypan blue assay (Wagstaff et al., 1999). In response to this severe stress, no protection was conferred by wt α-Syn. However, both mutants led to an increase in cell death compared to control vector cells, as seen in Figure 3.12.a. In the case where the stress was milder heat shock (46°C for 20 min and 1h recovery), there was statistically significant decrease in cell death in the wt α-Syn cells compared to control cells ($p<0.05$), while the mutants were again deleterious, leading to significantly higher cell death compared to control vector cells ($p<0.05$) (Figure 3.12.b).

(a)



(b)

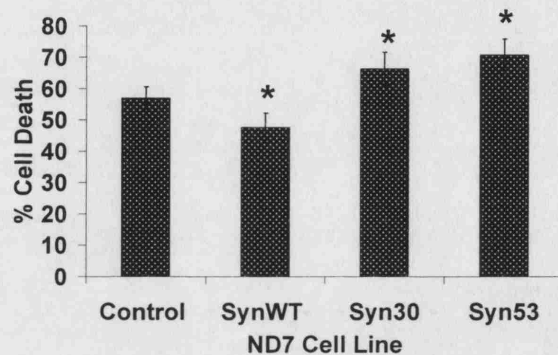


Figure 3.12 Effect of heat shock on cell death in ND7 cells expressing wild type or mutant α-Syn.

The levels of total cell death in control, wt and mutant A30P or A53T α-Syn expressing ND7 clones were assessed by the trypan blue exclusion assay. Cells were incubated in a 48°C **(a)** or 46°C **(b)** water bath for 20 min and then left in the 37°C incubator for 1h prior to total cell death assessment by trypan blue. One clone was used per cell line in three experiments. Values are the means ± SD of three independent experiments. The error bars represent standard deviations of the means calculated from the means of three counts for each sample (n=3). Asterisk (*) indicates significant difference in death compared with control cells ($p<0.05$).

3.5 Discussion

The findings presented in this chapter describe the establishment and characterisation of an *in vitro* model system where wt α-Syn and the disease-associated mutant forms are over-expressed. It was shown that the response of the cell lines to various death-inducing stimuli depends on both the type of stress and the form of α-Syn which is over-expressed.

There was a statistically significant reduction in cell death in ND7 cells over-expressing human wt α-Syn as compared to control cells (stably transfected with the vector alone) when cell lines were challenged by serum withdrawal in the presence of retinoic acid, after incubation with various dopamine concentrations or exposure to mild heat shock. However, following simulated ischaemia or treatment with staurosporine, wt α-Syn over-expression was observed to result in an increase in the levels of cell death as compared to control cells and these differences in cell viability were statistically significant. In the case of the two mutant forms of α-Syn, cell death was always enhanced compared to control cells and the differences in cell death between control and mutant cell lines were statistically significant for all the stresses that were tested.

The above results suggest a deleterious function of both mutant forms of the protein. When the cell lines expressing the two mutant forms of α-Syn were compared against each together there was no clear trend as to which mutant form is the most severe as the increase in cell death depended on the stress that was applied. In general it could be said that the A53T mutant was shown to be slightly more severe during dopamine induced cytotoxicity (small but significant difference compared to A30P cells after 24 h incubation with 62 μM dopamine). This might be due to a differential gain of function by the different mutants, making the A53T mutant unable to perform α-synuclein's potential role in dopamine handling and /or storage, whereas wt α-Syn protects ND7 cells since it might have such a role (see also discussion below). Under any of the other stresses the A30P mutant was occasionally slightly more toxic

that A53T, but such differences in cell death were not statistically significant. Overall, the above data support the proposed hypothesis that the disease-associated α-Syn mutations confer a gain of function and /or loss of a protective function of this protein, leading to deleterious effects for the cells. The data also suggest that, at least in this particular neuronal cell line, wt α-Syn can modulate cell death differently depending on the stressful condition, whereas both mutant forms uniformly enhance susceptibility to cell death.

The stresses that have been used here have been widely used by others and they are relevant to PD in a manner that serum deprivation simulates deprivation of survival signals to neurons, dopamine induced oxidative stress is relevant to the dopaminergic neuronal environment and staurosporine is a well studied apoptotic agent (a microbial alkaloid with an indolocarbazole structure - a potent PKC inhibitor that also suppresses the activities of many other protein kinases). The use of simulated ischaemia has not been reported before but it is a physiological stress that was used to challenge ND7 cells and induce cell death, therefore it allows assessment of the effect of α-Syn on ND7 cell survival (Wagstaff et al., 1999). Heat shock has been used in the past to demonstrate the protective role of hsp70 and hsp90 in ND7 cells under such conditions (Mailhos et al., 1994) and here it has been used in order to assess the effects that α-Syn has under these conditions, although it is unclear whether the cell death resulting is apoptotic on non-apoptotic. Since apoptosis as well as non-apoptotic cell death are known to be implicated in neurodegeneration (reviewed in Chapter 1, Introduction), all the stresses utilized in this study have been previously shown to induce programmed cell death in ND7 cells or other neuronal cell lines and were thus chosen based on their suitability for this experimental approach.

Cell viability in the experiments described in this chapter was assessed throughout by the trypan blue exclusion assay. However the assessment of apoptotic cell death by TUNEL staining and annexin V / propidium iodide staining and FACS analysis will be presented as part of the further investigation of these phenomena within Chapter 4.

As mentioned in the introduction of this chapter whilst α-Syn is a protein with an unclear physiological role, there is an accumulating body of evidence to support the idea that the mutations appearing in the few familial cases of PD are deleterious in most model systems studied. The system described in this chapter utilized a well characterized neuronal cell line in which the exogenous expression of α-Syn forms has a differential effect on neuronal cell survival upon distinct challenges that lead to mainly apoptotic cell death. It has been previously shown that the apoptosis induced by serum withdrawal appears to represent the response of cycling cells to a differentiation-inducing stimulus to which they are unable to respond correctly (Howard et al., 1993). It might be possible that α-Syn, whose expression is characteristic of differentiated neuronal cells, may allow cells which would otherwise die, to differentiate correctly (although this hypothesis was not tested by examining the surviving differentiated cells) whilst not having a protective effect against events such as oxidative stress-free radical generation, which occur during simulated ischaemia followed by re-oxygenation.

The results of the present study show that wt α-Syn, but neither of the mutants, protects cells from dopamine induced oxidative stress. ND7 cells are not dopaminergic but here we show that exogenous dopamine has a significant effect on their survival, which depends on the form of α-Syn they express. A53T mutant is the most sensitive to this challenge and it is possible to speculate that this might be due to loss of the potential wt α-Syn protective function under this challenge. For instance, Hashimoto et al. (2002) showed that α-Syn protects cells from oxidative stress by inactivation of JNK via increased expression of JIP-1b/IB1, although we did not investigate such possibilities in our model. The impairment of dopamine storage and the role of mutant α-Syn have been recently reviewed and supports our observations in that the mutations and particularly the A53T could abolish a potential dopamine storage assisting role of α-Syn, hence leading to free cytosolic dopamine accumulation and therefore severe oxidative stress (Lotharius and Brundin, 2002).

A number of *in vivo* studies also somewhat support the findings presented here. Exposure of mice to paraquat or MPTP is followed by an α-Syn up-regulation that appears to be part of a neuronal response to toxic insults (Manning-Bog et al., 2002; Vila et al., 2000). Manning-Bog et al. (2003) found that over-expression of wt or A53T α-Syn in mice protects against paraquat-induced neurodegeneration and also that these animals have increased hsp70 in the chatecholaminergic brain regions, where α-Syn is over-expressed (hsp70 co-localises with α-Syn in neuronal cell bodies within the substantia nigra). This report utilises the paraquat-treated mouse model and the findings, although different with ours, partly agree with a protective *in vivo* role of wt α-Syn. As reviewed in the Introduction (Chapter 1) and by Orth and Tabrizi (2003), there are various transgenic and knockout mice of α-Syn and generally both the mutant forms of α-Syn are toxic for dopaminergic neurons.

Furthermore, (Tanaka et al., 2001) showed that *in vitro*, PC12 cells expressing mutant A30P α-Syn showed increased sensitivity to apoptotic cell death when treated with sub-toxic concentrations of lactacystine, a well known exogenous proteasome inhibitor. Apoptosis was accompanied by mitochondrial depolarization and elevation of caspase-3 and -9, and was blocked by cyclosporine A. Such observations are in agreement with our observations, i.e. A30P mutant α-Syn is toxic for the cells under all stresses (assessed by trypan blue assay here and as described in Chapter 4 by TUNEL and Annexin V/PI).

The findings presented here, suggest that wt α-Syn can protect ND7 cells against some death-inducing stimuli, whilst having a damaging effect in the response to other stimuli. Wt α-Syn has been suggested to have either no effect on the response to cell death (Ko et al., 2000; Tanaka et al., 2001), a protective effect against death-inducing stimuli (da Costa et al., 2000; Lee et al., 2001) or to enhance cell death (Iwata et al., 2001; Saha et al., 2000; Zhou et al., 2000). The results of the present study can be compared with those reported by other groups (da Costa et al., 2000; Alves et al., 2002; Lee et al., 2001). In the above studies wt α-Syn protects from staurosporine induced death (da Costa et

al., 2000; Alves et al., 2002) whereas we see increased death during this stress. However, Lee et al. (2001) found that wt α-Syn protects from serum withdrawal but not staurosporine induced death which is in agreement with our findings. Mutant α-Syn, does not protect under various stresses including serum withdrawal (Lee et al., 2001), which is in agreement with the present study.

In addition, mutant α-Syn has been shown to increase susceptibility to dopamine toxicity in the human dopaminergic SH-SY5Y cell line and human-derived HEK293 cells, whereas the wt had no protective effect (Junn and Mouradian, 2002; Tabrizi et al., 2000). The contrast between the effect of α-Syn on death induced by serum withdrawal or oxidative stress is of particular interest since both these stimuli have been shown to induce up-regulation of endogenous α-synuclein *in vitro* (Choi et al., 2001; Tanji et al., 2001) and *in vivo* (Manning-Bog et al., 2002). So, the results presented in this thesis may resolve the discrepancies in the literature by supporting the hypothesis that the effect of wt α-Syn depends on the stress (see Zourlidou et al., 2003 in the appendix).

In this Chapter it was shown that altered levels of α-Syn or expression of PD-associated mutants have an effect on ND7 cell survival in response to a number of stresses, but not under normal growth conditions. The fact that the cell lines were clonally selected might be one reason why there is no apparent difference in growth or survival under normal culture conditions whereas only upon stress the cell lines respond differently. It is known that the dexamethasone-responsive expression system that was utilized here is slightly leaky due to the presence of endogenous steroid molecules in the tissue culture medium and would therefore result in the expression of low levels of α-Syn in the absence of dexamethasone. However, western blot analysis revealed that levels of α-Syn protein were almost undetectable, in the clones used for the experiments, in the absence of dexamethasone. One potential problem that arises with all experiments involving the construction of stable cell lines is variation in both the levels of expression of the transgene and the final phenotype of the different clones. Both phenomena are likely to be a result of

site of integration of DNA sequence of the transgene within the cell genome. However, this was compensated by utilizing multiple clones per construct to validate the data obtained for each stress as is standard practice when studying stable cell lines.

Another limitation of this system is the fact that ND7 cells are not primary cells or dopaminergic, which would be more relevant to the disease associated mutations, although there are numerous studies describing interesting effects with α-Syn in even non-neuronal or non-mammalian systems (for review see Chapter 1). ND7 cells are derived from a fusion of rat dorsal root ganglion neurons and mouse neuroblastoma cells and they retain a lot of the characteristics of neurons. They are useful due to the fact that they are well characterized, there are numerous stresses known to induce them to undergo apoptotic or non-apoptotic cell death, they grow well in culture and when differentiated they have characteristics of neurons (Wheatley et al., 1992). As a consequence of the above, an additional limitation is the fact that the ND7 cell line is not human, whereas the cDNAs of α-Syn expressed are human. This could complicate the analysis of data; however, there have been numerous studies where trans-species experiments have been successful in the past (for examples see Darios et al., 2003 and Katzir et al., 2003).

Therefore, having established the described effects of wt or mutant forms of α-Syn here, the following chapter attempts to answer to the question of whether α-Syn-associated toxicity can be reduced or even abolished by exogenous over-expression of various hsps with the help of highly efficient HSV-based viral vectors. The experimental evidence provided in the next chapter suggests a novel neuroprotective role of hsp27 in the model of α-Syn-associated toxicity, which was described here.

CHAPTER 4

The Neuroprotective Effects of Heat Shock Proteins in an *In Vitro* Model of α-Synuclein-Induced Toxicity

4.1 Introduction

The previous chapter dealt with the establishment and characterisation of an *in vitro* model of α-Syn-induced toxicity, where (wt) α-Syn has either a protective effect or enhances cell death, depending on the specific death inducing stimulus, whereas over-expression of either A30P or A53T mutant α-Syn always enhances cell death, regardless the cellular stress applied. Having established these effects, I next chose to study the potentially neuroprotective effect of various heat shock proteins (hsps) in this model system, utilising a highly efficient HSV-based gene delivery system (Wagstaff et al., 1999). This section introduces some of the literature findings that support a protective role of hsps in neurodegenerative disorders, however, a more extended review on hsps is provided in Chapter 1.

Neuroprotection is defined as an intervention that prevents, stops or slows neuronal degeneration and disease progression. Many factors have been involved so far in the aetiopathogenesis of Parkinson's disease (PD), providing candidate targets for a neuroprotective therapy. Among the possible neuroprotective approaches are vitamin E, ascorbate, iron chelators, NMDA receptor antagonists, co-enzyme Q10, creatine, Jun Kinase inhibitors and heat shock proteins (Olanow et al., 2004). The assessment of the protective effect of individual hsps had not been studied before in the context of α-Syn-related toxicity. This chapter provides data that support a novel way by which α-Syn-related toxicity and cell death can be mitigated by the over-expression of heat shock proteins, in particular by hsp27 (Zourlidou et al., 2004). As a result of these studies, hsp27 is suggested to have a novel neuroprotective role that awaits confirmation in *in vivo* systems.

It is known that aggregation and abnormal protein deposition are characteristics of many neurodegenerative disorders. Neurons appear to be particularly vulnerable to the toxic effects of mutant or misfolded protein and in fact their first line of defence against it is the molecular chaperones. Over-expression of hsp70, a chaperone upregulated in stress responses that refolds

misfolded protein, can reduce the toxicity of both mutant polyQ-containing proteins and α-Syn (Warrick et al., 1999; Auluck et al., 2002). These studies with α-Syn and hsp70 were performed in transgenic *Drosophila* and the hypothesis has not yet been tested in mammalian systems. In addition, some hsps (for example hsp70, hsp40 and hsp27) are found in Lewy bodies (the neuropathological hallmarks of PD) suggesting a possible link between hsps and α-Syn (a primary component of Lewy bodies) *in vivo* (Auluck et al., 2002; McLean et al., 2002).

Hsps have very important functions, including acting as molecular chaperones in physiological conditions or in response to stress (Jakob and Buchner, 1994; Kiang and Tsokos, 1998). A recent review by Parcellier et al., (2003a) highlights the role of hsps in: (1) intracellular "house keeping" by helping folding nascent or misfolded proteins, (2) interacting with components of the programmed cell death machinery, upstream and downstream of mitochondrial events and (3) having an important role in selected protein degradation under stress by the proteasome. As reviewed in Chapter 1, it is only in the last few years that some reports have started to elucidate the mechanisms by which hsps modulate cell death. For instance, hsp27 prevents caspase-dependent cell death (reviewed by Concannon et al., 2003 and Parcellier et al., 2003a) and hsp70 functions as a major cellular defence molecule against protein aggregation (Glover and Lindquist, 1998) and prevents both caspase-dependent and caspase-independent apoptosis. It also became apparent that such anti-apoptotic functions of hsps may be distinct from their role as chaperones. In addition, a "protein triage" machinery has been proposed in which hsps and the ubiquitin/proteasome system co-operate and modulate apoptosis, but further studies are necessary for a better understanding of the underlying mechanisms.

Hsp27 expression is known to protect cells from death under various stresses (Wagstaff et al., 1999; Benjamin and McMillan, 1998) and to interfere with apoptosis by preventing caspase activation by direct interaction with cytochrome c released from mitochondria (Bruey et al., 2000). Over-expression of hsp27 protects against apoptotic cell death triggered by various stimuli such

as oxidative stress, staurosporine, ligation of the Fas/Apo-1/CD95 death receptor, and cytotoxic drugs. Various distinct mechanisms have been proposed to account for such an anti-apoptotic activity. Hsp27 could increase the anti-oxidant activity of cells by decreasing reactive oxygen species (Mehlen et al., 1996a). In a study by Wyttenbach et al. (2002), hsp27 suppresses polyQ-mediated cell death, protecting from an increase in reactive oxygen species caused by huntingtin, in a cellular HD model. In a more recent study hsp27 was shown to rescue pathological hyperphosphorylated tau-mediated cell death in the human cortical neuronal cells HCN2A and also to bind to pathological hyperphosphorylated tau but not non-phosphorylated tau in human AD brain (Shimura et al., 2004). The formation of such a complex leads to a decrease of its concentration by assisting its degradation and dephosphorylation.

However, no studies have used over-expression of hsps to examine their protective effect against α-Syn-mediated cell death in a mammalian system. In the present study, herpes simplex viruses were utilised in order to assess whether hsp27, hsp70, hsp56 or a constitutively active mutant form of HSF-1 have any protective effect against various stresses applied in an *in vitro* system in which α-Syn or the disease-associated mutants, A30P and A53T α-Syn, are stably over-expressed. Over-expression of (a) hsp40 in combination with hsp70 or (b) hsp27 in combination with hsp70 or (c) constitutively active mutant HSF-1, were also tested in this system for some of the stresses. Cells were subjected to serum removal, dopamine, simulated ischaemia, staurosporine and heat shock in order to compare their response to these distinct death stimuli, in the presence or absence of over-expressed hsps and at multiple time points.

These stresses lead to mainly apoptotic cell death that was assessed by the trypan blue assay, TUNEL staining and annexin V / propidium iodide (PI) staining and FACS (fluorescence-activated cell sorting) analysis (see discussion of this chapter for further analysis). The trypan blue assay is an easy and relatively reliable and inexpensive method of total cell death assessment and it is based on the fact that cell membrane permeability to trypan blue dye depends

on membrane integrity loss of non-viable cells. TUNEL is a commonly used technique for the detection of apoptosis that utilises TdT-mediated X-dUTP nick end labeling (where X is a suitable conjugated label such as rhodamine or FITC), although its specificity is not absolute, since DNA fragmentation is common in other types of cell death. Annexin V / PI staining was used as an additional method of assessing apoptosis in our system. Developed by Vermes et al. (1995), it is a method that is based on the change of the architecture of the plasma membrane during apoptosis, independent of the initiating trigger that leads to almost complete absence of PS in the outer leaflet of the plasma membrane. Annexin V binds, in the presence of calcium ions, to phospholipid membrane when PS is present. Annexin V as a FITC conjugate in combination with PI (PI enters necrotic cells and intercalates with DNA) can distinguish between viable (annexin V- PI -), apoptotic (annexin V+ PI-) and secondary necrotic cells (annexin V+ PI +) or cellular debris (annexin V- PI +).

In order to test the potentially neuroprotective role of various hsps in this model system, it was important to utilize a highly efficient hsp gene delivery method. HSV-based viral vectors were therefore chosen to be used in the present study. These viral vectors have been developed specifically for delivery to neuronal cells and their use has been reported in both *in vitro* and *in vivo* studies (Wagstaff et al., 1999; Brar et al., 1999; Kalwy et al., 2003). It is out of the scope of this section to discuss the suitability and effectiveness of HSV-based gene delivery, as this was described in section 1.7.2 of Chapter 1 and it has been reviewed by Coffin and Latchman, 2001; Davidson et al., 2003 and Goins et al., 2004.

Briefly, the viruses used in the present study, offer a high efficiency means of specific gene delivery to primary neuronal cells and to ND7 cells (90-100% transduction efficiency). The viral vector that was used in the present study was constructed in our laboratory by Dr Marcus Wagstaff and was named 17+pR19 (Wagstaff et al., 1998). The transgenes (Chinese hamster hsp27, rabbit hsp56, human inducible hsp70, β-galactosidase or GFP) in this type of virus are driven by the human CMV-IE promoter which was inserted immediately

downstream of the LAT P2 promoter. The expression cassette (Figure 4.1) was inserted into the non-essential 2 kb LAT region of the HSV-1 genome of an IE2-deleted mutant HSV-1 strain (IE2 is the essential gene that encodes ICP27; HSV-1 strain 17+; GenBank™ accession number HE1CG) (Wagstaff et al., 1999). ICP27 and ICP4 are the IE genes that are absolutely essential for viral replication. ICP27 was therefore deleted in this virus type. As a result, these viruses were designed to produce a less cytopathic, latent, non-replicating viral mutant tested to be suitable for gene delivery (Wagstaff et al., 1999; Kalwy et al., 2003). Those viruses were grown in B130/2 cells which complement for ICP27 (Howard et al., 1998).

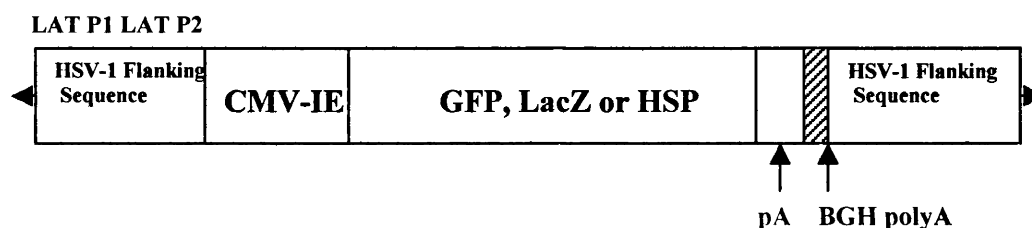


Figure 4.1 The pR19 cassette utilised in the 17+ virus constructs.

This expression construct was inserted in the non-essential LAT region of the IE2 deleted mutant HSV-1 DNA. The CMV-IE promoter is driving the transcription of the transgene and the bovine growth hormone polyadenylation sequence. The use of the LAT P1 and LAT P2 promoters with the CMV-IE promoter is thought to aid expression during viral latency in neurons.

The hsp40 virus was constructed by David Hay (Medical and Molecular Genetics, GKT School of Medicine, King's College London) which was a more disabled virus named 1764 27- 4- . This virus lacks ICP4 and ICP27, is deleted for the endogenous LAT P2 regions (to prevent recombination with the inserted LAT P2-containing expression cassette pR20.5 outside the LAT region) and also has ORFP deleted and a mutation inserted in VP16 and ICP34.5. ICP4 is the major viral transactivator protein and deleting it

dramatically reduces the expression of most of the rest approximately 80 HSV-1 genes. This viral mutant was developed because the ICP4 deletion mutant was still highly cytotoxic due to products of regulatory IE genes ICP0, ICP22, ICP27. So, it was thought to be more effective to also delete ICP27 and mutate VP16 (which mutation abolishes transactivation of IE genes but does not influence its structural role), as reported by Lilley et al., 2001. The pR20.5 cassette consists of a central LAT P2 element flanked by two heterologous promoters (CMV-IE and RSV) arranged in a back-to-back orientation, allowing simultaneous expression of hsp40 (under the Rous Sarcoma Virus - RSV-promoter) and GFP (under CMV-IE promoter). This cassette was inserted into a plasmid so that it allows insertional inactivation of the gene encoding vhs, which has been shown to have a role in pathogenesis and latency (Strelow and Leib, 1995). Since VP16 was mutated, this virus type was grown in complementing cell lines (M49), which over-express ICP4, ICP27 and the equine HSV-1 VP16 homologue, in media supplemented with HMBA, which also complements for VP16 (for an account of those viral vectors see Lilley et al., 2001).

