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Characterisation of