Having an available highly efficient HSV-1-based vector system for hsp gene delivery in neuronal cells, and also having established and characterised a series of inducible, stable cell lines expressing various α-Syn forms, proved very useful in the present study. As shown in this chapter, it allowed us to test the hypothesis of whether α-Syn-related toxicity in mammalian neuronal cells subjected to various stresses can be suppressed by individual or by combinations of heat shock proteins (hsp27, hsp40, hsp70, hsp56) or by over expression of a constitutively active HSF-1 mutant, that induces expression of various hsps in ND7 cells.

The following sections of this chapter briefly describe the infection method of the cell lines by the viruses, the characterisation of heat shock protein expression in the cell lines and then describe the results obtained after applying each death stimulus and quantifying cell death. Lastly, the significance of the findings is discussed, along with advantages and limitations of the system.

4.2 Viral Infections of Cells

4.2.1 Complementing Cell Lines of Viruses

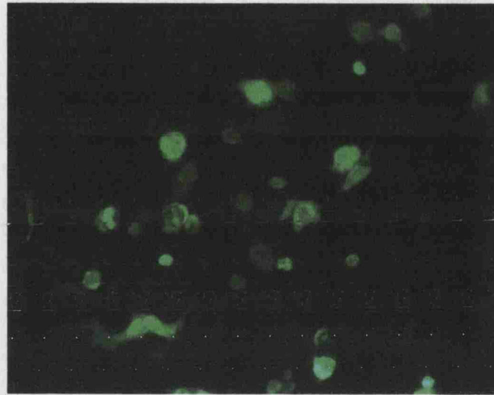
The viral stocks used in this study were grown and prepared as described in Chapter 2, section 2.3. B130/2 BHK cells (for 17+27- virus type) or M49 cells (for 1764 27-4- virus type) were used for this purpose, as they are complementing to the disabled viruses and assist lytic viral growth (Howard et al., 1998; Thomas et al., 1999). This allows the production of large amounts of the virus that can subsequently be purified and concentrated in high titre stocks. Each time a new high titre stock was prepared, western blotting analysis was carried out after infection of the non-complementing ND7 cells in order to reconfirm over-expression of the transgene.

4.2.2 Microscopy of Virally Infected ND7 Cells and Western Blot Analysis

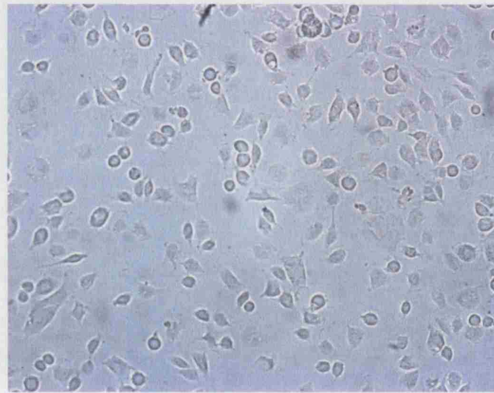
ND7 cells were infected as described in section 2.3.4 of Chapter 2 and then they were left for 24 h to allow over-expression of the transgene prior to treatment. Nevertheless, GFP was visible even earlier than 12 h post-infection, and continued being expressed until the end of all types of treatment. Examples of infected cells are shown in Figure 4.1. The 17+pR19 type of HSV-vector has been previously shown to successfully infect ND7 cells and produce high amounts of hsps (Wagstaff et al., 1999). As seen in Figure 4.2, ND7 cells infected with either a control GFP, hsp27 or hsp70 expressing virus exhibited high expression of the transgene after 24h. The GFP virus allows quick assessment of the percentage of cells expressing GFP but the hsp expressing viruses, apart from the hsp40 virus that co-expresses GFP, do not contain a visual marker and therefore it is assumed that infecting the cells with the same amount of virus as with the GFP virus at the same m.o.i, will result in the same transduction efficiency as they are the same type of viruses. For the experiments where annexin V / PI were used to assess cell death, a LacZ virus (of the same type as the GFP and hsp viruses) was utilised instead of GFP because the GFP fluorescence would interfere with the FITC conjugated annexin V readings.

Hsp56 as well as HSF-1 viruses were also shown to over-express the respective transgenes, as determined by western blotting (Figure 4.4.a and b). The constitutively active mutant HSF-1 expressing virus was shown in the past (Wagstaff et al., 1998) to induce, in the absence of stress, the expression of various hsps in ND7 cells, such as hsp70 and also hsp27 and hsp32 at a smaller degree, whereas hsp90, 56 and 60 levels remain unchanged. The hsp40 virus (which is more disabled than the 17+pR19 viruses) that was used in a small number of experiments expresses relatively high amounts of hsp40 in ND7 cells, as shown by western blotting, using a specific to human hsp40 anti-hsp40 antibody, (Figure 4.4.c), but only when the cells are super infected with a less disabled virus such as a 17+pR19 type virus, and this will be discussed later. Cells were infected at an m.o.i (multiplicity of infection) of ten for lanes (3) and (4) and an m.o.i of five for each virus for lanes (5), (6) and (7), so that in total is always an m.o.i of 10. At 24h post infection GFP expressed from the fully disabled virus could only be visualised in cells that were harvested and run in lanes (5), (6) and (7), as a result of super infection with a non-GFP expressing 17+ virus (less disabled, LD). It should be noted that the hsp40 virus (fully disabled, FD) contains a cassette that allows simultaneous hsp40 and GFP expression.

(a)

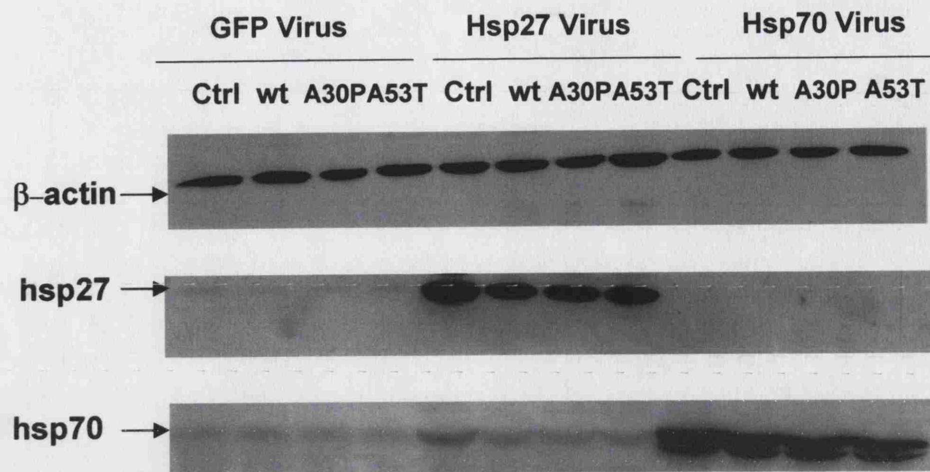


(b)



<u>Figure 4.2</u> **Gene delivery of GFP or heat shock proteins using HSV-based viral vectors.**

ND7 cells 24 h after infection with GFP virus **(a)** and hsp27 virus **(b)** at 10 m.o.i. Cells are of normal morphology. There is not a visual marker for the overexpression of hsp in these viruses but the expression levels were assessed by means of Western blotting (shown later in Figures 4.2 and 4.3) after the cells had been infected at the same m.o.i with either GFP or LacZ or hsp expressing viruses.



<u>Figure 4.3</u> Heat shock protein over-expression in ND7 cells expressing wild type and mutant α-Syn at 24h post infection with recombinant HSV-based vectors expressing GFP, hsp27 or hsp70.

Control cells, wt and mutant A30P and A53T α-Syn expressing cells were infected at an m.o.i of 10 with either of the recombinant viruses over-expressing GFP, hsp27 or hsp70. Western blotting was carried out using anti-β-actin, anti-hsp27 and anti-hsp70 antibodies. There is no significant induction of expression of hsp as a result of infection with the virus itself (see GFP control virus) and there is high expression of the transgenes, all driven by the CMV-IE promoter.

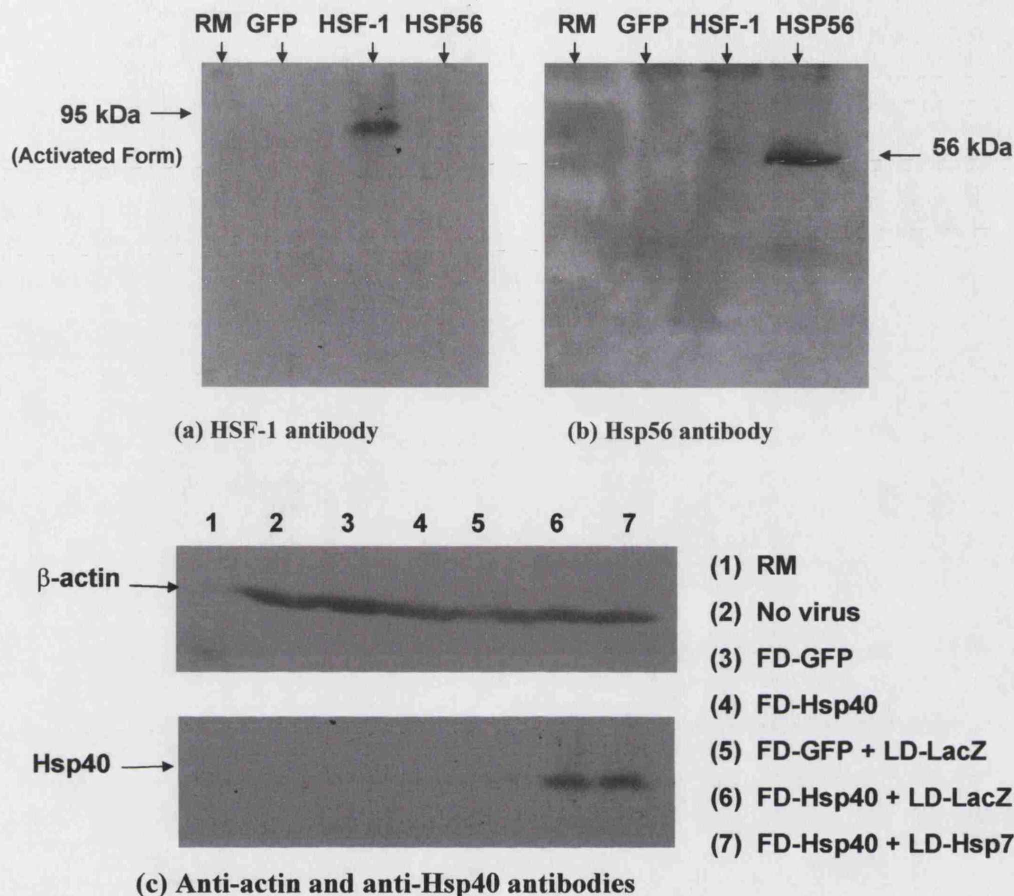


Figure 4.4 Detection of heat shock protein over-expression in ND7 cells by Western blotting at 24 h post-infection with recombinant HSV-based vectors.

Membranes were probed with **(a)** an anti-HSF-1 rabbit polyclonal antibody and **(b)** an anti-hsp56 goat polyclonal antibody. There is no significant induction of expression of hsp or HSF-1 due to infection with the virus itself (see GFP control virus). Expression of the transgenes is driven by the CMV-IE promoter. **(c)** Expression of hsp40 in ND7 cells by a fully disabled (FD) virus can only be achieved by super infection with a less disabled virus (LD). Membranes were probed with anti-β-actin or specific to human hsp40 goat polyclonal antibodies. The m.o.i used for each lane is mentioned in section 4.2.2, RM: rainbow marker.

4.3 α-Syn Cell line Responses to Stress after Exogenous Heat Shock Protein Over-expression

Having characterised the expression of wt or mutant α-Syn in ND7 cells (see Chapter 3) and having demonstrated that hsps can be over-expressed in those cells by viral vectors, the next step was to utilise this system and test whether hsps can confer neuroprotection against the toxic effects of α-Syn forms *in vitro*. This section therefore describes the results obtained after testing for the protective effect of individual hsps in the model system. It should be noted here that, for practical reasons, the following experiments were carried out using one or two different clones per cell line and in some experiments one of the mutants was used. It has been shown in Chapter 3 that although there was some variation between clonal cell lines, they responded similarly to the various stresses. In all cases, this is indicated for each treatment in the relevant section.

Similarly to Chapter 3, the findings are presented below according to the type of stress which was used, with the difference that for each stress the cell death assessment was carried out not only with the trypan blue assay but also with TUNEL and annexin V/ PI staining and FACS analysis. This proved to be particularly useful as it provided some indication of the cell death induced by the stresses, which was mainly apoptotic, and also provided reconfirmation of the observations by alternative cell death assessment methods, as noted in the introduction of this chapter.

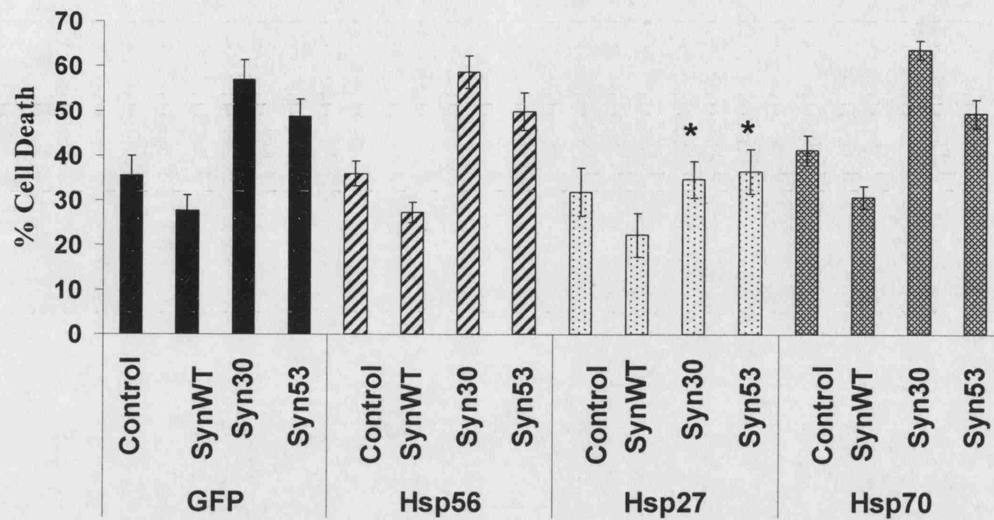
4.3.1 Serum Removal

Therefore, serum removal (1 μM retinoic acid was also always present in serum free media, see Methods and Materials) was applied to the various cell lines for various lengths of time (12 h, 24 h and 48 h). As shown in Figure 4.5 there is a protective effect by hsp27 against the damaging effects of the α-Syn mutants but this is not observed with GFP, hsp56 or hsp70. Under this particular stress wt α-Syn protects the cells (see Chapter 3) but hsp27 does not seem to

further enhance this protective effect at the time points tested. It is noted that the reduction in cell death as a result of hsp27 over-expression, compared to the respective GFP cell line, is approximately 30% for the mutants A30P and A53T at the 24h time point (Figure 4.5.a), 48% at 12 h and 29% at 48h (Figure 4.5.b). Those differences are statistically significant ($p<0.001$) and are indicated by the asterisks.

The TUNEL assay was then performed and TUNEL-positive cells were count in a blind fashion. This revealed the same protective effect of hsp27 but not hsp70 to A30P mutant expressing cells at 24 h from serum removal (Figure 4.6.a shows experimental data and Figure 4.6 shows representative examples of TUNEL positive cells). Annexin V / Propidium Iodide (PI) FACS analysis at 12 h (Figure 4.6.b) confirmed that apoptotic cell death is significantly decreased in control, wt and mutant α-Syn (67.6% reduction in the mutant) cell lines over expressing hsp27 ($p<0.001$) but not LacZ or hsp70. As detailed in Chapter 2, at least 20,000 cells were acquired using a flow cytometer and were gated based on forward activated light scatter (FS) and side scatter (SS) characteristics. The percentage of apoptotic cells, shown in the B4 region of a representative example of a plot in Figure 4.6.c (annexin V positive / PI negative cells) was used in order to summarise the results on the graph in Figure 4.6.b.

(a)



(b)

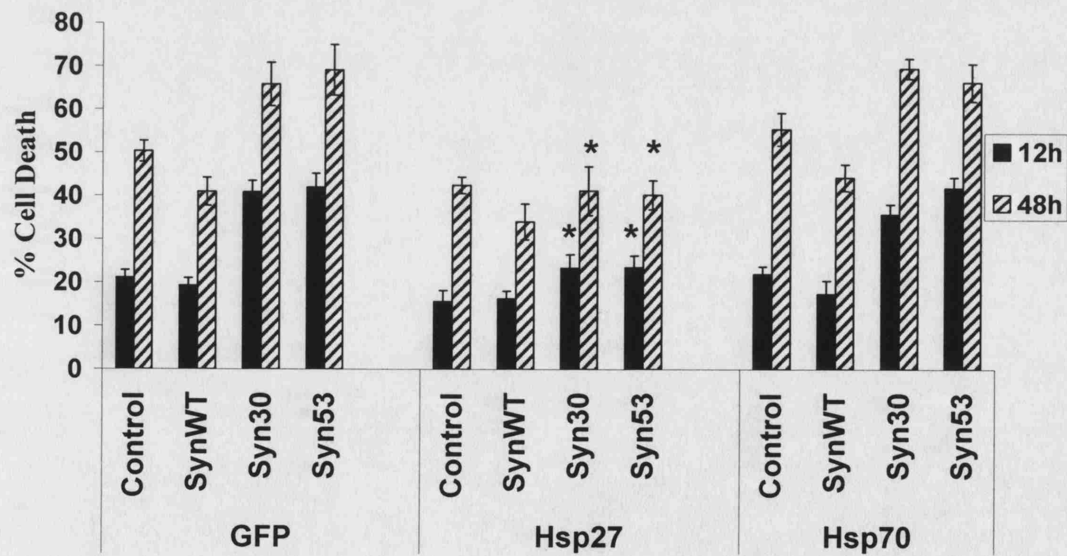
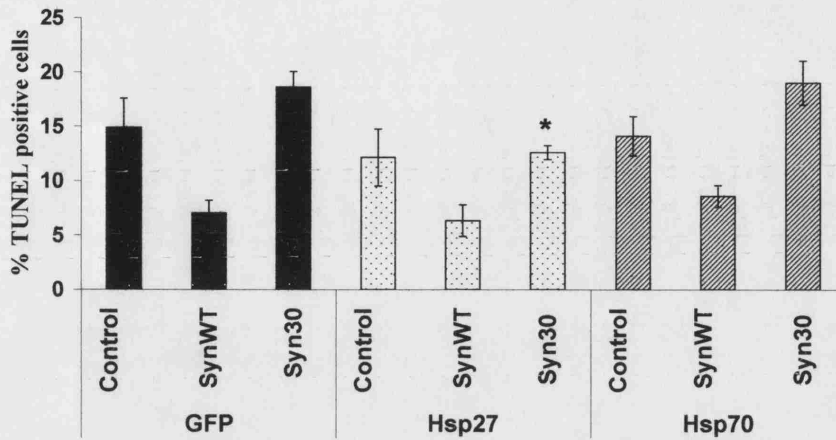


Figure 4.5 ND7 cell death following exogenous heat shock protein or GFP overexpression and at various lengths of time from serum withdrawal.

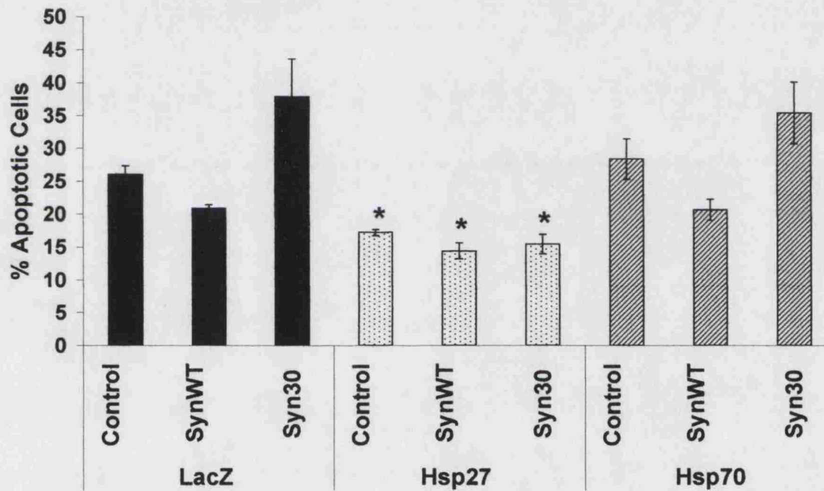
(a) ND7 cell death following 24 h serum removal after infection with HSV vectors expressing hsps. The proportion of cell death was assessed with the trypan blue exclusion assay, after 24 h of serum deprivation of engineered ND7 cells expressing wt or mutant α-Syn. Cells were infected with hsp viruses 16 h prior to serum withdrawal. Bars represent mean ± S.D. calculated for triplicate counts per sample (n), n=7. Significant differences were calculated using a Bonferroni test after one-way ANOVA ($p<0.001$). * indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

(b) Total cell death after 12 h and 48 h of serum withdrawal of engineered ND7 cells expressing wt or mutant α-Syn and exogenous hsps. A single clone per cell line was used in three independent experiments (n=3) and statistically significant differences were calculated as above (* indicates statistically significant difference between means, when comparing with the respective GFP expressing cell line, $p<0.001$).

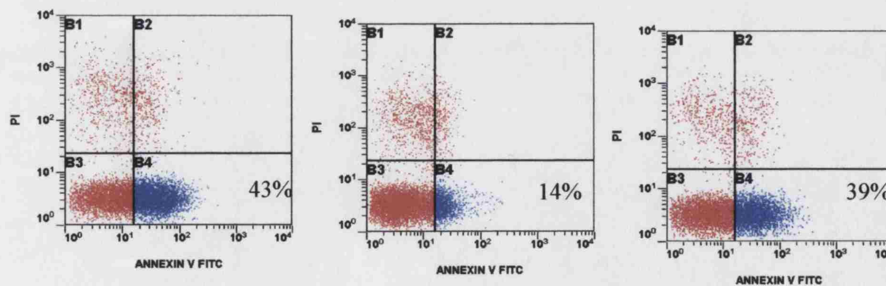
(a)



(b)



(c)



(i) Syn30/LacZ **(ii)** Syn30/hsp27 **(iii)** Syn30/hsp70

Figure 4.6 Apoptotic cell death as assessed by TUNEL and annexin V / PI FACS analysis at different time points from serum removal.

(a) Percentage of TUNEL-positive cells counted after 24 h of serum removal in three different fields in three independent experiments (n=3). Bars represent mean ± S.D. calculated for triplicate counts per sample (n). Significant differences were calculated as above and * indicates significant difference between means, when comparing with the respective GFP virus infected cell line ($p<0.001$).

(b) Flow cytometry of phospholipid redistribution by annexin V / PI assay at 12 h after serum withdrawal. The engineered cell lines had been infected 16 h prior to stress at m.o.i. of 10 with control LacZ or hsp expressing viruses. Bars represent mean ± S.D. calculated for n=2. Significant differences were calculated as above ($p<0.001$). * indicates statistically significant difference between means, when comparing with the respective LacZ virus infected cell line.

(c) Plots (i), (ii) and (iii) are representative examples of flow cytometry data (summarised above) that depict the level of apoptotic cell death (seen in region B4) in the mutant A30P α-Syn cells overexpressing LacZ, hsp27 or hsp70, respectively. B1 region includes debris and nuclei (annexin V - / PI +) and B3 live cells (annexin V- / PI -), whereas the B2 region includes late apoptotic/secondary necrotic cells (annexin V + / PI +).

(a) **(b)**

Control Vector TUNEL +ve cells

A30P α-Syn TUNEL +ve cells

<u>Figure 4.7</u> Representative images of ND7 cells stained positively with TdT-mediated dUTP nick end labelling (TUNEL).

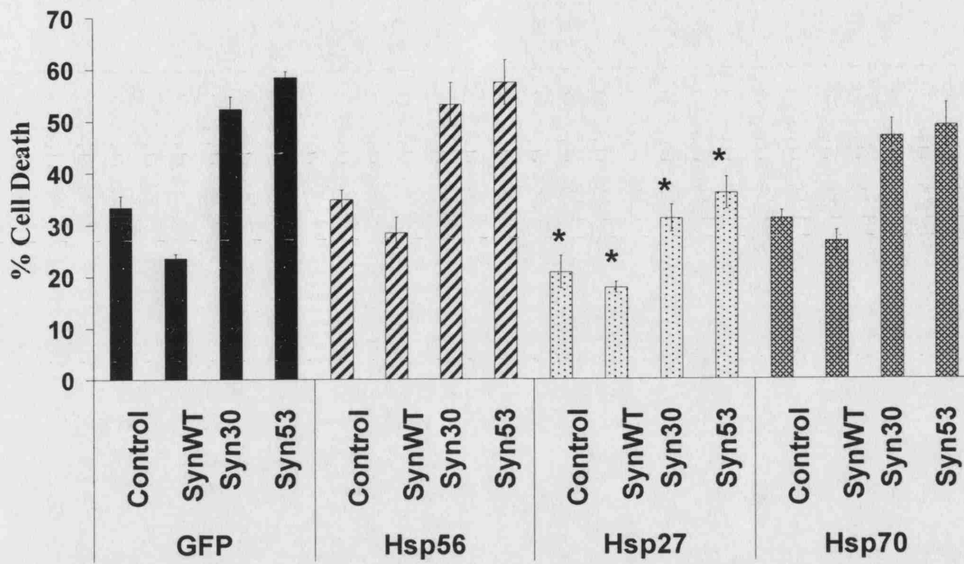
Rhodamine-conjugated nucleotides label the fragmented DNA in cells undergoing apoptosis, in this case 24 h serum removal, and blind counts of three random fields per slide were counted in order to determine the percentage of TUNEL positive cells adhered to the slide of **(a)** control-vector cells or **(b)** A30P α-Syn expressing cells. Only the highly intense fluorescent cells were counted as positive.

4.3.2 Dopamine

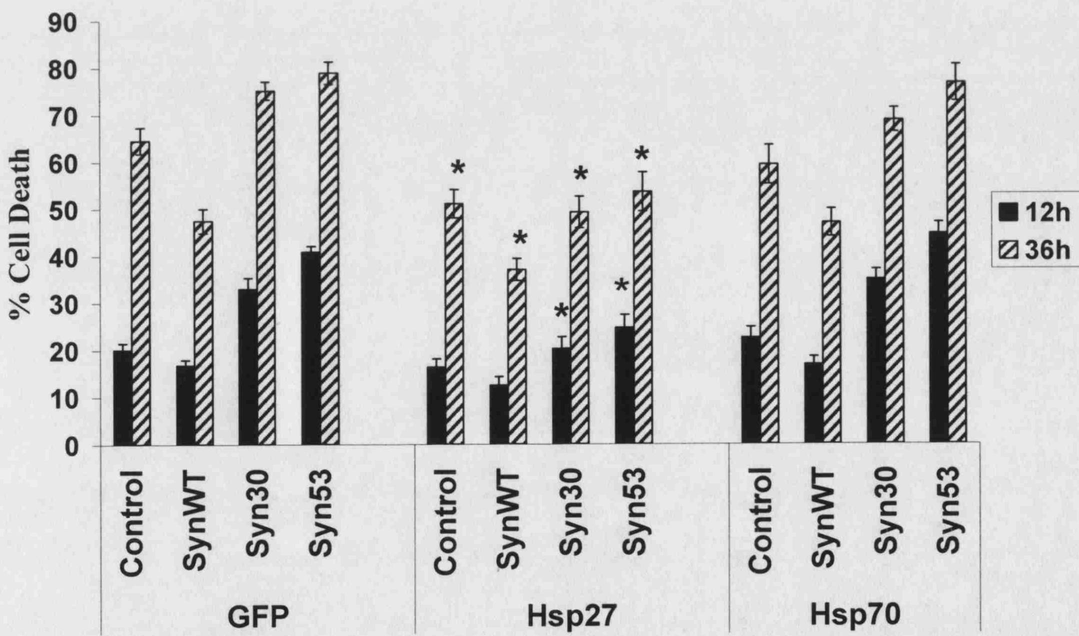
When the cells were challenged with 62 μM dopamine for 24 h (Figure 4.8.a) hsp27 again could be seen to protect all cell lines by significantly reducing cell death compared to the respective GFP cells ($p<0.001$), in particular in A30P and A53T cell lines (up to 42.3% cell death reduction). Similarly at 12 h or 36 h (Figure 4.8.b) there is significantly less cell death in the hsp27 expressing cell lines when compared to the respective control GFP ($p<0.001$). Approximately 32.6% reduction in death was observed in the mutant α-Syn cell lines (36h). Under this treatment wt α-Syn cells are protected compared to control (see Chapter 3) and therefore the difference in cell death between hsp27-wt α-Syn over-expressing cells and GFP-wt α-Syn is less pronounced, compared to the reduction in cell death in the case of either of the mutant α-Syn cell line. Hsp56, like hsp70 did not confer protection to any of the cell lines.

In addition, TUNEL staining reconfirmed the trend of hsp27 but not hsp70 or GFP being protective for the cells expressing A30P α-Syn at 24 h (Figure 4.9.a) whilst annexin V/ PI staining indicated a significant suppression in apoptotic cell death by hsp27 but not hsp70 or LacZ over expression in all cell lines at 24 h ($p<0.001$), for instance 76.7% reduction in apoptotic cell death in mutant α-Syn / hsp27 expressing cells compared to mutant α-Syn / LacZ cells (Figure 4.9.b).

(a)



(b)

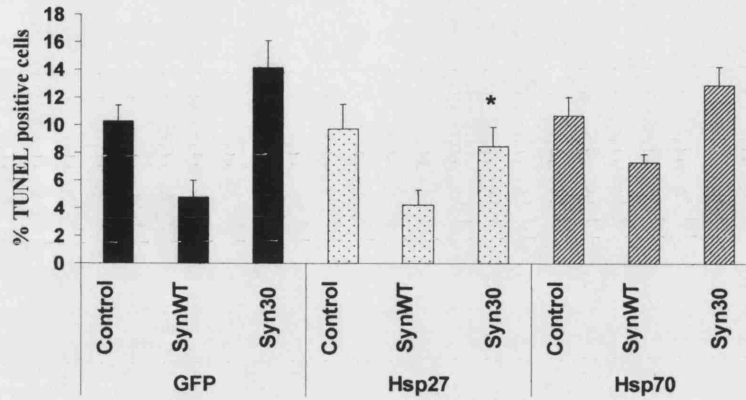


<u>Figure 4.8</u> ND7 cell death at various lengths of time with 62 μM dopamine incubation, following exogenous heat shock protein or GFP overexpression.

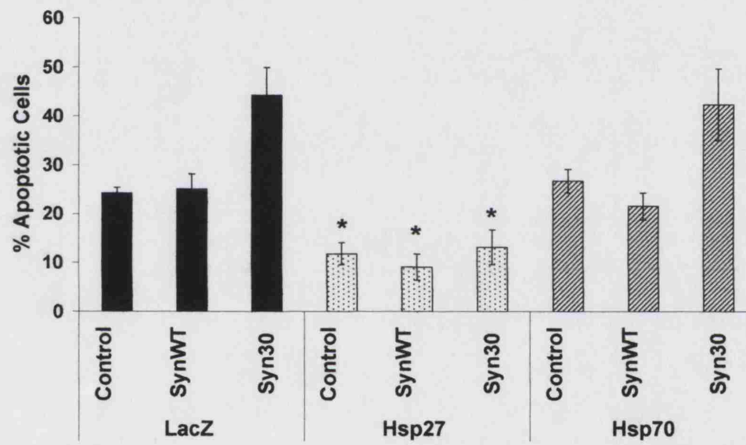
(a) ND7 cell death following 24 h 62 μM dopamine incubation after infection with HSV vectors expressing hsps. Dopamine induced cell death of the engineered ND7 cells expressing wt or mutant α-Syn. Cells were again infected with hsp viruses 16 h prior to stress. Bars represent mean ± S.D. calculated for triplicate counts per sample (n). n=4. Significant differences were calculated using a Bonferroni Multiple Comparison's *t* test after one-way ANOVA ($p<0.05$). * indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

(b) Total cell death after 12 h and 36 h incubation with 62 μM dopamine of ND7 cells expressing wt or mutant α-Syn and exogenous hsps. One clone per cell line was used in three independent experiments (n=3) and statistically significant differences were calculated as above (* indicates statistically significant difference between means, when comparing with the respective GFP expressing cell line, $p<0.001$).

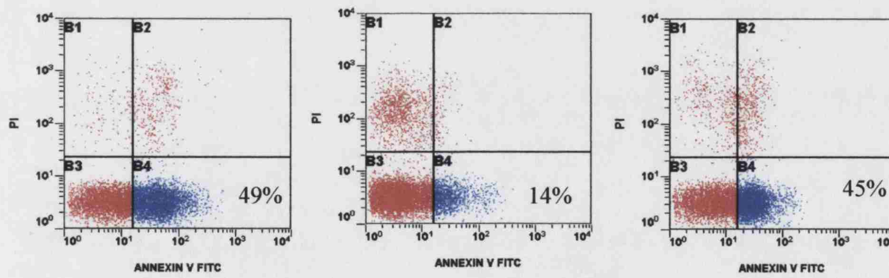
(a)



(b)



(c)



(i) Syn30/LacZ **(ii)** Syn30/hsp27 **(iii)** Syn30/hsp70

Figure 4.9 Apoptotic cell death as assessed by TUNEL and annexin V / PI FACS analysis at 24 h of 62 μM dopamine incubation.

(a) Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to overexpress hsps or GFP 16 h prior to treatment with dopamine for 24h. Bars represent mean ± S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.001$. * indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

(b) Flow cytometry of phospholipid redistribution by annexin V / PI assay at 24 h dopamine incubation. The cell lines had been infected 16 h prior to stress at 10 m.o.i. with control LacZ or hsp expressing viruses. Bars represent mean ± S.D. calculated for n=3. Significant differences were calculated as above ($p<0.001$). * indicates statistically significant difference between means, when comparing with the respective LacZ virus infected cell line.

(c) Plots (i), (ii) and (iii) are representative examples of flow cytometry data (summarised above) that show the percentage of apoptotic cell death (seen in region B4) in the mutant A30P α-Syn cells overexpressing LacZ, hsp27 or hsp70, respectively.

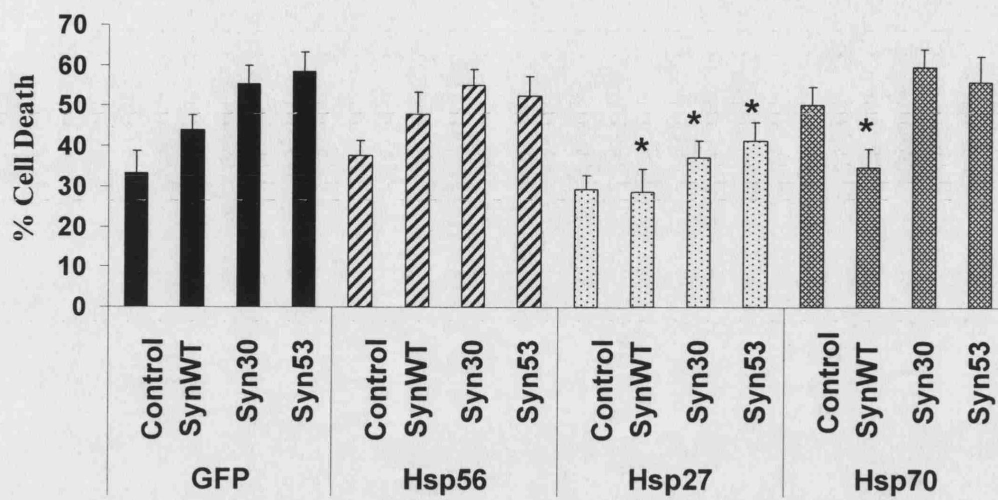
4.3.3 Simulated Ischaemia

The next treatment that was tested was various time points of simulated ischaemia (for method details see Chapter 2). Again hsp27 seemed to have a protective effect at 3 h ischaemia / 24 h reoxygenation which reduces cell death in wt and A30P and A53T mutant cell lines by approximately 34% whereas hsp70 surprisingly reduces cell death only in wt α–Syn expressing cells by approximately 24% (Figure 4.10.a). It should be noted here that, as shown in Chapter 3, wt α-Syn increases cell death under ischaemia compared to control vector but at a lesser extent as compared to the mutant forms. Hsp70 over-expression is shown here to protect those wt α-Syn cells by reducing cell death by 24% as compared to GFP expressing cells. This small but statistically significant protective effect by hsp27 is also profound at 4 h ischaemia / 24 h reoxygenation but is smaller at 2 h ischaemia / 24 h reoxygenation. Overall, as seen in Figure 4.10.b, hsp27 significantly reduces cell death in the various cell lines by 30% - 44% when compared to GFP virus-infected cells ($p<0.001$), whereas hsp70 reduces, but with a smaller efficiency, cell death in wt α-Syn cells ($p<0.001$) such that the cell death percentage becomes similar to control cells. The same is true for hsp27, where clearly the amount of cell death in all different synuclein over-expressing cell lines becomes similar to (2 h time point) or lower than (4 h time point) that observed in control vector-GFP cells.

However, when apoptotic cell death was assessed with either TUNEL staining or annexin V / PI at 3 h ischemia / 24 h reoxygenation the above results were reconfirmed in the case of hsp27 but not of hsp70 (Figure 4.11). When compared with the respective GFP cell line, hsp27 but not hsp70 significantly reduced cell death by approximately one third. TUNEL positivity in control, wt α-Syn and A30P α-Syn cells (Figure 4.11.a). The same trend was reconfirmed by annexin V / PI analysis in 2 experiments (Figure 4.11.b and c) i.e. hsp27 is reducing apoptotic cell death by approximately 60% in A30P α-Syn cells when compared with the respective LacZ cells ($p<0.001$), whereas hsp70 does not seem to reduce apoptotic cell death in any of the cell lines, which contrasts with

the observations in Figure 4.10 where α-Syn cells are protected by hsp70 in the trypan blue assay of total cell death ($p<0.001$).

(a)



(b)

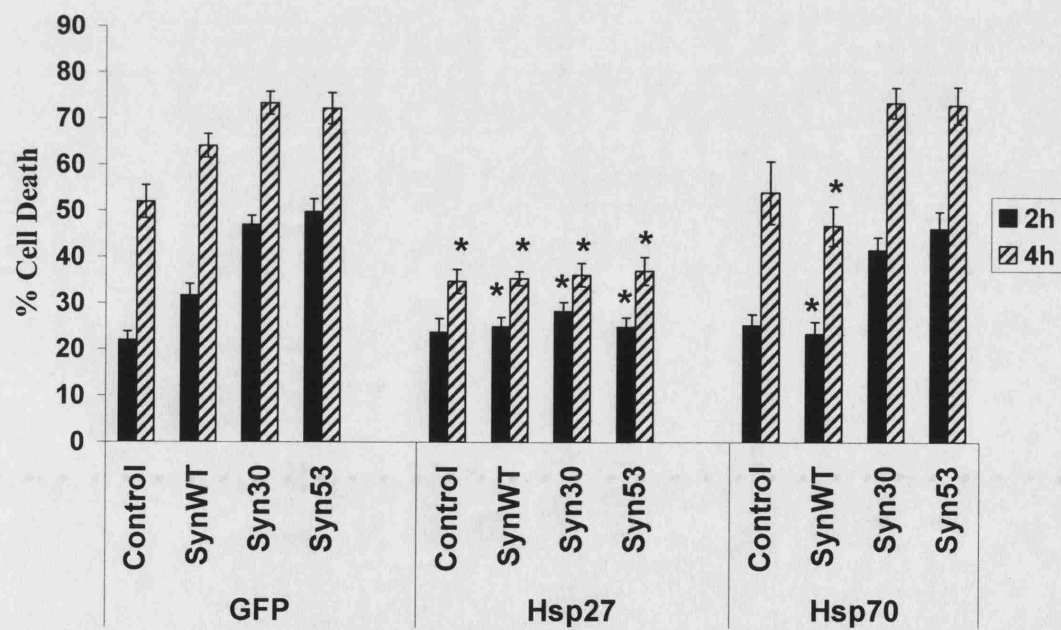
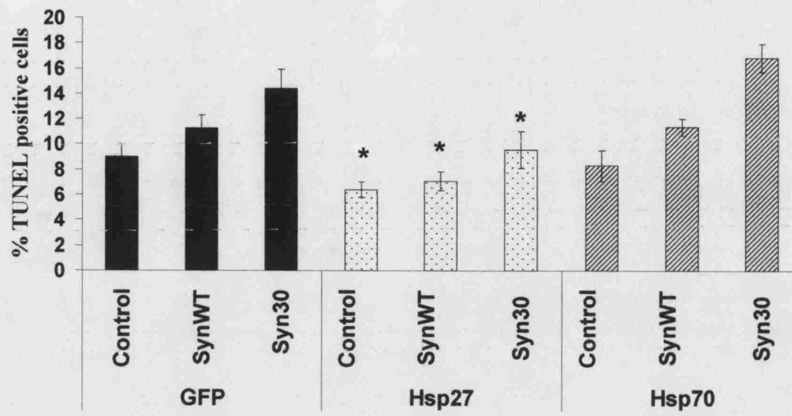


Figure 4.10 ND7 cell death at various time points of simulated ischaemia, following exogenous heat shock protein or GFP overexpression.

(a) Proportion of cell death after 3 h ischaemia and 24 h reoxygenation of engineered ND7 expressing wt or mutant α-Syn that had been infected with HSV vectors expressing hsps. Cells were infected with hsp viruses 16h prior to stress. Bars represent mean ± S.D. calculated for triplicate counts per sample (n). n=10. Significant differences were calculated using a Bonferroni test after one-way ANOVA ($p<0.05$). * indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

(b) Total cell death after 2 h ischaemia / 24 h reoxygenation and 4h ischaemia / 24 h reoxygenation of ND7 cells expressing wt or mutant α-Syn and over-expressing hsps. One clone per cell line was used in three independent experiments (n=3) and statistically significant differences were calculated as above (* indicates statistically significant difference between means, when comparing with the respective GFP expressing cell line, $p<0.001$).

(a)



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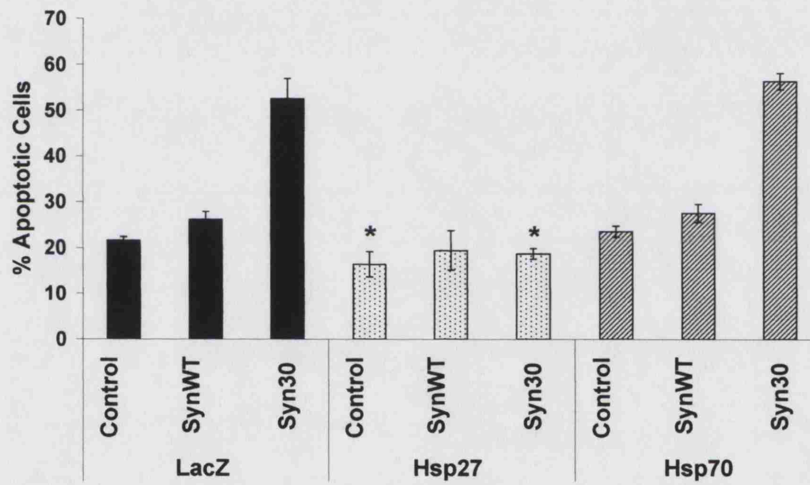
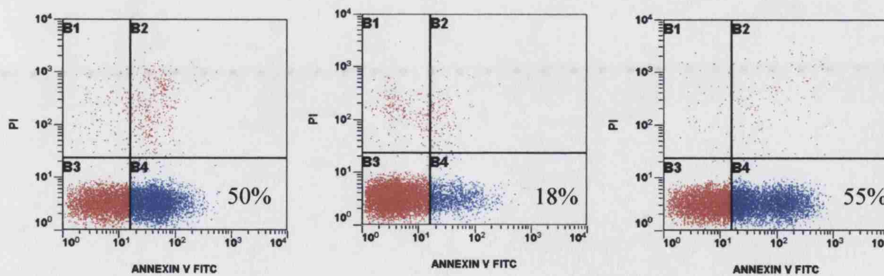


(c)



(i) Syn30/LacZ **(ii)** Syn30/hsp27 **(iii)** Syn30/hsp70

Figure 4.11 **Apoptotic cell death as assessed by TUNEL and annexin V / PI FACS analysis at 3 h of simulated ischaemia followed by 24 h reoxygenation.**

(a) Percentage of TUNEL-positive cells counted in three different fields in two experiments. Cell lines were infected with viruses to over-express hsps or GFP 16 h prior to 3h simulated ischaemia / 24 h reoxygenation. Bars represent mean ± S.D. calculated for triplicate counts per sample (n=2). Significant differences were calculated using a Bonferroni Multiple Comparison's *t* test after one-way ANOVA and * indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line. Hsp27 significantly suppresses apoptotic cell death in control ($p<0.05$), wt and A30P α-Syn expressing cells ($p<0.001$), compared with the respective GFP expressing cell line.

(b) Annexin V / PI assay after ischemia. The cell lines had been infected 16 h prior to stress at 10 m.o.i. with control LacZ or hsp expressing viruses. Bars represent mean ± S.D. calculated for n=2. Significant differences were calculated as above ($p<0.001$). * indicates statistically significant difference between means, when comparing with the respective LacZ virus infected cell line.

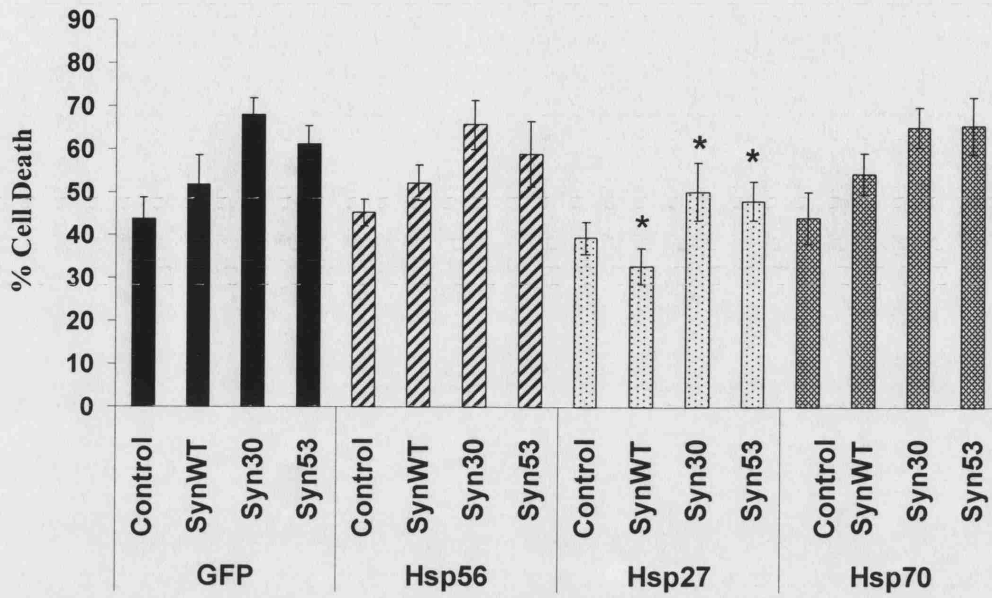
(c) Plots (i), (ii) and (iii) are representative examples of flow cytometry data (summarised above) showing the percentage of apoptotic cell death (seen in region B4) in the mutant A30P α-Syn cells over expressing LacZ, hsp27 or hsp70, respectively.

4.3.4 Staurosporine

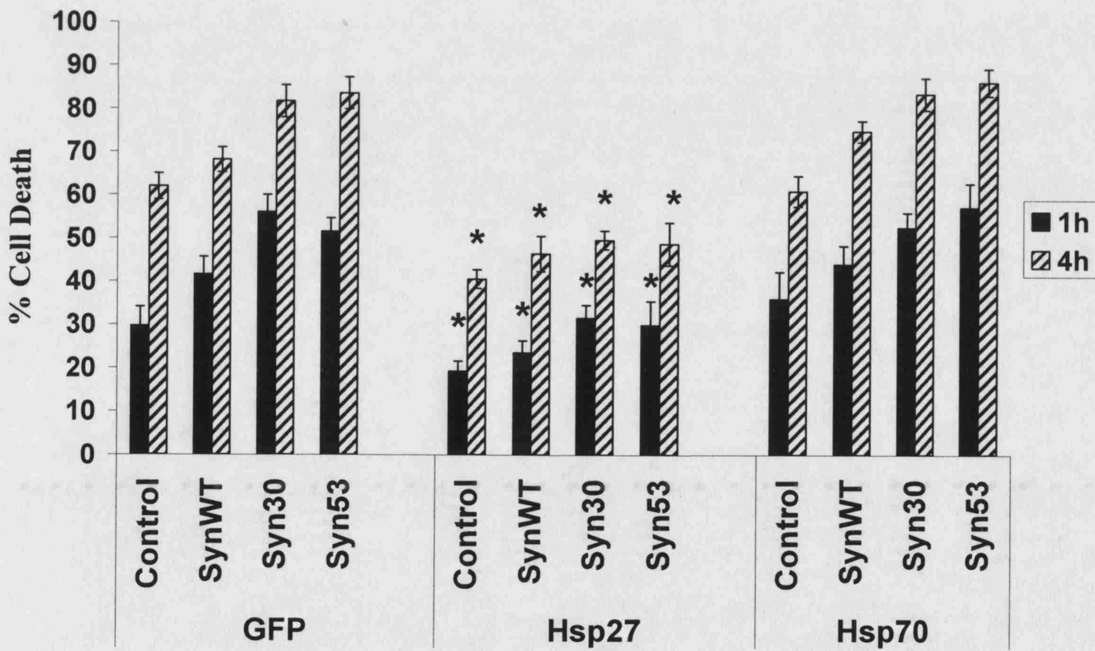
Cells were next treated with 1 μM staurosporine for 1 h, 2 h, or 4 h. Once more hsp27 was protective in wt and either of the mutant α-Syn cells (all forms of synuclein are shown to be deleterious under this stress, at a different extent each), leading to up to 30% cell death reduction (2 h) or up to 42% reduction (4 h) (Figure 4.12.a and b). It should be noted that hsp27 significantly reduces cell death levels of wt or mutant cells up to death levels similar to control vector-GFP cells (1 h) or even further at 4 h ($p<0.001$). In contrast, hsp70 has no effect in reducing cell death.

The above observations were reconfirmed, as before, by apoptotic cell death assays in which TUNEL positivity at 2 h treatment is reduced significantly by hsp27 ($p<0.001$) and this reduction is approximately 41% for control cells, 60% for wt α-Syn and 67% for A30P α-Syn cells (Figure 4.13.a). Annexin V / PI analysis in a small number of experiments reconfirms the same trend of protection by hsp27 in all cell lines where there is a significant reduction in cell death in control, wt and mutant α-Syn cells infected with the hsp27 virus, compared to LacZ virus ($p<0.001$) (up to 60% reduction in apoptotic cell death by hsp27 compared to LacZ) (Figure 4.13.b). Hsp70 expression in the various cell lines showed no reduction in apoptotic cell death as assessed with TUNEL and annexin V / PI staining.

(a)



(b)

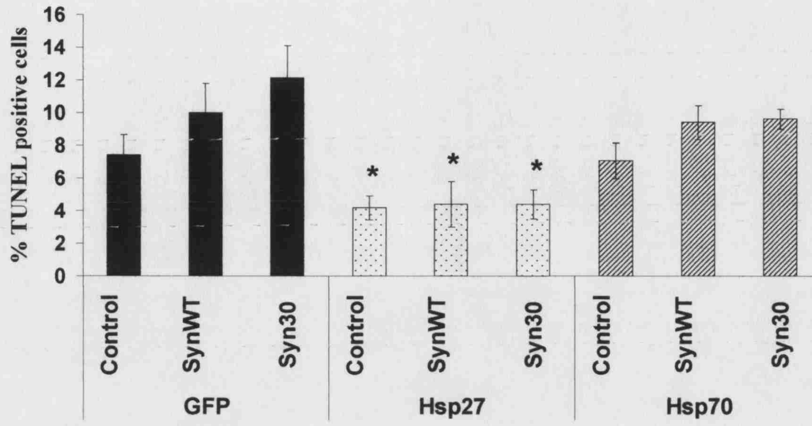


<u>Figure 4.12</u> ND7 cell death at various time points of 1 μM staurosporine incubation, following exogenous heat shock protein or GFP over expression.

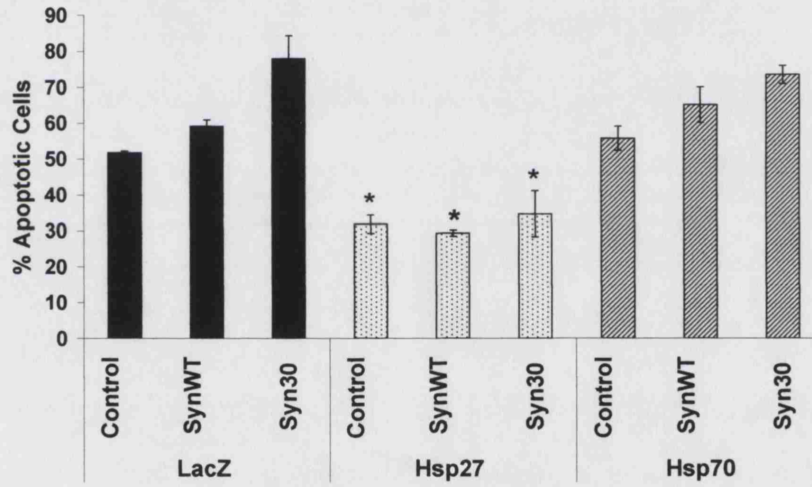
(a) ND7 cell death following 2 h 1 μM staurosporine incubation after infection with HSV vectors expressing hsps. Cell death was assessed with the trypan blue exclusion assay, after incubation of ND7 cells expressing wt or mutant α-Syn with staurosporine. Cells were infected with hsp viruses 24 h prior to stress. Bars represent mean ± S.D. calculated for triplicate counts per sample (n). n=9, data pooled from 2 clones per cell line. Significant differences between means were calculated using a Bonferroni Multiple Comparison's *t* test after one-way ANOVA ($p<0.05$). * indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

(b) Percentage of cell death of ND7 cells expressing wt or mutant α-Syn and over-expressing hsps after incubation with 1 μM staurosporine for 1 h and 4 h. One clone per cell line was used in three independent experiments (n=3) and statistically significant differences were calculated as above (* indicates statistically significant difference between means, when comparing with the respective GFP expressing cell line, $p<0.001$).

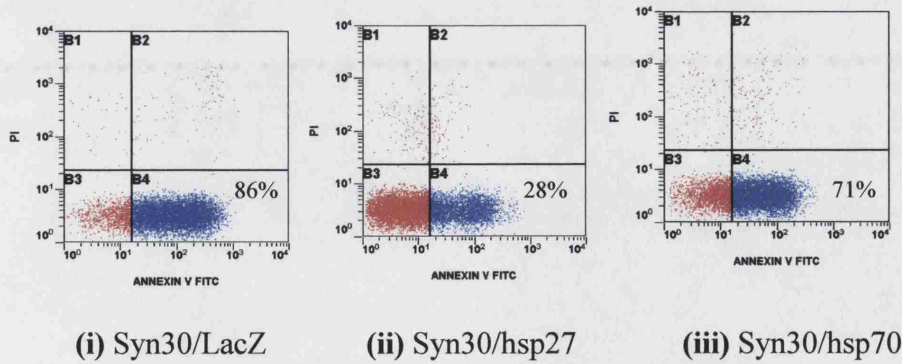
(a)



(b)



(c)



(i) Syn30/LacZ **(ii)** Syn30/hsp27 **(iii)** Syn30/hsp70

Figure 4.13 Apoptotic cell death as assessed by TUNEL and annexin V / PI FACS analysis at 2 h staurosporine incubation.

(a) Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to overexpress hsps or GFP 24 h prior to treatment with 1 μM staurosporine for 2 h. Bars represent mean ± S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.001$. * indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

(b) Annexin V / PI assay after staurosporine induced apoptotic cell death. The cell lines had been infected 24 h prior to stress at 10 m.o.i. with control LacZ or hsp expressing viruses as before. Bars represent mean ± S.D. calculated for n=2. Significant differences were calculated as above ($p<0.001$). * indicates statistically significant difference between means, when comparing with the respective LacZ virus infected cell line.

(c) Plots (i), (ii) and (iii) are representative examples of flow cytometry data (summarised above) showing the percentage of apoptotic cell death (seen in region B4) in the mutant A30P α-Syn cells over expressing LacZ, hsp27 or hsp70, respectively.

4.3.5 Heat Shock

In contrast to the results described above, under severe heat shock (20 min heat shock at 48°C followed by 1 h recovery at 37°C), both hsp27 and hsp70 could be seen to confer protection from cell death, as assessed with the trypan blue assay, in all cell lines, leading to great reduction in death compared to control which varies between 46.6% to 50% (Figure 4.14). This difference is statistically significant for all cells lines ($p<0.001$). It is notable that the cell death in all cell lines expressing hsp27 or hsp70 is reduced to levels much lower to control-GFP cells and also that the extent of protection by hsp27 and hsp70 is very similar, as seen in Figure 4.14. Once again, hsp56 does not confer protection in any of the cell lines and cell death is similar to GFP expressing cells.

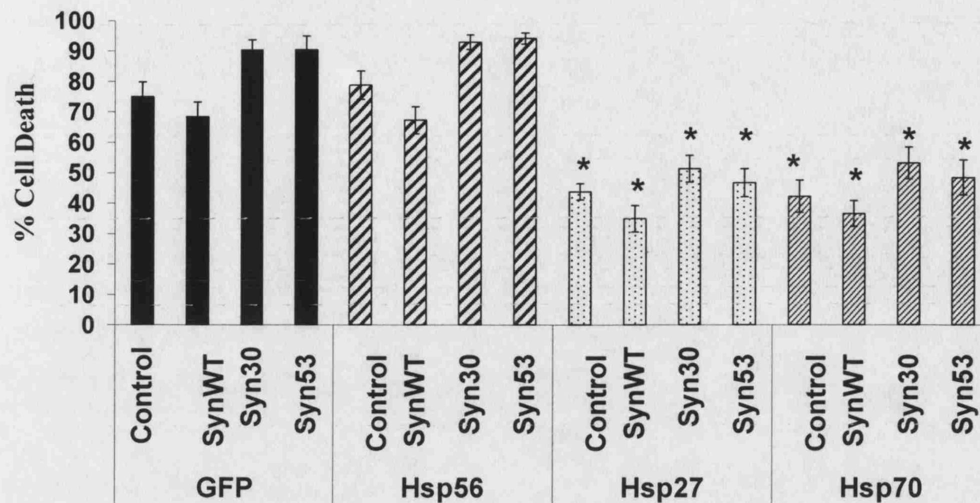


Figure 4.14 Cell death after severe heat shock of engineered ND7 cells over-expressing α-Syn forms and either GFP, hsp27 or hsp70.

One clone per cell line was incubated for 20 min at 48°C followed by 1 h recovery at 37°C and cell death was immediately assessed with the trypan blue assay. Bars represent mean ± S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.001$. * indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

4.3.6 Effect of a Constitutively Active HSF-1 Mutant-Expressing Virus or Combinations of Heat Shock Protein Expressing Viruses

Lastly, it was attempted to test the hypothesis whether combining the over-expression of different hsps by co-infecting cells with different viruses or HSF-1 over-expression under a particular stress, such as 48 h serum withdrawal, would have a protective effect or even be more efficient in protection compared to single hsp over-expression. Only one stress (48 h serum withdrawal) was chosen in order to test this hypothesis in one clone per cell line. So far, hsp27 has been shown to be protective in cells expressing either wt or mutant α-Syn under all stresses tested (the effects were reconfirmed by total and apoptotic death assays). However, hsp70 was protective only under severe heat shock (assessment of total cell death) in all cell lines and also in wt α-Syn cells exposed to 2 h, 3 h or 4 h ischaemia with 24 h reoxygenation (this effect was relatively small and was reconfirmed by total cell death assay but not apoptotic assays, therefore hsp70 might not protect from apoptosis but only from necrosis).

Figure 4.15 illustrates that hsp27 over-expression alone has a similar protective effect compared to over-expression of hsp27 and hsp70 together in cells. Cell death is reduced by up to approximately 43% in A30P and A53T mutant α-Syn cells in the case of hsp27 alone and by up to 20% in the case of hsp27 and hsp70 co-expression (note that for the co-infection half the m.o.i was used for either virus up to a total of an m.o.i 10). The differences in cell death due to hsp27 alone or hsp27 and hsp70 co-expression when compared to GFP-expressing cells are statistically significant ($p<0.001$) and this is indicated by the asterisks in the respective cell lines in Figure 4.15. Also, when comparing mutant α-Syn expressing hsp27 alone to mutant α-Syn, hsp27 and hsp70 co-expressing cells there is no statistically significant difference in cell death.

HSF-1 delivered at the same m.o.i as the other viruses is mildly protective for either control vector cells or wt α-Syn cells exposed to serum removal (approximately 10-12% cell death reduction which is statistically

significant, $p<0.001$) but not for mutant A30P or A53T α-Syn expressing cells. Overall, under this particular stress, control-vector cells are mildly protected by hsp27 alone, hsp27 and hsp70 co-expression and HSF-1, wt α-Syn cells are protected by hsp27 and hsp70 co-infection or HSF-1 and A30P and A53T α-Syn cells are protected by hsp27 or hsp27 and hsp70 co-expression. The effect of co-expression of hsp27 and hsp70 is very similar to over-expression of hsp27 alone.

Finally, hsp40 was tested as a potential and necessary hsp70 co-factor in conferring protection under stress in the various cell lines (see Chapter 1 for literature on hsp40/hsp70). This experiment was carried out by using two different types of viruses, one of which is a 17+pR19 virus expressing either hsp70 or GFP, described in the Introduction, and a fully disabled 1764 27- 4- pR20.5 GFP/LacZ or hsp virus that allows co-expression of hsp40 and GFP simultaneously or GFP and LacZ. However, as found after infection of ND7 cells with the fully disabled virus the transgene expression is very poor unless the cells are super infected with the less disabled 17+ virus. The reason why this happens is not fully investigated but it is speculated that this is due to an active repression of both HSV and non-HSV promoters by a host cell specific factor which is an effect that is masked in less disabled viruses by the presence of some IE gene products (Preston and Nicholl, 1997). Because hsp40 was only available in a fully disabled virus backbone (a kind gift by David Hay), the only solution for over-expressing hsp40 sufficiently in ND7 cells was to use it in combination with an equal amount of 17+ virus which would trigger induction of the transgene.

As a control, a fully disabled virus expressing simultaneously GFP and LacZ was used in combination with a less disabled LacZ virus, therefore having delivered the appropriate virus amounts, one should be able to see the GFP produced by the fully disabled virus after 24 h. Similarly, a less disabled LacZ virus delivered with the fully disabled hsp40-GFP co-expressing virus, allowed expression of GFP and hsp40 from the fully disabled virus and this was detectable 24h post infection. It was assumed that GFP production meant hsp40

production, as their cDNAs were under the same LATP2 promoter (GFP - CMV - LATP2 - RSV - HSP40).

Figure 4.16 shows that hsp40 over-expression in the various cell lines has the same effect on cell death, after 48 h serum withdrawal, as GFP or hsp70. Also, combination of the over-expression of the hsp70 with hsp40 virus had the same effect as single hsp over-expression or GFP, therefore no protective effect was evident by this combination of hsp over-expression. As a positive control, hsp27 virus was used at 5 m.o.i with a fully disabled GFP-LacZ expressing virus at 5 m.o.i (total virus at 10 m.o.i) and reconfirmed that under these conditions of stress and over-expression of hsp27, cell death is significantly reduced in control-vector and in mutant α-Syn cells ($p<0.001$) as well as wt in α-Syn expressing cells ($p=0.025$) when compared to the respective GFP virus infected cells (again 5 m.o.i of each fully disabled and 17+ type of virus were used).

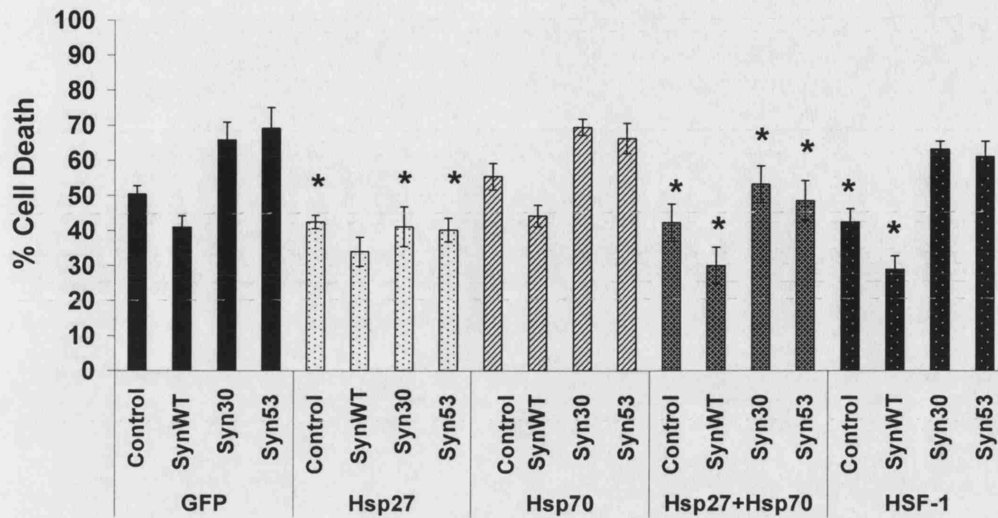
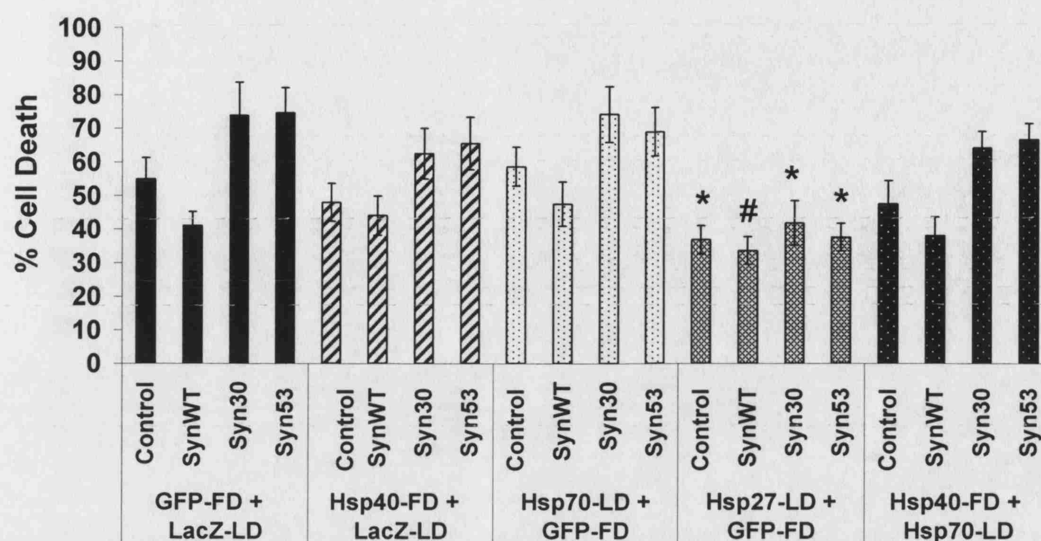


Figure 4.15 ND7 cell death following exogenous hsp or HSF-1 or GFP over expression and 48h serum withdrawal.

Hsp27 and hsp70 co-infection with viruses was performed at 5 m.o.i for each virus and for all the rest viruses 10 m.o.i were used for a single infection, 16h prior to stress induction. Cell death was immediately assessed with the trypan blue assay. Hsp27 virus and hsp70 virus alone were used as well as a GFP control virus so that the difference in cell death reduction can be compared. Bars represent mean ± S.D. calculated for triplicate counts per sample n (n=3) and significant differences were calculated as previously ($p<0.001$). * indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.



<u>Figure 4.16</u> ND7 cell death following exogenous heat shock protein or GFP over expression at 48 h from serum withdrawal.

The proportion of cell death was assessed with the trypan blue exclusion assay, after 48h of serum deprivation of engineered ND7 cells expressing wt or mutant α-Syn. Cells were co-infected with combinations of hsp or GFP viruses 16h prior to stress. In each infection one virus is fully disabled and the other is a 17+ type virus, at 5 m.o.i each. Bars represent mean ± S.D. calculated for triplicate counts per sample (n), n=3. Significant differences were calculated using a Bonferroni Multiple Comparison's *t* test after one-way ANOVA. * ($p<0.001$) or # ($p=0.025$) indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

4.4 Discussion

This chapter describes the protective effect of heat shock proteins in the *in vitro* model system of α-Syn-associated toxicity that was described in Chapter 3. HSV-based viral vectors were utilised in order to efficiently express various heat shock proteins. In summary, the α-Syn expressing cell lines were infected with the viral vectors or the relevant control viruses and subsequently the cells were subjected to various treatments as in the experiments described in Chapter 3, including serum withdrawal, dopamine, simulated ischaemia, staurosporine and heat shock. The response of the cell lines to the above death inducing stimuli was quantified by different methods of cell death assessment, such as a general death assay based on trypan blue exclusion and apoptotic cell death assays based on TUNEL or annexin V / PI staining.

Briefly, the results indicate that hsp27 protects the mutant α-Syn cell lines from cell death induced by serum removal, whereas wt α-Syn cells are not generally further protected by hsp27 compared with GFP cells (i.e. wt α-Syn protects from serum withdrawal, as shown in Chapter 3, but hsp27 does not further protect these cells from serum withdrawal). Hsp27 also protects most of the cell lines, in particular those expressing both mutant forms of α-Syn, from cell death induced by dopamine, simulated ischaemia, staurosporine or severe heat shock. Therefore, hsp27 seems to reduce cell death in the cell lines, which was assessed at various time points and by apoptotic or general cell death assessment methods for each stress. Such protective effects were stronger in the mutant α-Syn expressing cell lines.

However, hsp70 had no protective effect on any of the cell lines after serum withdrawal, dopamine or staurosporine challenge or simulated ischaemia, with the exception of a mild protective effect only in wt α-Syn expressing cells exposed to ischemia. It should be noted that wt α-Syn causes enhanced cell death in response to this stress and therefore it can be postulated that hsp70 only protects when wt is damaging, but this again depends on the stress. This was only shown by a general cell viability assay and not by apoptotic cell death

assays. In addition, hsp70 was shown to uniformly enhance viability at a similar extent as hsp27 in all cell lines after severe heat shock. When hsp70 is expressed in combination with hsp27, cell death after 48h serum deprivation is decreased to levels similar to those by hsp27 overexpression alone, leading to the conclusion that hsp70 does not further enhance the protective effect conferred by hsp27 under this stress. Hence, it could be concluded that the protective effect of hsp70 in this model is more restricted compared to that of hsp27 and it is unlikely that hsp70 has an anti-apoptotic effect in this system; rather an anti-necrotic effect is possible.

HSF-1 was seen to exert a relatively small protective effect on the empty vector transfected cells or wt α-Syn cells 48 hours after serum withdrawal, and had no effect on the mutant α-Syn expressing cells. Hsp56 had the same effect as GFP or LacZ under all stresses, allowing us to conclude that this heat shock protein exerts no protective effects in this model system and serves as an additional control for the respective experiments. Lastly, in an attempt to test if hsp40 over-expression is necessary for hsp70-mediated neuroprotection, hsp40 (which stimulates the ATPase activity of hsp70) was used in combination with hsp70 over expression, and showed no effect in enhancing the viability of any of the cell lines after serum removal induced apoptosis.

Hsp56 is a component of the steroid receptor complex and plays a role in protein folding (Pratt and Welsh, 1994). Its overexpression has not been studied in neurons apart from a report by Wagstaff et al. (1999) where hsp56 was found to have only a small protective effect from thermal and ischaemic stress in DRG neuronal cells but not ND7 cells. Hence, it is not surprising it does not have any effect in our system, but it remains a useful negative control in our experiments. On the other hand, HSF-1 has not been extensively studied here, but from the results obtained it seems as if it does not protect mutant α-Syn cell lines in our system. This might be due to insufficient levels of overexpression of hsps as a result of its action. As shown by Wagstaff et al. (1998), western blotting analysis showed that in ND7 cells viral gene delivery

of the constitutively active mutant HSF-1 leads mainly to hsp70 overexpression and also hsp27 and hsp32 to a smaller degree, whereas hsp90, 56 and 60 levels remain unchanged. They also found that the individual 17+pR19 hsp viruses all produce higher amounts of their respective hsp gene products compared to those induced by the 17+pR19 HSF-1 virus. Of course this transcription factor might stimulate transcription of other genes by interacting with elements of their promoters and this may lead to certain protein production that can influence the net effect of HSF-1 in our system.

Importantly, our results with hsp27 demonstrate for the first time that hsp27 overexpression uniformly confers protection from death induced by a range of different stresses at several time points post-stress-induction in mammalian neuronal cells over-expressing wt or mutant α-Syn. These findings are in agreement with several reports in which hsp27 is protective against a number of stresses in different model systems. Previously, Wagstaff et al. (1999) demonstrated that neuronal cells can be protected by hsp27 but not by hsp70 against apoptotic stimuli, but the exact mechanism of protection against these stimuli is unclear. Wyttenbach et al. (2002) in a cellular model of HD showed that hsp27 suppressed polyQ-mediated cell death, protecting from the increase of reactive oxygen species caused by huntingtin. Such a defensive role of hsp27, which can be separate from its anti-apoptotic activities, is possible in our system and needs to be further investigated. More recently, an interesting report demonstrates that hsp27 has a neuroprotective role against pathological hyperphosphorylated tau-induced apoptotic cell death in a human cortical neuronal cell line and also that hsp27 binds with hyperphosphorylated tau in AD brain, facilitating ubiquitin-independent degradation and/or dephosphorylation of this species of tau by conformational changes (Shimura et al., 2004).

The findings described in the current study could be explained in part by the evidence that hsp27 has multiple anti-apoptotic actions, for instance hsp27 is known to bind to cytochrome c, after its release from the mitochondria, and pro-caspase-3 thus preventing apoptosome formation and events downstream of mitochondrial damage, as well as blocking Daxx-mediated

apoptosis (see review by Concannon et al., 2003 and Parcellier et al., 2003a). The precise mechanism of how hsp27 mediates its protective effect in various situations is very complex and not completely clear, for instance hsp27 can act together with various intrinsic survival promoting factors, such as other heat shock proteins but this needs further investigation.

The anti-apoptotic actions of hsp27 in this system are very important, in view of the results obtained by TUNEL and Annexin/PI staining (and will be discussed further in Chapter 5 in view of further findings), but it cannot be excluded that there could also be an involvement of the chaperone activity of hsp27, which might assist proper folding of potentially aggregated or misfolded mutant or wt α-Syn or protein degradation in a ubiquitin-independent manner as shown by Shimura et al. (2004) in their tau system or by Parcellier et al. (2003a) in a system where hsp27 under stress favors ubiquitinated and phosphorylated I-κBα (main inhibitor of NF-κB) proteasomal degradation. The latter finding suggests a novel hsp27 function that provides another explanation of its antiapoptotic activities through the increase of NF-κB activity.

There is also evidence demonstrating that hsp27, as well as other hsps, co-localize with α-Syn in the Lewy bodies of PD brains (McLean et al., 2002) and therefore there might be an important link between hsp27 and α-Syn *in vivo*. However, in the same study, hsp70 and other hsps, but not hsp27, were able to suppress the *in vitro* aggregation of α-Syn in a model that utilised neuroglioma cells. Aggregation in the present system was not studied as it was observed that the α-Syn antibody did not work well on the non-denatured epitope under the conditions tested resulting in intense background staining. For this reason, priority was given to the cell death assessment and protection.

The fact that hsp70 is not very efficient in protecting the cell lines from apoptotic cell death in our system is not in agreement with reports that show protection from apoptosis in non-neuronal cell systems (Samali and Cotter,

1996). Auluck et al. (2002) also demonstrated that directed co-expression of wt or mutant α-Syn and human hsp70 in *Drosophila* prevented α-Syn-associated dopaminergic neuronal loss, although the LB-like inclusions in α-Syn expressing flies did not change in morphology, number or distribution upon hsp70 co-expression. Interfering with the endogenous chaperone activity of the constitutively expressed fly hsp70 was neurotoxic and the same group proposed that α-Syn-mediated neurotoxicity is as a result of interference with chaperone activity (Auluck et al. 2002).

More recently, the first evidence for an effect of hsp70 on reducing high molecular weight and detergent insoluble α-Syn species in an *in vivo* mammalian model was reported (Klucken et al., 2004). In this study, where α-Syn and hsp70 transgenic mice were crossed, there is evidence that hsp70 reduces high molecular weight α-Syn and the detergent insoluble species of α-Syn (but it does not change the total amount of soluble monomeric α-Syn). The same study reports protection from α-Syn toxicity by hsp70 (which is not in agreement with findings of the present study) in an *in vitro* model of α-Syn aggregation as well as reduction of detergent insoluble α-Syn species. Further studies are necessary though, to explain the mechanisms of the above effects and whether hsp70 affects the viability of dopaminergic neurons of these mice. Given the fact that the work by Auluck et al. (2002) suggested that in *Drosophila* hsp70 protection from α-Syn toxicity can be dissociated from prevention of α-Syn aggregation, it would be interesting to see if hsp70, apart from reducing aggregation, could also ameliorate dopaminergic cell death and improve the phenotype of the *in vivo* model described by Klucken et al. (2004).

The stimulus-dependent protection of wt α-Syn expressing cells by hsp70 in our system is difficult to be directly compared with the above findings where hsp70 has a protective role against toxicity of wt α-Syn in a non-mammalian system (Auluck et al., 2002) or in H4 neuroglioma cells (Klucken et

al., 2004), but may support our suggestion that wt α-Syn may differentially modulate cell death in different stressful situations (Zourlidou et al., 2003).

It is possible that hsp70 is not very effective because hsp70 was expressed after and not simultaneously with α-Syn in the ND7-derived cell-lines. If it is assumed that α-Syn confers toxicity in this system due to protein aggregation-related events, then delivering hsp70 after aggregation has occurred will not help, since it is known that hsp70 does not refold already misfolded/denatured proteins. Indeed, hsp104 (acting with hsp40 and hsp70 to rescue previously aggregated proteins) is the only hsp known to refold already aggregated/misfolded proteins (Glover and Lindquist, 1998). Hsp104 is the most crucial thermotolerance-related protein in yeast capable of disaggregating prions (Kushnirov et al., 2000). It has homology with torsinA, a protein that is present in LBs (Shashidharan et al., 2000) and was shown to act as a molecular chaperone suppressing α-Syn aggregation (McLean et al., 2002) and protect against various cellular insults (Shashidharan et al., 2004) in *in vitro* cellular systems. Therefore, various other combinations of hsps or the use of pharmacological agents, such as geldanamycin (see study by Auluck and Bonini, 2002) that induce their expression could be used to test for enhanced neuroprotection.

Another explanation for this limited protective effect of hsp70 was initially thought to be the fact that for protection to be detectable in our system, it may be essential that hsp40 (an hsp70 co-chaperone that binds to and stimulates the ATPase activity of hsp70) is co-expressed with hsp70. This was investigated by dual-infection of the various cell lines using a virus expressing hsp40 together with the hsp70-expressing virus. Numerous studies have revealed that chaperone members hsp70 and hsp40 inhibit self-assembly of poly-Q proteins into amyloid- like fibrils (Muchowski et al., 2000) or suppress both their aggregation and toxicity (Sherman and Goldberg, 2001). However, the data presented in the present study suggests that no protective effect is conferred in our model system by the co-expression of hsp70 and hsp40. This

could be due to cell type dependency of the protective effect of hsp overexpression. Alternatively, a hypothesis could be that the disease-associated mutants of α-Syn not only enhance its death-inducing properties but also render it refractory to hsp70 or hsp70 and hsp40-mediated neuroprotection. It also possible that a potential ATP-depletion in these cells under the stresses used does not allow hsp70 to function, since its activity is ATP-dependent. In contrast, hsp27 under the same conditions protects due to its activity which is not ATP-dependent. However, it is notable that hsp70 has some protective effect over the damaging effect of wt α-Syn, but this is stress specific, i.e. it protects under conditions of simulated ischaemia, as shown by total death assays but not by apoptotic assays, but not from staurosporine induced apoptosis. Therefore, it can be postulated that hsp70 might play a role in protecting from non-apoptotic cell death in this system.

In addition, the finding that hsp70 protects all the ND7-derived cell lines from severe heat shock (20 min heat shock at 48°C followed by 1 h recovery at 37°C) to a similar extent to hsp27, indicates a stress specific response. Therefore, it can be postulated that over-expression of hsps confer protection which is not only cell type dependent, but also stress specific. It is possible that the stress itself induces other gene expression alterations that cause an effect in combination with the hsp that is being over-expressed or independently. The fact that hsp70 has a limited neuroprotective effect compared to hsp27 in the system described here cannot be explained in detail here as further experiments are essential; hence any of the above explanations remain speculative.

Although the cellular model presented here is not the ideal cellular model to study PD - mainly because of its non-dopaminergic origin - it remains a very useful mammalian neuronal system where an interesting effect of α-Syn and the PD-associated mutants has been observed (Chapter 3). This is an advantage compared to the reported use of various non-neuronal or non-mammalian systems. It has allowed testing for the first time the protective effect

of various hsps against α-Syn-induced cell death in a mammalian neuronal system. Most importantly, this system helped to conclude that hsp27 is a potent neuroprotective agent against mutant α-Syn-associated toxicity; this is the first report to show mitigation of wt and mutant α-Syn neurotoxicity in an *in vitro* mammalian neuronal system (Zourlidou et al., 2004).

The distinct death stimuli used at various time intervals (serum withdrawal, dopamine, ischaemia, staurosporine and heat shock) were selected on the basis of having been previously characterised in ND7 cells (as explained in Chapter 3) and being representative stresses inducing both apoptotic and necrotic cell death in neuronal cells, which are modes of neuronal death implicated in PD pathology (see review by Tatton et al., 2003). In addition, there is accumulating evidence that protein misfolding and proteasome system activation occur during apoptosis (Soldatenkov et al., 1998). Therefore, the apoptotic stimuli used here represent some of the situations occurring in PD, which is protein misfolding and proteasome system activation that can also happen during the course of apoptosis (see reference above). The highly efficient gene delivery of hsps by viral vectors, on the other hand, assists the study of the potentially protective effects by one or more hsps and the biochemical dissection of mechanisms of protection in this system.

The experiments which involved cell death assessment by annexin V / PI showed that the majority of dead cells are apoptotic (annexin V positive but PI negative) and very few are secondary necrotic cells that have lost their cell membrane integrity and uptake PI (annexin V and PI positive). The ability to exclude dyes such as trypan blue or PI is a property of cells that have an intact plasma membrane (i.e. early phases of apoptosis). On the other hand, early necrotic/late apoptotic cells have lost membrane integrity and therefore easily stain with the above dyes. It should be noted here also that after certain passage numbers ND7 cell lines were difficult to maintain, as expression of the transgenes was eventually lost and therefore only a small number of experiments assessing cell death using annexin V and FACS analysis were

performed, although these were enough to provide an indication to the trend observed earlier in terms of protection by hsp27.

As explained in the Introduction of this Chapter, annexin V staining was performed according to Vermes et al. (1995). The time between shifting from an early apoptotic stage with intact cellular membrane integrity (see cells in B4 square of FACS plots) to a stage where this membrane integrity is lost (late apoptotic or secondary necrotic cells in B2 square of FACS plots) can be variable and depends on the cell type and the experimental conditions. Cells were harvested and incubated with annexin/PI for 15 min and subsequently analyzed within 30 min time by flow cytometry. When cells were harvested for the trypan blue assay the cell death assessment was more time consuming because of the larger sample numbers that had to be counted in a haemocytometer. This means that the samples had to be left on ice for more time and, therefore, it is a possibility that apoptotic cells might lose their membrane integrity relatively faster and uptake the trypan blue dye, resulting in high number of dead or dieing blue cells in the relevant experiments.

TUNEL on the other hand, was performed on adhered cells on glass slides and therefore after the end of the treatment period the media were aspired, the cells were washed, fixed and permeabilised prior to TUNEL, which labeled fragmented DNA in the cell nuclei undergoing apoptosis. As a result many of the cells that had died and were already detached form the plate at the end of the treatment were not counted. However, by comparing the percentage of TUNEL positive cells per cell line we can get a fairly accurate estimate of the relative levels of apoptotic cell death in these different cell lines. It is usually suggested in the literature that different methods of cell death assessment should be applied before one can conclude on certain research questions, as well as assessing cell death at multiple time points.

From the present study it becomes clear that no single method of cell death quantification is precise and totally informative and that conclusions should be drawn very carefully after reconfirming a trend in cell death

susceptibility by alternative methods. It would have been easier and less time consuming, although more costly, to perform FACS analysis with TUNEL and chose a fluorimetric assay instead of the trypan blue assay (that is associated with cellular toxicity and generally is not a very sensitive method). Trypan blue has also the disadvantage of color intensity variation (amount of dye uptake varies depending on cell state) and this can lead to under or over estimation of the dead cells. In order to generate reliable and unbiased data it is therefore important to set the same thresholds at all experiments, in which cell counts are taken in a blind fashion by the same researcher.

Overall, both trypan blue and TUNEL assays (in cells in suspension and on adhered cells on slides, respectively) have been extensively used and remain valid and reliable assays for cell viability and apoptotic cell death assessment respectively. By comparing the cell death of a cell line to the appropriate control cell line, an effect due to treatment can be observed by the relative differences, as it is the case in the present study. Annexin and PI use with flow cytometry is also a reliable technique for quantification of apoptosis and necrosis. This method was used here as an alternative way of reconfirming the observed trend in cell death observed by the other methods and also the type of death, which is mainly via apoptosis.

Finally, some other points that need to be considered include the fact that the α-Syn cDNA is human and the disease mutations are found in humans with autosomal dominant PD, whereas the ND7 cell line is rat/mouse hybrid, hsp27 is from hamster, hsp56 from rabbit, hsp40 from human and hsp70 is the human inducible hsp70. However, hsps are highly conserved proteins and also trans-species experiments are frequently performed (i.e. human hsp70 over-expressed in mice and so on). For example, in a report with α-Syn transgenic *Drosophila*, human α-Syn is expressed in the fly (that does not have an α-Syn homologue), but it produces intracytoplasmic inclusions similar to LBs in PD brains. In the same study, the human inducible hsp70 is co-expressed with α-Syn and protects against dopaminergic neuronal loss (Auluck et al., 2002). It is

difficult to predict if the species' origin of the cDNAs used in the present study are of significance but they need to be considered.

In summary, this body of work has demonstrated for the first time that mammalian neuronal cells over-expressing wt and the disease-associated mutant forms of α-Syn are protected from various death stimuli by exogenous hsp27, providing a novel neuroprotective role for hsp27 against α-Syn induced neurotoxicity. Further studies are necessary in order to clarify the mechanisms of neuroprotection, since there is clearly a potential therapeutic role of hsps in many neurodegenerative diseases. In particular, manipulation of endogenous cellular defence mechanisms such as the heat shock response, through nutritional antioxidants or pharmacological compounds, may represent an approach to therapeutic intervention in neurodegeneration. The following Chapter attempts to suggest a possible mechanism for this neuroprotection by investigating the role of hsp27 in pathways of apoptotic cell death upstream/downstream the mitochondrion.

CHAPTER 5

Investigation of the Mechanism of Action of Hsp27-mediated Protection in an *In Vitro* Model of α-Synuclein-Induced Toxicity

5.1 Introduction

The previous chapter dealt with the characterisation of the protective effect of heat shock proteins in an *in vitro* model of α-Syn-induced toxicity. This chapter provides data that attempt to address a possible mechanism for the protection conferred by hsp27, as this hsp was shown to uniformly protect from cell death under the various stresses used in the previous chapter.

The data presented in the previous chapters suggest that, at least in the cellular model system described in this thesis, apoptosis seems to play an important role in the cellular loss provoked by various stressful stimuli; it was therefore hypothesised that hsp27 might confer protection due to its anti-apoptotic properties (reviewed in Chapter 1 and Chapter 4) and hence, apoptotic pathways at the level of the mitochondrion and downstream of the mitochondrion were investigated. Mitochondria are important organelles, because they generate energy for the cell and also because of the major role they play in apoptosis (Kroemer and Reed, 2000; Desagher and Martinou, 2000). Moreover, a number of studies have linked mitochondrial dysfunction to PD. The observation that MPTP produces a Parkinsonian syndrome in experimental animals and humans through interference with energy production in the mitochondria and a resultant increase in the production of oxygen free radicals led to studies of mitochondrial function in the brains from patients with PD. Schapira et al. (1992) found that complex I activity was selectively reduced in the substantia nigra of patients with PD. Swerdlow et al. (1996) also reported a significant reduction in complex I activity in platelets from patients with PD. A strong link has also been established between exposure to the pesticide rotenone, a well-defined and specific inhibitor of complex I, and Parkinson's disease (Jenner, 2001; Di Monte, 2003).

Overall, in both PD in humans as well as in animal models of PD mitochondrial dysfunction seems to play a central role in the pathogenesis of the disease (reviewed in Chapter 1). In the context of the study presented here, there are a small number of reports suggesting that, at least in *in vitro* model systems,

α-Syn may exert some of its toxic effects in neuronal cells *via* alterations in the mitochondria (Hsu et al., 2000; Tanaka et al., 2001; Sherer et al., 2002). It was therefore decided that the present study should be extended to investigate if this hypothesis is a possibility in the model described here and whether hsp27 acts by interfering with such pathways, having already established that hsp27 reduces toxicity in this system. The strategies employed aimed firstly to investigate the involvement of the caspase cascade, and secondly to utilize mitochondrial selective probes in order to examine possible alterations in the mitochondrial membrane potential, $\Delta\Psi m$.

At early stages of apoptosis the disruption of active mitochondria is evident. This disruption includes changes in the mitochondrial membrane potential, $\Delta\Psi m$, and alterations to the oxidation-reduction potential of the mitochondria (see review by Ly et al., 2003). $\Delta\Psi m$ is the electrochemical gradient resulting from the respiration-driven, electron transport chain-mediated pumping of protons out of the inner mitochondrial membrane and is indispensable for driving the ATP synthase. The changes in $\Delta\Psi m$ are thought to be due to the opening of the mitochondrial permeability transition pore that allows ions and small molecules to pass. The resulting ion equilibration leads in turn to the decoupling of the respiratory chain and cytochrome c release into the cytosol. In fact, the opening of the mitochondrion permeability transition pore causes an increase of the permeability of mitochondrial membrane and the release of several types of apoptogenic factors from the intermembrane space, such as cytochrome c, apoptosis inducing factors (AIFs), procaspases and Ca^{2+}. These apoptotic factors can either activate caspases or independently degrade the intranuclear chromatin, or interact with other Ca^{2+}-dependent proteins (see reviews by Kroemer and Reed (2000) and Ly et al. (2003).

In situ, $\Delta\Psi m$ is a sensitive indicator for the energetic state of the mitochondria and the cell (Brand et al., 1994) and many cationic fluorescent probes have been used in combination with flow cytometry or fluorescence microscopy to measure $\Delta\Psi m$. The experiments presented here utilize two cell-permeant mitochondrion-selective fluorescent dyes, MitoTracker Green FM

(MTG) and tetramethylrhodamine methyl ester (TMRM). The MTG probe is essentially non-fluorescent in aqueous solutions and only becomes fluorescent once it accumulates in the lipid environment of mitochondria, whilst the uptake of TMRM is dependent on the mitochondrial membrane potential. It is possible therefore to measure by FACS the fluorescent intensity in the various α-Syn cell lines that have been stressed and obtain a measurement of the relative reduction in mean fluorescence intensity, which can be interpreted as an indication of the mitochondrial membrane potential dissipation (Zoratti and Szabo, 1995). The application of the protonophore carbonyl cyanide chlorophenylhydrazone (CCCP) at certain concentrations collapses the $\Delta\Psi m$ and is therefore used as a positive control for such experiments.

The results presented here provide evidence for the involvement of caspase activation and reduction in the mitochondrial membrane potential ($\Delta\Psi m$) as a result of α-Syn toxicity in the model described, while exogenous hsp27 over-expression suppresses such toxic effects. The results are evaluated and discussed and further experiments are suggested in order to fully understand the mechanism and site(s) of action of hsp27 in the context of α-Syn toxicity.

The aims of this chapter are therefore listed below:

(a) To address the role of caspases in this system by using specific caspase inhibitors and compare their effect on cell survival with the effect of hsp27 over-expression. (b) To utilise mitochondrial specific fluorescent probes (MTG and TMRM) and FACS analysis in order to examine possible changes in the mitochondrial membrane potential ($\Delta\Psi m$) in these cell lines in the absence or presence of hsp27 over-expression under stress. (c) To evaluate the potential of the anti-apoptotic properties of hsp27 in this particular system and suggest alternative mechanisms of action that should be tested.

5.2 The Effect of Hsp27 and Caspase Inhibitors on Wild Type and Mutant α-Syn-induced Cell Death

The observed protective effects by wt α-Syn, following serum withdrawal and dopamine induced toxicity (described in Chapter 3), and by hsp27 under all stresses (Chapter 4) in the described cellular model required further investigation. Initially a pan-caspase inhibitor (Z-VAD-FMK) and caspase-8 and caspase-9 specific inhibitors (Z-IETD-FMK and Z-LEHD-FMK, respectively) were employed to investigate whether inhibition of all or parts of the caspase cascade could influence the amount of survival in the α-Syn cell lines. Serum withdrawal was chosen as the stress stimulus given the differential responses between the wt and A30P mutant α-Syn cell lines described in the previous chapters. These experiments were extended to investigate whether protection conferred by the exogenous expression of hsp27 involved modulation of the caspase pathways.

Whilst all three caspase inhibitors produced no significant effects on cell survival in the wt α-Syn cell lines following serum withdrawal, as illustrated in Figure 5.1, it was found that Z-VAD (the pan-caspase inhibitor) and specific inhibitors of both caspase-8 and caspase-9 suppress cell death in the A30P mutant α-Syn cell lines to the level observed in the control cell lines, and this difference in cell death is statistically significant ($p<0.001$). Similarly, the degree of protection observed in these A30P cells following caspase inhibition was similar to that afforded by the exogenous expression of hsp27 in these cell lines (cell death in A30P α-Syn cells infected with hsp27 virus was reduced up to 46% compared to these same cells infected with control GFP virus). It should be noted here that the cell death was assessed by both trypan blue exclusion assay (Figure 5.1) and annexin V / propidium iodide staining (Figure 5.2) and that only the A30P mutant was tested because the A53T α-Syn lines had to be discarded as they were no longer expressing the transgene.

Again, wt α-Syn cells were shown to be protected by the over-expression of α-Syn under this particular stress (as reported in Chapter 3) and

no further suppression of death was observed in these cells upon the expression of hsp27 (Chapter 4). Interestingly, when hsp27 was over-expressed in these cells in combination with addition of any of the three caspase inhibitors, there was no indication of an additive protective effect, since there is no statistically significant difference in cell death between cell lines expressing hsp27 and the respective cell lines expressing hsp27 in the presence of caspase inhibitors. In addition, caspase inhibitor only treated cells have no significant difference in cell death compared to hsp27 expressing cells ($p<0.05$, significant differences not depicted in figure) (Figure 5.1).

Similar results were also obtained in repeated experiments in which the percentage of apoptotic cells was assayed using Annexin V / propidium iodide staining following the same protocol and FACS settings as described in Chapter 4 (Figure 5.2). It is clear that upon the application of all three caspase inhibitors to LacZ virus infected-A30P cell lines there is again a significant reduction in cell death (up to 40% reduction in apoptotic cells), compared to their respective LacZ / control cells ($p<0.05$). Furthermore, no synergy in reducing the number of apoptotic cells was observed following the application of the caspase inhibitors to the A30P cell lines that were over-expressing hsp27. In addition, in each treatment group, apoptotic cell death in mutant α-Syn expressing cells is not significantly different in between them, with the exception of GFP expressing / A30P α-Syn cells that have a significant difference in apoptotic cell death compared to all the A30P cells with different treatments ($p<0.05$).

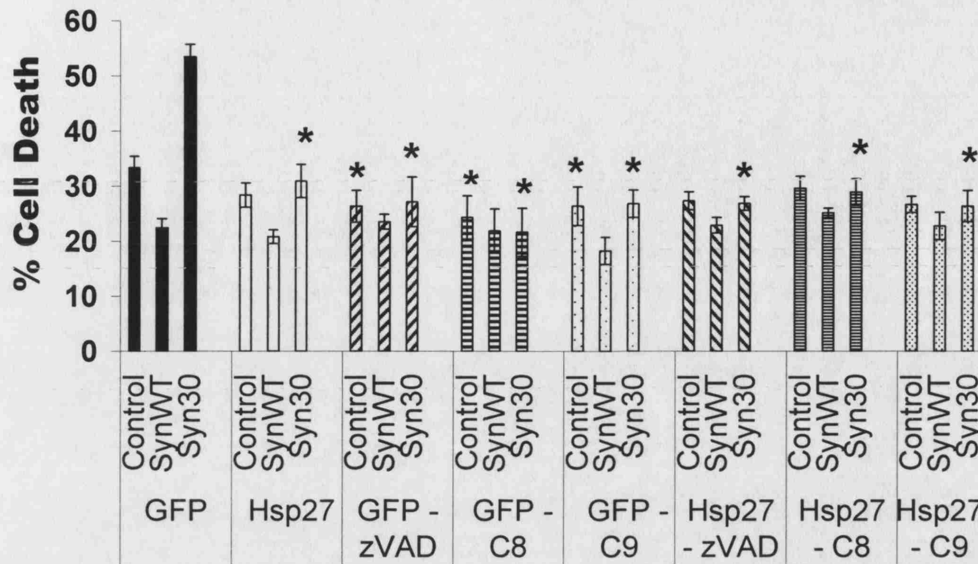


Figure 5.1 Levels of cell death of ND7 cells after infection with GFP or hsp27 expressing viruses or incubation with caspase inhibitors and following 24 h serum removal.

Control vector transfected cells, wt and A30P α-Syn transfected cells were infected with GFP or hsp27 expressing viruses 16 h prior to incubation with Z-VAD, caspase-8 (C8) or caspase-9 (C9) inhibitors and serum free media with DMSO vehicle where appropriate. Cell death was assessed by the trypan blue exclusion assay in three independent experiments. Bars represent mean ± S.D. calculated for triplicate counts per sample n (n=3) and significant differences were calculated using a Bonferroni Multiple Comparison's *t* test after one-way ANOVA. * indicates statistically significant difference between means, when comparing with the respective control-vector, wt and A30P α-Syn GFP virus infected cell line ($p<0.001$).

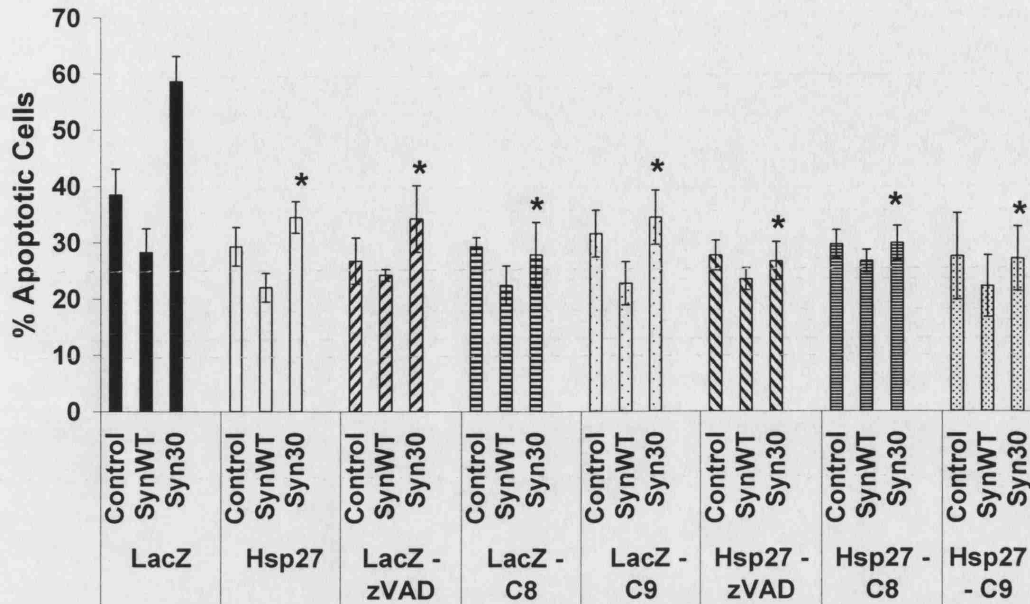


Figure 5.2 Percentage of apoptotic cell death of engineered ND7 cells after infection with LacZ or hsp27 expressing viruses or/and incubation with caspase inhibitors followed by 24 h serum removal.

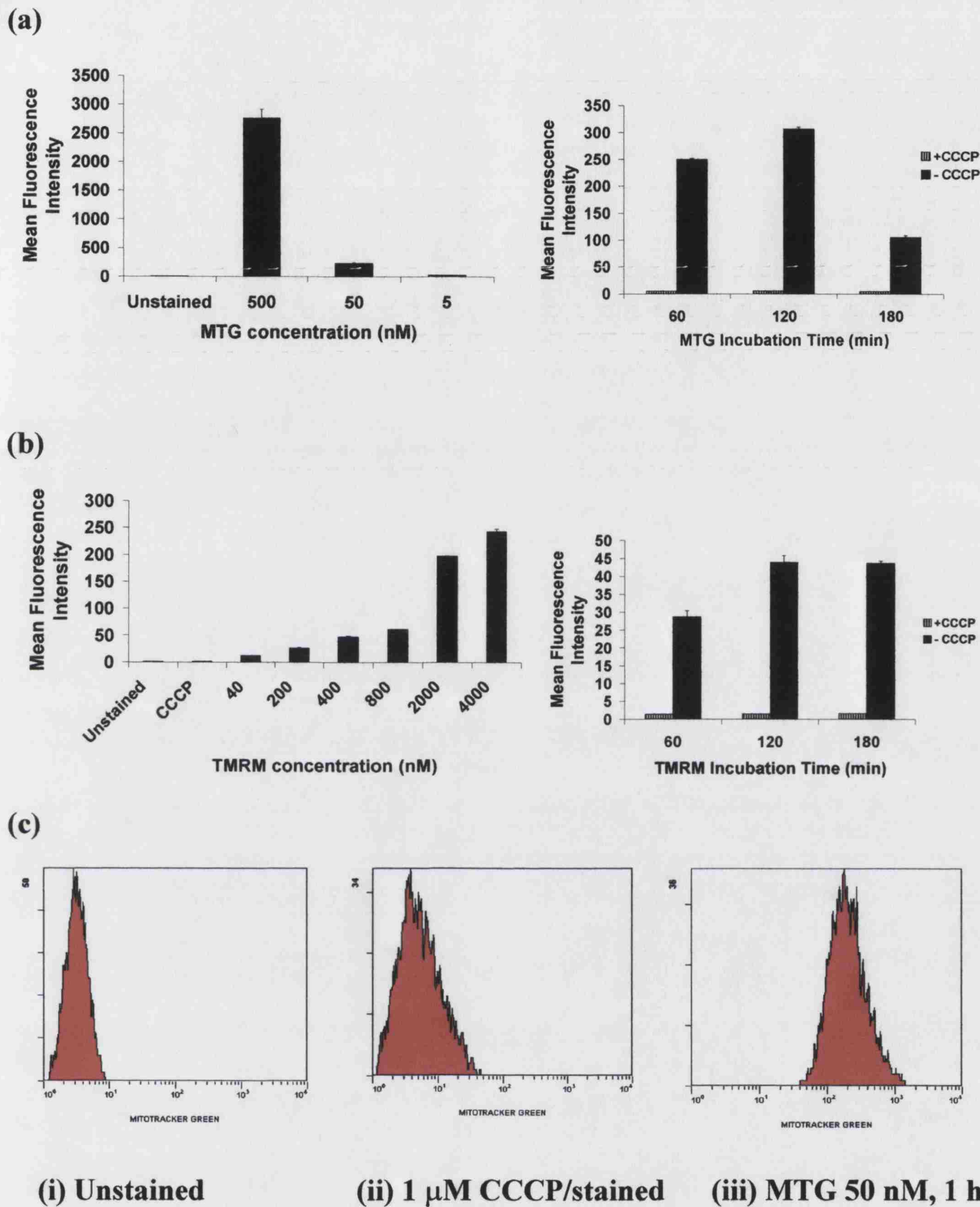
Control vector transfected cells, wt and A30P α-Syn transfected cells were infected at 10 m.o.i. with control LacZ or hsp27 expressing viruses 16 h prior to incubation with Z-VAD, caspase-8 (C8) or caspase-9 (C9) inhibitors and serum free media with DMSO vehicle where appropriate. Apoptotic cell death was assessed by flow cytometry of phospholipid redistribution by annexin V / PI assay at 24 h from serum removal in two independent experiments, in order to confirm the findings in Figure 5.1. Bars represent mean ± S.D. calculated for four counts per sample n (n=2, N=8) and significant differences were calculated as above with $p<0.05$. * indicates statistically significant difference between means, when comparing with the respective control-vector, wt and A30P α-Syn LacZ virus infected cell line.

5.3 Changes in the Mitochondrial Membrane Potential in α-Syn Expressing Cells Under Stress

Along with the efforts described in the previous section, the mitochondrial membrane potential ($\Delta\Psi m$) was also studied in order to determine whether the enhanced mutant α-Syn-induced toxicity is associated with a reduction in the $\Delta\Psi m$. There are a number of reagents commercially available for the study of changes in the $\Delta\Psi m$ during apoptosis; two of them (MTG and TMRM) were used for the purposes of this study. These mitochondria-selective fluorescent probes have not previously been used with FACS analysis of ND7 cells and it was necessary therefore to optimise the amount of dye used and the incubation time required for this analysis. The FACS analysis settings have been described in Chapter 2.

5.3.1 MTG and TMRM Probe Optimisation for Engineered ND7 Cells

As detailed in Chapter 2, green (525nm) and orange (575nm) fluorescence emissions were collected using logarithmic amplification. The mean fluorescence intensity (MFI) was determined after exclusion of debris by using 7AAD. The disruption of the mitochondrial membrane potential that occurs in early apoptotic stages leads to reduction in the MFI. The mean values of MFI were used for each sample in the graphic representation of changes in $\Delta\Psi m$. The data were therefore analysed using the MFI in order to determine the reduction in fluorescence relative to the depolarisation of the mitochondria. The mitochondrial uncoupler CCCP was used throughout as a control for the irreversible disruption of the mitochondrial membrane potential. At least 20,000 cells were acquired using a flow cytometer and the cells were gated as described in Chapter 2. Figure 5.3 shows the determination of the dye concentrations and incubation times used in subsequent experiments. The concentrations were selected on the basis that they provided adequate fluorescent signal without cellular toxicity and for MTG it was 50 nM, whereas for TMRM 400 nm. Both dyes were added in the cell culture media one hour prior to wash and flow cytometry.



<u>Figure 5.3</u> Determination of the incubation time and concentration of fluorescent probes used in ND7 cells.

The smallest possible concentration and incubation time was selected provided that it could give adequate fluorescence to be measured and this was **(a)** 50 nM for MTG for 1 h and **(b)** 400 nM for TMRM for 1 h. Panel **(c)** illustrates examples of representative FACS data for MTG staining, which are similar to those obtained with TMRM staining (not shown here).

5.3.2 The Effect of Wild Type or Mutant α-Syn on the Mitochondrial Membrane Potential of Cells Exposed to Various Stresses

Having determined the optimum experimental conditions for the two probes, a series of experiments were carried out to determine whether the expression of either form of α-Syn exerted an effect, in the presence or absence of the stress stimulus, on the mitochondrial membrane potential by utilising two clones per cell line/construct as assayed with MTG. Because the MFI values obtained in different experiments are occasionally variable, thus masking any changes, the mean MFI values from each experiment are normalized by being expressed relatively to their respective control-vector transfected ND7 cells. Data of this type have been previously reported in the same fashion (Mocanu et al., 2001).

In these experiments it was observed that, in stresses under which wt α-Syn is protective (serum withdrawal or incubation with dopamine) cells exhibit an increased MFI (interpreted as an increase in their $\Delta\Psi m$) compared to control vector transfected cells, whereas A30P α-Syn cells show a decrease in $\Delta\Psi m$. These differences in the relative MFI of different cell lines are illustrated in Figure 5.4 and are statistically significant, as indicated by the asterisk ($p<0.001$). The results presented in Figure 5.4 suggest that control cells and wt α-Syn cells have healthier mitochondria than the cells expressing A30P α-Syn. CCCP was used as a control to disrupt the mitochondrial membrane potential and consistently had a devastating effect on MFI (data not shown). Following a challenge with 1μM staurosporine, where wt α-Syn is toxic, no significant alteration in the mean MFI as compared to control was observed. In contrast, a significant reduction in MFI was observed in A30P cell lines when challenged with staurosporine compared to control or wt α-Syn cells ($p<0.001$). In addition, mutant A30P α-Syn cells exhibit reduced MFI, i.e. mitochondrial membrane potential disruption also in the absence of stress, which might be due to the presence of stress under those particular culture conditions, in which cells are induced by dexamethasone to over-express the mutant protein.

The trends shown here therefore extend the previous findings demonstrating the differential effect of wt α-Syn on cell death and the damaging effect of A30P mutant in response to a variety of stressful stimuli (Chapter 3).

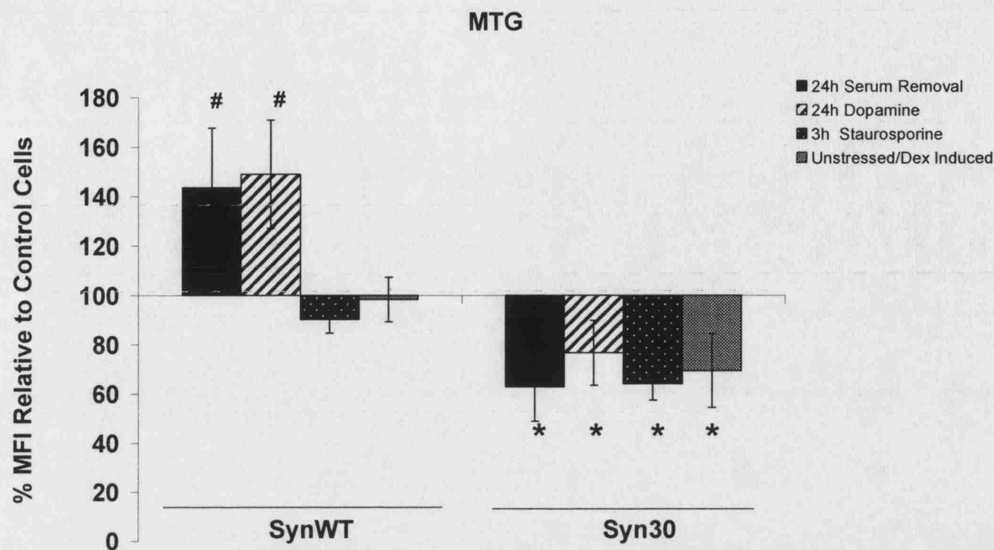


Figure 5.4 Mean fluorescence intensity of wt and A30P α-Syn cells relative to control vector transfected ND7 cells.

Two different clones from each control-empty vector, wt and A30P mutant α-Syn expressing cell lines had been treated with 24 h serum removal, 24 h 62 μM dopamine, 3 h 1 μM staurosporine or incubated with normal growth medium, prior to incubation with MTG for 1 h followed by FACS analysis. Mean Fluorescence Intensity (MFI) values are expressed as a percentage of the respective control vector ND7 cells. Bars represent mean value ± S.D. calculated for triplicate counts per sample (n=4). Significant differences were calculated using a Bonferroni test after one-way ANOVA ($p \leq 0.05$). # indicates statistically significant difference between relative means, when comparing stressed SynWT cells or stressed Syn30 cells to the respective unstressed cells. Significant differences (indicated by *, $p < 0.001$) between SynWT and Syn30 cells within one treatment were calculated by using a two-tailed *t*-test for paired samples.The mitochondrial uncoupler CCCP was always used as a control to collapse the mitochondrial membrane potential (not shown here).

5.4 The Effect of Hsp27 Over-expression on the Mitochondrial Membrane Potential in α-Syn Expressing Cells Under Stress

To investigate whether hsp27 overexpression leads to an increase in the MFI (hence in the $\Delta\Psi m$) of cells expressing wt or mutant α-Syn, prior to a tested example stress (serum withdrawal or staurosporine), two dyes were used in parallel in two clones from each cell line. The data were pooled in order to be analysed (Figure 5.5). Two dyes are utilised here, since the reliability of mitochondrial selective fluorescent probes and their accuracy in measuring $\Delta\Psi m$ have been subject to criticism (Duchen et al., 2003) as discussed later. For reasons of simplicity not all calculated significant differences are depicted in the figures.

Using MTG, it is clear that following serum withdrawal SynWT cells infected with LacZ virus have a statistically significant increase in MFI compared to either control and A30P α-Syn cell lines infected with LacZ virus ($p<0.001$, not indicated in Figure 5.5.a). Conversely, a significantly lower MFI was observed in LacZ / Syn30 cell line compared to LacZ / control cell line ($p<0.001$).

The same trends were observed when these cell lines were infected with hsp27 virus instead of LacZ (Figure 5.5.a). In addition, by comparing the MFI of hsp27 expressing cells with the respective LacZ cells, it was shown that there is a statistically significant difference between the MFI of all cell lines ($p<0.001$), indicating that hsp27 has a beneficial effect by increasing $\Delta\Psi m$ in these cells under this particular stress (Figure 5.5.a). These increases in MFI due to hsp27 expression were approximately 33% for control cells, 22.2% for wt α-Syn and 32% for the mutant A30P.

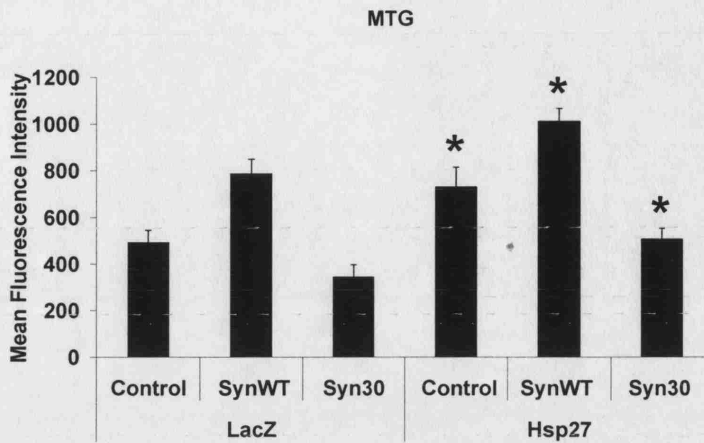
When using TMRM (Figure 5.5.b), statistical analysis of the results of three experiments indicate that no significant difference in the MFI values between LacZ virus infected control cells and hsp27 virus infected control cells. At the same time a significant 40.5% increase in MFI (hence in $\Delta\Psi m$) was

observed in mutant A30P cell lines infected with hsp27 virus when compared to those infected with control virus ($p<0.001$). The same is true for wt α-Syn cell lines infected with hsp27 virus when compared to those infected with control virus (23.3% increase in MFI), although the increase in MFI was not as dramatic as in the mutant cell line ($p=0.016$).

These findings, which will be discussed later, suggest that there is only a slight difference in the results obtained by using different dyes. Overall, there is a clear trend showing that hsp27 helps the cells to maintain a relatively higher $\Delta\Psi m$ compared to cells expressing a control protein instead under the same conditions.

Similarly, Figure 5.6 illustrates the findings after incubation with 1 μM staurosporine for 6 h. Under these conditions, wt α-Syn is known to be deleterious compared to control vector cells but at a lesser extent than mutant α-Syn. As shown in Figure 5.6, data obtained with both dyes revealed that wt α-Syn expression in cells infected with the control virus have a significantly lower relative MFI compared to these same cells when infected with hsp27 virus ($p<0.001$ for MTG and $p=0.02$ for TMRM). More importantly, A30P cell lines infected with hsp27 virus have significantly higher MFI (hence $\Delta\Psi m$) compared to these same mutant cell lines infected with control virus ($p<0.001$ for both MTG and TMRM). These increases in relative MFI are 44.5% (MTG) or 25% (TMRM) for wt α-Syn and 53.8% (MTG) or 76% (TMRM) for the mutant A30P (Figure 5.6). The above percentages of relative MFI increase are obtained by comparing groups infected with LacZ control virus and groups infected with hsp27 virus and they are indicative of a beneficial effect due to hsp27 over-expression.

(a)



(b)

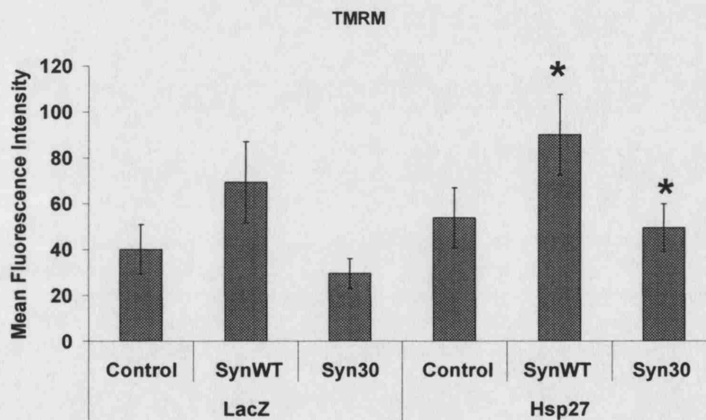
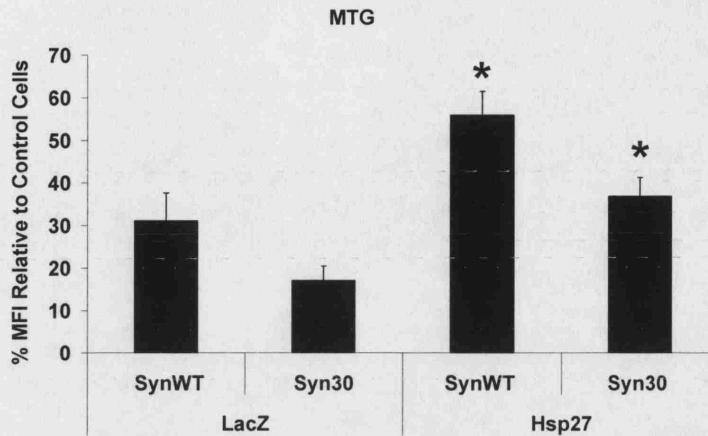


Figure 5.5 Mean fluorescence intensity determined by FACS analysis in cells incubated with mitochondria specific fluorescent probes 24 h after serum withdrawal.

Mitotracker Green (MTG) **(a)** and tetramethylrhodamine methyl ester (TMRM) **(b)** were added in the serum free media of cells the had been previously infected, 16 h prior to serum withdrawal, with either a control LacZ expressing virus or an hsp27 expressing virus. Two clones were used from each engineered cell line and two different probes were used simultaneously. Bars represent mean ± S.D. calculated for triplicate counts per sample n (n=3). Significant differences were calculated using a Bonferroni Multiple Comparison's *t* test after one-way ANOVA ($p<0.001$). * indicates statistically significant differences in means compared to the respective LacZ expressing cell line.

(a)



(b)

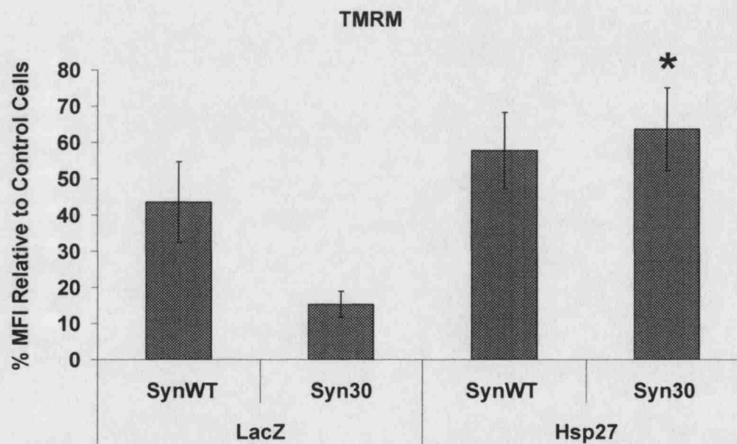


Figure 5.6 Mean fluorescence intensity of wild type and A30P α-Syn cells relative to control vector transfected ND7 cells expressing LacZ or hsp27 after staurosporine treatment.

Control-empty vector, wt and A30P mutant α-Syn expressing cell lines had been treated with 1 μM staurosporine for 6 h. One hour prior to FACS analysis **(a)** MTG and **(b)** TMRM were added in the cells simultaneously. Mean Fluorescence Intensity (MFI) values are expressed as a percentage of the respective control vector ND7 cells. Bars represent relative mean value ± S.D. calculated for triplicate counts per sample (n=3). Significant differences were calculated using a Bonferroni Multiple Comparison's *t* test after one-way ANOVA ($p<0.001$).* indicate significant difference when comparing with the respective LacZ expressing cells.

5.5 Discussion

In an attempt to identify a mechanism of protection by hsp27, the serum withdrawal protocol was chosen initially to test whether the use of specific caspase inhibitors and/or hsp27 over-expression could reduce the levels of cell death, particularly in the mutant A30P α-Syn expressing cells. The results presented in section 5.2 indicate that following serum withdrawal the mutant A30P α-Syn increases toxicity in ND7 cells by potentially increasing the activation of components of the caspase cascade in these cells, leading to increase in cell death. This sensitivity is abolished by inhibiting either caspase-8 or caspase-9 or by over-expressing hsp27. Interestingly, although A30P α-Syn expressing cells were markedly protected by either caspase inhibitors or by hsp27 over-expression, there was no synergy or additive effect between hsp27 and the caspase inhibitors for further protection to occur. Hence, it seems likely that the protection afforded by hsp27 in this model is at least in part a result of it impinging upon the pathways regulating the enhanced caspase-dependent cell death due to mutant A30P protein over-expression.

This study is the first to link hsp27-mediated protection from α-Syn induced toxicity with the anti apoptotic properties of hsp27 and extends previous reports that caspases play a role in α-Syn mediated toxicity. For instance, Alves da Costa et al. (2002) showed that in TSM1 neuronal cells α-Syn drastically lowers basal and staurosporine-stimulated caspase-3 immunoreactivity and activity and that 6-hydroxydopamine fully abolishes the α-Syn-mediated reduction of caspase-3 activity and reverses the associated decrease of p53 expression. Previously, Tanaka et al. (2001) showed that A30P α-Syn expression in PC12 cells leads to increased sensitivity to apoptotic cell death upon lactacystine treatment and this is characterized by elevation of the levels of caspase-3 and caspase-9. This latter study also identified a relationship between mutations in α-Syn and mitochondrial dysfunction, which is in agreement with the findings described in this chapter.

Caspases, apart from playing a role in α-Syn mediated toxicity have also been implicated in the pathogenesis of PD. *In vitro* and *in vivo* models of PD as well as studies in post-mortem PD brains have suggested a role for apoptotic cell death (reviewed in Chapter 1). Caspases are important components of the apoptotic cascade and there are two studies that demonstrate their involvement in PD. A study by Hartman et al. (2000) concluded that caspase-3 is a vulnerability factor for pigmented dopaminergic neurons, activated caspase-3 is increased in PD brain and more importantly it precedes chromatin condensation during apoptosis. In a second study by Viswanath et al. (2001) the role of caspases was addressed by using MPTP-treated mice, dopaminergic cells in culture and human post-mortem PD brain. MPTP elicits sequentially cytochrome c release, caspase-9, caspase-3 and caspase-8 activation and Bid cleavage in the substantia nigra of MPTP-treated mice, which are attenuated in the general caspase inhibitory protein p35 transgenic mice. Activated caspase-8 and caspase-9 are also found in the dopaminergic neurons MPTP-mice and PD patients. In this context, and relating it to our findings demonstrating the anti-apoptotic properties of hsp27 and caspase inhibitors, neuroprotective strategies utilising caspase inhibition can be envisaged, although it should be noted that there are potential side effects of neoplasia or induction of autoimmune disorders. Besides, the long-term viability and functionality of dopaminergic neurons rescued by caspase inhibitors remain to be determined.

In explaining the protective effect by hsp27 in our model, it should be noted that hsp27 has been shown to be a potent anti-apoptotic protein in a variety of systems (reviewed in Chapter 1 and Chapter 4). For instance, Kalwy et al. (2003) showed that, in an *in vivo* model of epilepsy (in which systemic administration of kainic acid causes neuronal loss particularly in the hippocampus) exogenous hsp27 protects from kainic acid-induced neuronal loss that is, at least in part, due to apoptosis. In addition, transgenic hsp27 mice have reduced kainate-induced seizure severity and mortality rate and reduced neuronal death in the CA3 region of the hippocampus, which are associated with a marked attenuation of caspase-3 induction (Akbar et al., 2003).

One of the proposed mechanisms of such activity is that demonstrated by Mehlen et al. (1996a) according to which hsp27 can increase the anti-oxidant defence of cells by decreasing reactive oxygen species. Hsp27 suppressed polyQ associated death (but not aggregation) by decreasing reactive oxygen species in cells expressing mutant huntingtin (Wyttenbach et al., 2002). In addition, hsp27 seems to neutralize toxicity due to oxidized proteins and this is via a mechanism modulated by its phosphorylation status but independent from interaction with cytochrome c. Garrido et al. (1999) showed that hsp27 inhibits cytochrome c dependent activation of procaspase-9 by directly sequestering cytochrome c released from mitochondria, whereas Bruey et al. (2000) reported that *in vitro* and *in vivo* non-phosphorylated large oligomers of hsp27 are required for hsp27 anti-apoptotic activity and that cell-cell contacts induce the formation of such oligomers. Hsp27 has also been reported to directly interact with Daxx and prevent apoptosis (Charette et al., 2000), to interact with capsase-3 (Concannon et al., 2001), and more recently to facilitate phosphorylated and ubiquitinated I-κBα proteolytic degradation (Parcellier et al., 2003b) and to bind pathological hyperphosphorylated tau and facilitate its degradation without ubiquitination (Shimura et al., 2004).

Therefore, for the first time, hsp27 was shown to reduce α-Syn associated toxicity in mammalian neuronal cells under various death stimuli with supporting evidence that this is due to its anti-apoptotic properties. Other pathways such as oxidative stress pathways upstream from or in parallel to caspase activation may also mediate neuronal cell death in this model. It is possible that hsp27 interferes also with such pathways, for a protective effect to occur. It should be noted, however, that in the present study as well as in various reported studies, although caspase inhibition / hsp27 overexpression can protect by successfully preventing morphological aspects of cell death, functional recovery in these cells is uncertain.

An additional interesting finding in the cellular model studied here is that the mitochondria seem to be affected by the expression of mutant A30P α-

Syn but not by wt α-Syn or control vector under several stress conditions such as serum removal, dopamine incubation or, at a smaller extent, under 24 h induction of α-Syn by dexamethasone without stress. This was assessed by measuring the mitochondrial membrane potential decrease by utilising FACS analysis and a mitochondrial-specific fluorescent probe (MTG). Whilst relating events involving the mitochondria with mutant α-Syn-induced neurotoxicity it is not possible to determine if the mitochondrial defects (possibly due to a mutant α-Syn-related mechanism) are a cause of cell death or if these defective mitochondria are one of the consequences of an already abnormal cell that is caused by A30P α-Syn toxicity acting directly on other neuronal components/pathways. The above disparity would be addressed by performing further experiments involving a detailed study of the mitochondria and signaling pathways in connection with α-Syn and at the present time only speculations could explain the mitochondrial membrane potential decrease in cells expressing mutant α-Syn. However, a clear trend was confirmed in this system, allowing proceeding with testing the hypothesis whether hsp27 overexpression leads to a relative increase of $\Delta\Psi m$ that correlates with the cell death data described in the previous chapter.

As mentioned previously, Tanaka et al. (2001) demonstrated that PC12 cells over-expressing mutant A30P α-Syn, in addition to having decreased proteasome activity, they are also more sensitive to mitochondria-dependent apoptosis. This study utilised a dual emission fluorescent dye (JC-1) depending on the mitochondrial membrane potential and concluded that in the presence of the proteasome inhibitor lactacystine A30P α-Syn cells have more depolarised mitochondria compared to wt α-Syn expressing cells, whereas cyclosporine A inhibits mitochondrial depolarisation and apoptotic death in those cells. In the present study, different fluorescent dyes are used combined with FACS analysis. Similarly, cyclosporine A (which binds to a mitochondrial form of cyclophilin and inhibits the mitochondrial permeability transition which induces mitochondrial depolarization and participates in the initiation of apoptosis) could have been used along with hsp27 over-expression to see whether there is a

restoration of the normal mitochondrial membrane potential after insult in the mutant A30P α-Syn expressing ND7 cells. Nevertheless, our results agree with those of the above study, regarding the mutant protein, in that these cells have increased mitochondria depolarization under stress or even under normal growth conditions compared to control vector or wt α-Syn expressing cells.

There are also a few other studies that highlight an important link of mitochondrial impairment, α-Syn and oxidative stress and justify our choice of studying mitochondrial depolarization in relation with α-Syn in the present system. Sherer et al. (2002) established a chronic, cellular model based on treating human SK-NM-C cells with the pesticide and inhibitor of complex I, rotenone. Four weeks of rotenone exposure lead to increased SDS-insoluble α-Syn levels, oxidative stress including DNA and protein damage, glutathione loss, elevation of apoptotic markers such as cytochrome c release from mitochondria and finally enhanced H_2O_2 sensitivity and apoptosis. Dopamine neurons are thought to exist in a state of constant oxidative stress partly due to H_2O_2 generation. Chinopoulos and Adam-Vizi (2002) also showed that mitochondria with mild complex I defects depolarize gradually upon H_2O_2 treatment, by using JC-1 as a mitochondrial probe.

Finally in a recent report of considerable interest, Song et al. (2004) showed that there is substantial evidence for a link between α-Syn over-expression and mitochondrial dysfunction in MPTP treated mice. Transgenic α-Syn over-expressing mice treated sub acutely with MPTP had enlarged and grossly deformed mitochondria with occasional electron dense inclusion bodies and also neuritic abnormalities and small perinuclear α-Syn inclusions specific to the striatum and the substantia nigra. In other words, over expression of α-Syn enhances toxicity of an otherwise non toxic MPTP treatment. However, the exact interrelationship between α-Syn and mitochondria is yet unknown but there is speculation (see commentary by Beal, 2004) suggesting a possible interaction of α-Syn with the outer mitochondrial membrane, α-Syn translocation into the mitochondria or even interference with transcription of

nuclear encoded mitochondrial genes. Hsu et al. (2000) also reported that overexpression of wt α-Syn in a hypothalamic neuronal cell line leads to α-Syn inclusion formation, structural and functional mitochondrial alterations and free radical formation.

The findings presented in this body of work extend the above studies as they show that the response of the cells in this model system is stimulus-dependent in that wt α-Syn is not always deleterious for the cells, in contrast to mutant α-Syn which is always deleterious irrespective of stress. Moreover, it is important to note that in the present system it was possible to show that hsp27 ameliorates such deleterious effects due to wt or mutant α-Syn related toxicity under stress.

The role of the mitochondrion in apoptosis has been extensively studied in recent years and it has been argued that it also has a contributory role in neurodegeneration. In many instances, permeabilization of mitochondrial membranes is a rate-limiting step of apoptotic or necrotic cell death and this has important consequences for the pathophysiology of cell death, as well as for its pharmacological control. Various studies demonstrate roles of the mitochondria in neuronal death and their involvement with PD or HD (Schapira, 1998; Hsu et al., 2000a; Sawa et al., 1999; Cooper and Schapira, 1997). Importantly, in a very recent study by Valente et al. (2004) mutations in *PINK1* (a mitochondrially located kinase associated with the PARK6 locus) are causative of hereditary early-onset PD. By using TMRM and FACS analysis, the effect of the mutations in *PINK1* on $\Delta\Psi m$ was studied and it was suggested that *PINK1* may protect neurons from stress-induced dysfunction of the mitochondria and apoptosis, which is an effect that is abolished by the disease-associated mutations. Studying the mitochondrial depolarization in connection with α-Syn in our system was useful because it helped to identify a potential link between α-Syn-induced toxicity/protection in this system and also test whether hsp27 has a protective role connected with the $\Delta\Psi m$.

Some caveats of this model include the fact that it is a highly simplified one where interactions that could take place *in vivo* are missed – although it could be argued that this is the case with all *in vitro* model systems and *in vitro* assays. Additionally, not all stresses were studied due to time limitations, but representative example stresses were used along with two clones per cell line and two different dyes for the study of $\Delta\Psi m$. It remains possible that different mechanisms of protection by hsp27 exist under different stresses due to different interactions.

There are also some limitations of the fluorescent probes used. TMRM is a membrane permeant cationic hydrophobic fluorophore that loads into polarized mitochondria electrophoretically due to the negative membrane potential of the mitochondria; hence, it is reversibly up taken by live cells. So, polarized mitochondria take up cationic TMRM, which is released after depolarization but TMRM does not label depolarized mitochondria. MTG is also a cationic fluorophore that accumulates electrophoretically into polarized mitochondria. Unlike TMRM, MTG binds covalently to intramitochondrial protein thiols and remains bound after depolarization. As a consequence, green MTG fluorescence persists inside mitochondria after depolarization and does not change with changes of mitochondrial membrane potential. There is little evidence that MTG alone or any single dye alone can provide a reliable quantitative measure of $\Delta\Psi m$ (Ferlini et al., 1998). To identify both polarized and depolarized mitochondria in living cells, cultured cells were co-loaded with green-fluorescing MTG and red-fluorescing TMRM in the present study.

However, there are several studies where dyes such as TMRM and Mitotracker dyes have been used with success, despite their limitations. Among them there is an elegant study by Deshmukh et al. (2000) in which caspase inhibition was found to extend the period before which a cell becomes fully committed to a fate of cell death in trophic factor-deprived sympathetic neurons and allowed recovery of neurons arrested after the loss of cytochrome c but not beyond the subsequent loss of mitochondrial membrane potential, which was

studied by using mitotracker orange staining, which is a similar compound to mitotracker green that was used in the studies presented in this thesis. By extrapolating those findings to our system it can be speculated that maybe the protection observed by caspase inhibition or hsp27 expression is due to a potential interference with mechanisms that relate to maintenance of $\Delta\Psi m$ and suppression of apoptosis.

Another group reports the use of TMRM in ND7 cells suggesting that a moderate decrease but not a rapid and complete loss of $\Delta\Psi m$ could induce changes characteristic of apoptosis (Gautier et al., 2000). In a study of the effects of sulfonylureas on $\Delta\Psi m$ of cardiomyocytes mitochondria, again TMRM and FACS analysis were used with success (Mocanu et al., 2001) as have been high-throughput measurements of $\Delta\Psi m$ in neuronal cell lines performed using TMRM and fluorescent plate reader (Wong and Cortopassi, 2002). TMRM in combination with confocal microscopy was used by Kindler et al. (2003) in a study of nitric oxide effects on mitochondria of a human neuronal cell line and primary rat neurons. Another useful study attempted to analyse mitochondrial free radical generation in animal models of neuronal disease, where MitoTracker Red and Green were successfully used (Kim et al., 2002). Finally, another example is the study described earlier on where JC-1 is used to confirm that increased apoptosis of Huntington's disease lymphoblasts is associated with repeat length-dependent mitochondrial depolarization (Sawa et al., 1999). Another study by Buckman et al. (2001) highlighted the limitations associated with the use of mitotracker labelling for semi-quantitative determination of $\Delta\Psi m$.

Overall it is suggested by the same authors that most mitochondrial dyes must be used with caution. For some of them such as TMRM and JC-1 a direct relationship has been demonstrated between $\Delta\Psi m$ and fluorescence intensity. Therefore, it becomes clear that mitochondrial selective probes are very useful in the study of $\Delta\Psi m$, and although they are very widely used, the results should be interpreted carefully and confirmed by multiple dyes/methods if possible.

In summary, there are two main conclusions emerging from the above studies:

(a) There is an enhanced A30P mutant α-Syn toxicity in ND7 cells, which is reflected by their increased susceptibility to apoptotic cell death compared to control cells, and this is partly suppressed by pharmacological caspase inhibition or hsp27 over-expression using an HSV-based vector. However, the two effects are not additive, suggesting that hsp27 interferes with enhanced caspase activation in this system.

(b) Studies of changes in the mitochondrial membrane potential of the cell lines indicate mitochondrial defects particularly in mutant A30P α-Syn cells and this effect can be mitigated by hsp27 over-expression prior to stress.

However, the reduction in $\Delta\Psi m$ could be responsible for triggering or enhancing apoptotic cell death or, alternatively, it could represent one consequence of the state of the cell undergoing programmed cell death mediated by other putative factors, possibly interacting with mutant α-Syn. This study cannot provide an answer to this, as we only have an observation that links apoptosis, α-Syn, hsp27 and mitochondria. Therefore, further experiments (see Chapter 6 for suggestions) are necessary but they were not performed due to time limitations.

It is highly likely that multiple pathways are involved in hsp27 protection, and it will be necessary to investigate the contribution of these other pathways, for instance the suppression of reactive oxygen species generation (as in a cellular HD model reported by Wyttenbach et al., 2002), to the effects reported here. In our model it would be useful to perform caspase activation assays to investigate if caspase processing varies in response to different stresses in the presence or absence of hsp27 over-expression, and to extend these investigations by transfecting dominant negative caspase constructs in the various α-Syn cell lines. A similar approach could be utilized to investigate other signal transduction pathways involved in cell survival such those involving Akt and p42/p44 MAP kinase. Furthermore, alternative fluorescent

probes, such as JC-1, could be used in combination with biochemical methods in order to further dissect the molecular mechanism by which α-Syn affects the mitochondria membrane potential of the cells and subsequently how hsp27 helps in partially restoring it.

Overall, the findings presented in this chapter do not provide sufficient evidence for the full understanding of the mechanism of toxicity of the disease-associated mutants of α-Syn or of the precise mechanism of action *via* which hsp27 exerts its protective effect. However, they clearly suggest (a) the involvement of caspase activation in the increased susceptibility to cell death induced by mutant A30P α-Syn (b) that exogenously expressed hsp27 impinges on the caspase cascade when suppressing cellular toxicity and (c) the possible involvement of hsp27 at the level of the mitochondrion. Further suggestions for future experiments utilising this simple cellular model or using more advanced systems to confirm these findings - such as in *in vivo* models - are discussed in the next chapter, along with a further evaluation of the present study.

CHAPTER 6

Discussion

The work presented in this thesis demonstrates the effectiveness of using a simple neuronal cellular model of α-Syn-induced toxicity and an efficient gene delivery method based on HSV-1 vectors, in order to test hypotheses relevant to PD-related pathogenesis and to the neuroprotective effects of heat shock proteins. This chapter aims to discuss general aspects, advantages and limitations of the work presented here as well as to suggest further experiments but more details are included in the individual Discussion sections of the respective Chapters 3, 4 and 5. Therefore, Chapter 3 described the establishment of the *in vitro* cellular model and the characterisation of its response to various stresses. Having demonstrated certain effects in this system, which result from the over-expression of wt or A30P and A53T α-Syn mutant forms, it was possible to proceed and ask if hsp over-expression could mitigate the deleterious effects of α-Syn under stressful conditions (Chapter 4) and then investigate potential mechanisms of action of hsp27 (Chapter 5).

The following sections will highlight how the findings presented in the last three chapters compare with reported findings in the literature, before summarising the overall advantages and the limitations of the model presented here and finally propose some further experiments extending the work of this thesis.

The results presented in Chapter 3 suggest that the exhibited differential effects on cell death modulation by wt α-Syn in neuronal cells depend on the nature of the stress imposed/applied, whereas both mutant forms are deleterious under all the stressed used (also see Zourlidou et al., 2003 in the appendix). This might resolve discrepancies in the literature on the protective / deleterious effect of wt α-Syn. Work by others (also discussed in Chapter 3) provided evidence that increased levels of wt α-Syn can be deleterious *in vitro* depending on the stress (Zhou et al., 2000; Hsu et al., 2000; Ostrerova-Golts et al., 2000; Iwata et al., 2001; Lee et al., 2001; Stefanova et al, 2003; Junn and Mouradian, 2002; Zhou et al., 2002; El Agnaf et al., 1998) or *in vivo* (Kirik et al., 2003; Masliah et al., 2000; Feany and Bender, 2000) or even in humans (Singleton et al., 2003; Farrer et al., 2004). The later findings are of particular

interest as it seems that α-Syn in normal brain must have a very important role and it only becomes toxic (a) if it is mutated, (b) if it is expressed at increased levels or (c) under certain environmental conditions known to cause PD accompanied by α-Syn and other protein aggregation in LBs and LNs (pesticide exposure, iron etc).

In contrast to the above findings, wt α-Syn was reported to be protective in our studies on serum withdrawal and dopamine-induced toxicity and in other studies *in vitro* (Hashimoto et al., 2002; da Costa et al., 2000; Lee et al., 2001) and *in vivo* (Manning-Bog et al., 2003). Mutant α-Syn, on the other hand, increased toxicity in the system studied here under all the stresses used. Numerous other groups also found that the disease mutants A30P and A53T are deleterious *in vitro* (Tanaka et al., 2001; Lee et al., 2001; Tabrizi et al., 2000; Saha et al., 2000; Ostrerova-Golts et al., 2000) or *in vivo* (Giasson et al., 2002; Kirik et al., 2003; Klein et al., 2002). There has also been a study that found protection against paraquat-induced toxicity by both wt and A53T mutant in a transgenic mouse model (van der Putten et al., 2000; Feany and Bender, 2000).

As a summary, comparison of this model to mice, fly and *in vitro* PD models (reviewed in Chapter 1 and discussed in Chapter 3) suggests that it generally agrees with a number of studies on the role of mutant α-Syn to enhance toxicity, while the novel finding is that wt α-Syn can be protective or toxic in different situations (Zourlidou et al., 2003). Subsequently, the later observation was confirmed by similar work by others (Jensen et al., 2003; Wersinger and Sidhu, 2003). In the first study, there was no protective effect for the A30P and A53T mutants, but a differential cytoprotective role of wt α-Syn against oxidative stress, which varies according to expression levels. In the second study, wt α-Syn, but not its mutants, protected dopaminergic cells from the parkinsonism-inducing drug MPP^+, by preserving mitochondrial function, and from rotenone (complex I inhibitor) and 3-nitroproprionic acid (complex II inhibitor), whereas it did not protect from 6-hydroxydopamine, hydrogen-peroxide, or the beta-amyloid peptide, A-beta (Wersinger and Sidhu, 2003). The authors suggested that the normal physiological role for α-Syn may change

during development while the present study suggested a modulatory role for wt α-Syn which varies under different conditions.

Chapter 4 presented experimental data that give a novel neuroprotective role to hsp27 against PD-associated α-Syn mutant forms expressed in neuronally derived cells (see also in the appendix Zourlidou et al., 2004). Hsp27 has a potent protective effect against mutant α-Syn-induced toxicity under all the pro-apoptotic and oxidative stresses used. This is a novel finding in the context of PD models, and agrees with numerous studies in the literature that support the protective function of hsp27 against many stresses leading to apoptosis (see Chapter 5 discussion below).

Hsp27 is linked with PD only in a few studies so far, summarised below. Notably, hsp27 is a component of LBs in PD brain (McLean et al., 2002) together with α-Syn and other hsps. In the context of other neurodegenerative disease models, Wyttenbach et al (2002) elegantly showed that hsp27 has a potent protective effect in a polyQ cellular model in which increased ROS formation is evident due to polyQ expression, but it is suppressed by hsp27 expression. Finally, as recently reported by Shimura et al. (2004), hsp27 rescues pathological hyperphosphorylated tau-mediated cell death in a human cortical neuronal cell line. Interestingly, hsp27 was shown to bind to pathological hyperphosphorylated tau but not non-phosphorylated tau in human AD brain. The formation of such a complex *in vitro* leads to a decrease of its concentration by assisting its degradation and dephosphorylation.

However, in the experiments of the present study hsp70 did not have a strong protective role in most of the stresses used, whereas simultaneous hsp27 and hsp70 over-expression was as protective as hsp27 expression alone and hsp70/hsp40 co-expression did not reduce cell death. Hsp70 was shown to be protective in a significant study in a non mammalian system by Auluck et al. (2002), who reported the generation of α-Syn and hsp70 co-expressing flies with a reduction in dopaminergic neuronal loss compared to α-Syn expression flies (described in detail in Chapter 4). More recently Klucken et al. (2004)

demonstrated an effect of hsp70 on reducing high molecular weight and detergent insoluble α-Syn species in mice. In this study, where α-Syn and hsp70 transgenic mice were crossed, hsp70 reduces high molecular weight α-Syn and detergent insoluble species of α-Syn (but it does not change the total amount of soluble monomeric α-Syn). Finally, the same study showed protection from α-Syn toxicity by hsp70 expression in a neuroglioma cell line model of α-Syn aggregation (which is not in agreement with findings of the present study), although a protective effect in the mice was not reported.

Hsp70 and hsp40 over-expression in several *in vitro* and *in vivo* models of polyQ disease proved to be efficient in suppressing polyQ aggregation (Muchowski et al., 2000) or both aggregation and toxicity (Sherman and Goldberg, 2001) (additional evidence was discussed Chapter 1 and Chapter 4). In addition, over-expression of human inducible hsp70 in a mouse model of SBMA ameliorated phenotype and reduced the levels of aggregated and monomeric mutant AR in neuronal nuclei (Adachi et al., 2003). In a SCA1 model over-expression of hsp70 did not prevent the formation of nuclear inclusions in Purkinje cells but suppressed degeneration and improved motor performance (Cummings et al., 2001). Phenotype rescue, as measured by survival, rotarod analysis and gait analysis, was seen on homozygous over-expression of hsp70 and also hsp70 reduced the levels of both aggregated and soluble AR detected in the nucleus. Hsp70 over-expression may assist in degrading the mutant AR protein through the ubiquitin-proteasome system. However, hsp70 over-expression was less efficient in HD models (Hansson et al., 2003; Hay et al., 2004). Transgenic cross of R6/2 mice to a line over-expressing hsp70 (Hansson et al., 2003) resulted in broadly similar findings as in the study by Hay at al. (2004). No differences were apparent in clasping phenotype or survival in the mice. Hsp70-R6/2 double transgenics showed a delay in the reduction of body weight but hsp70 overall failed to suppress R6/2 neuropathology as measured by overall brain weight, volume of striatum and diameter of medium spiny neurons.

Why hsp70 does not protect in the present system is not clear. As discussed later on in the section with suggested further experiments, there might be a temporal element in the expression of hsps in order for protection to be evident (i.e. hsps should be over-expressed before α-Syn in the system). One can argue that it is possible that the levels of hsp expressed by the viral vector were too low or too high for protection to occur in this system (adverse effects could arise by excess hsp levels and molecular crowding effects). However, the hsp27 was shown to be protective at the same expression levels as hsp70, and this was demonstrated for multiple time points and various stresses in this system.

Alternatively, hsp70 might not be protecting in this system because its function becomes impaired due to ATP depletion which might occur under the conditions used. It should also be noted that, as shown in Chapter 5, under stress and α-Syn over-expression the mitochondrial membrane potential is significantly reduced and this means reduced mitochondrial activity, energy depletion and possibly a resulting reduced chaperone activity. This, combined to a situation of enhanced cellular stress leading to enhanced protein misfolding, and finally death due to an apoptotic cascade, might account for the observed effects. It is known that hsp70 function is ATP-dependent (its ATP-ase activity is stimulated by hsp40, which was also used in the present study), but hsp27 function is not. So, maybe in the event of possible energy depletion during the applied stresses, hsp27 can still confer protection due to preservation of its normal chaperone function, while hsp70 alone or hsp70 and hsp40 co-expression cannot function properly and protect the cells.

Another parameter that has to be considered for the reason why hsp70 is not protecting under most of the stresses used here might be the fact that the levels of essential co-chaperones in the cells are insufficient. An attempt was made towards this possibility by testing hsp40 overexpression using a viral vector (Chapter 4). However, the levels of cell death remained unaltered compared to control virus infected cells.

In the model presented here it seems that apoptosis is the main mode of cell death induced by the stressful stimuli. Although aggregation or protofibril (prefibril) formation by α-Syn was not investigated here, it is likely that hsp70 or hsp70 and hsp40 co-expression did not protect from apoptosis, since their main role is connected with protein folding; this might not be an issue in the model here i.e. there might not be any aggregation related toxicity. Chaperones such as hsp70 were suggested to prevent the formation of the toxic protofibrils (but they do not act on mature fibrils, Muchowski et al., 2000) or accelerate their conversion to non toxic aggregates that can be degraded by the proteasome. However, the interactions at such level are not yet well understood. Maybe hsp27 was shown here to be protective under all stresses because in this particular model α-Syn increases sensitivity of the cells by other mechanisms, involving the caspase-dependent pathway of apoptosis, rather than exerting deleterious effects due to a toxic pre-fibril formation-associated mechanisms (see further experiments). Therefore, hsp27 protected against enhanced apoptotic cell death, whereas hsp70 protected only against non apoptotic cell death under the conditions described and at the chosen time points.

The work presented in Chapter 5 provides some evidence that suggests (a) that the mechanism of action of hsp27 in reducing susceptibility to cell death is by interfering with the enhanced caspase activation in cells in which mutant A30P α-Syn is expressed and (b) one potential site of action is the mitochondrion, since hsp27 seems to help maintaining Ψm in the presence of mutant α-Syn and this correlates with the data on suppression of apoptotic cell death. A detailed mechanism of how Ψm is maintained due to exogenous hsp27 expression is unknown. Nevertheless, and although speculative, it is more likely that hsp27 protects due to its involvement in more than one sites/pathways and this should be further investigated.

The results support the idea that it is the anti-apoptotic activity of hsp27 which is responsible for protection against death. The involvement of apoptosis in PD and also the role of hsps in suppressing apoptotic pathways were discussed extensively in Chapter 1 along with the role of mitochondria in

both PD and apoptosis. In summary, work by others strongly supports that in PD models and in PD brain apoptosis occurs (Tatton et al., 2003; Vila and Przedborski, 2003), while hsp27 is capable of suppressing it (Concannon et al., 2003; Parcellier et al., 2003a; Beere, 2004).

To further investigate the involvement of hsp27 over-expression in modulating apoptotic cell death pathways, quantification of the $\Delta\Psi m$ was performed for two distinct stresses (serum withdrawal and staurosporine). However, hsp27 was able, through an unknown mechanism, to mediate events that maintained the mitochondrial membrane potential in these cells over-expressing α-Syn and which are also exposed to apoptotic stimuli. Therefore, in addition to inhibition of caspases in a similar manner/extent compared to pharmacological inhibition, hsp27 also had an advantageous effect on the maintenance of a relatively high mitochondrial membrane potential. Although the findings presented in Chapter 5 do not provide a full understanding of the mechanism of protection in the model of α-Syn related toxicity, they provide significant evidence on the interference of hsp27 with caspases and apoptotic cascade events upstream the mitochondria. It is possible that multiple sites of action are involved as well as multiple activities by hsp27 are involved at the same time. For instance, apart from suppressing apoptosis (Beree et al., 2004), hsp27 may act by (a) stabilising actin and interacting with cytoskeleton components, aiding to the stability of the cytoskeleton (see review of hsp27 roles in Chapter 2), (b) suppressing ROS generation as in the report by Wyttenbach et al. (2002), (c) assisting proteasomal degradation of unidentified substrates up to date, as in the study by Parcellier et al. (2003a), in which for the first time hsp27 was shown to assist ubiquitinated and phosphorylated I-κBα (the inhibitor of NF-κB) proteasomal degradation.

Finally the importance of the mitochondrion in this system seems obvious in the present study. Bcl2 members can preserve or disrupt mitochondrial integrity and release cyt c, smac/Diablo and AIF. Activated initiator caspases like caspase-8 and caspase-9 are responsible for events like mitochondrial damage, so it is possible that hsp27 interacts with them and hence

preserves the mitochondrial membrane potential and suppresses cell death under the stresses tested.

The role of the mitochondrion in PD is becoming increasingly important (see Chapter 1 review on PINK1, DJ-1 and mitochondrial defects in PD). Therefore, the preliminary findings presented here on hsp27 acting protectively by maintaining the mitochondrial membrane potential are of particular interest and need to be further extended.

In vitro cellular models have been extensively used in the field of neurodegenerative disorders and have provided a vast amount of valuable knowledge. Some of these findings were extended by conducting further *in vivo* studies or they were confirmed in studies with human post mortem material. In the same way, the methodology used here is not novel but the model system presented here overall contributed to field; this will be explained next along with some of its limitations.

The limitations that accompany this *in vitro* model system are mainly due to the fact it is an *in vitro* system utilising a non human hybrid cell line. However, such limitations do not necessarily rule out the significance of the findings, but they have to be extended in other systems.

Neurodegenerative diseases like Parkinson's are very complex and not completely understood at the molecular pathological level. There are also difficulties imposed by the fact that they are age related and progressive diseases where usually more than one parameter accounts for their pathology, including environmental triggers and genetic predisposition. So, the extent to which a simple cellular model can assist the understanding of the disease pathogenesis is relatively limited. However, by reviewing the knowledge of the biology of hsps and of α-Syn, the cellular model system presented here was able to demonstrate a novel neuroprotective role for hsp27 on α-Syn-related toxicity. The role of hsp27 in protecting in this system is not surprising, in view of evidence on hsp27 anti-apoptotic functions and protection from ROS and

several other stimuli in non neuronal and neuronal cells or *in vivo* systems (see review in Chapter 1). Hsp70 was also shown to have some anti-apoptotic activities (reviewed by Beere, 2004) but it is not known why in contrast to hsp27 it still does not protect as efficiently here.

As discussed in the previous relevant chapters, advantages of the system presented here include the fact that ND7 are well characterised neuronal mammalian cells, the system is characterised by relative inducibility of the transgenes (Chapter 3), although some leaky expression can occur by the MMTV promoter. The advantages of the inducibility of the system are profound particularly when the expressed protein is potentially toxic and this would cause problems during clonal selection. The viral vectors used in the present studies proved to be an invaluable tool for highly efficient hsp gene delivery in neuronal cells (90-100% transduction) as discussed in Chapter 4. Strong evidence on the protective effect of hsp27 against various apoptosis-inducing stresses was provided by three different methods of assessing quantitatively cell death (trypan blue exclusion assay, TUNEL and annexin/propidium iodide staining), as shown in Chapter 4. Briefly, it should be noted here that these are valid and widely used methods for the assessment of cell death (all three) or more specifically for the assessment of the apoptotic index (TUNEL and annexin/PI). Finally, mitochondrial membrane potential change ($\Delta\Psi m$) quantification by utilising two different mitochondria selective florescent probes and FACS analysis shed some light on the mechanism of toxicity by α-Syn and the interference of hsp27 at this level (extensively discussed in Chapter 5).

If α-Syn was expressed by different (stronger) promoters or its expression was induced more strongly by higher dexamethasone concentrations (using the same cells and applying the same stresses for the same length of time), it could be concluded whether the same differential modulation of cell death was observed. In addition the use of human dopaminergic cells (SH-SY5Y) or primary striatal or other neuronal cultures could be used to confirm the findings of the present study. Since the role of the proteasome in this system was not studied (i.e. the use of proteasome inhibitors), mainly because of time

limitations and because it has been extensively studied in other *in vitro* systems, it would be interesting to perform experiments involving proteasome activity assays and see if hsp27 rescues from a potentially toxic proteasomal impairment.

The study of signalling pathways in the present system could also be informative. For instance the PKB/Akt pathway involvement in relation to α-Syn protection and hsp27 (Hashimoto et al., 2004; Seo et al., 2002) can be investigated in the present system by immunobloting lysates of cells incubated under the presence or absence of stress and hsp27 or GFP control virus expression, using antibodies that specifically recognise the phosphorylated forms. In addition, in order to extend the study of the hsp27-mediated mechanisms that confer protection in our system, it would be interesting to see if there are enhanced levels of ROS as a result of α-Syn expression and if so, whether hsp27 suppresses their formation and hence susceptibility to death in our system, as seen in *in vitro* polyQ toxicity in the study by Wyttenbach et al. (2002).

In addition, some other stresses could have been used, such as those inducing oxidative damage, proteasome inhibitors, mitochondrial complex inhibitors and so on, as relevant agents to the disease pathology that have been extensively used by others. Finally, it could be of interest to look to into the mitochondrial activity by using for example commercially available assays, caspase activity assays, immunodetection of cytochrome c release and if this is blocked by hsp27, or to use cyclosporine A as positive control that restores the mitochondrial membrane potential and so on.

In addition, cells could have been infected with the hsp70 or the respective GFP virus and after 24h, when hsp transgene expression was shown to be high, add dexamethasone in order to induce α-Syn expression in the cell lines and finally stress the cells to see if in this case hsp70 protects. In the experiments presented here hsp70 was over-expressed in cells while α-Syn expression had already been induced by the addition of dexamethasone. This

experiment could exclude the possibility that maybe hsp70 does not protect the cells because of potentially aggregated α-Syn in the cells. It should be noted that hsp70 cannot refold already misfolded and aggregated proteins. In the study by Auluck et al. (2002) hsp70 and α-Syn were co-expressed and dopaminergic cell protection was observed.

Aggregation or protofibril (prefibril) formation by α-Syn was not investigated here so it could be further investigated, as absence of aggregation related toxicity would explain the inability of hsp70 to be ineffective in protecting a system where apoptosis suppression by hsp27 proved more effective. So, immunocytochemical studies or electron microscopy studies can shed some light in this aspect of the cellular model. Alternatively, by using anti-sense, dominant negative constructs or RNA interference technology, one can ask whether compromising the inherent hsp machinery in those cells sensitises the cells more to α-Syn over-expression in the presence of stress or not, as Auluck et al. (2002) showed in the fly.

Furthermore, along with the use of viruses to over-express efficiently individual or combined hsps in cells, drugs could have also been used in order to (a) initially characterise their effectiveness in hsp induction by the drug-induced stress response and (b) subsequently assess cytoprotection. Chaperones are multifunctional proteins and it is possible that depletion of chaperones may impair mechanisms which are independent of protein folding and there is evidence to suggest that the abundance and relative levels of chaperones may be critical in determining cellular signalling (Nollen and Morimoto, 2002; Young et al., 2003).

Pharmacological or other hsp induction strategies should aim to (a) replace potential "losses" of chaperones, such as losses due to their sequestration in aggregates, that might deplete the cellular pool of hsps needed for other functions in the cell and (b) induce hsps at higher than the normal levels to assist the cell to cope with stresses that would otherwise lead to malfunction or death. Pharmacological modulation of heat shock protein levels

may represent not only a possibility to test in the present *in vitro* system, but a potential therapeutic avenue in disorders like PD with prominent protein aggregation. Several compounds have been reported to initiate a heat shock response in various cell lines. Amongst these compounds, the structurally unrelated antibiotics geldanamycin and radicicol are of particular interest as the mechanism by which they initiate a heat shock response through inhibition of Hsp90 and activation of HSF-1 is reasonably well understood.

Finally, confirmation of the findings presented in this study could be obtained *in vivo* by crossing mice over-expressing α-Syn with available hsp27 transgenic mice (Akbar et al., 2003), in order to see if firstly the phenotype is altered and then, if this is the case, how hsp27 accomplishes this, by conducting biochemical analysis.

Whatever the future possibilities, the work presented in this thesis is of significance in the field of Parkinson's disease and heat shock protein biology. This is due to the fact that there is no effective treatment for PD and heat shock proteins represent therapeutic targets and potent therapeutic agents for the modulation of neurotoxicity in neurodegenerative disorders. This work has to be extended and validated in more complex systems *in vivo*, before definitive conclusions can be drawn. Besides, many aspects of α-Syn biology still remain puzzling and it is anticipated that further work will aid to the understanding of PD and its molecular pathogenesis, assisting the development of novel therapeutic interventions. Such strategies, among other neuroprotective or neurorestorative strategies, may involve manipulation of the heat shock response pharmacologically or by means of gene therapy.

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Appendix

from the body midline. Conversely, during anti-phase coordination, all three body segments were involved in a highly compatible isodirectional motion pattern that promoted stability. This latter coordination pattern was spontaneously adopted by all subjects whereas much more variation was observed during the in-phase mode. These findings converge with previous observations on spatial constraints whereby stationary head positions affected rotations of a steering device controlled by both upper limbs [9,10]. The present findings have theoretical as well as practical significance. On the one hand, they point to the important role of allocentric spatial constraints on homologous limb coordination and, more specifically, on the role of isodirectionality as an organizing principle that coexists with the egocentric constraint. This perspective is generally consistent with evidence for directional tuning of neurons in various brain areas according to the population vector principle [4,5]. It appears that simultaneous coding of movements involving differential directional specifications gives rise to neural interference between the effectors' control centres. On the other hand, the findings point to the critical role of voluntary head movements in daily recreational and work-related actions. Head rotations may bias the movements made in other body parts and vice versa, and may give rise to performance errors. Even though coordination research has so far predominantly focused upon the coordination between the upper and/or lower limbs, the inclusion of head movements in experimental protocols may provide a significant advance in developing our understanding of coordination constraints, their coalition, and the preferred coordination patterns to which they give rise. This approach complements the study of muscular-skeletal, biomechanical, cognitive, and neural constraints on interlimb coordination [3,18].

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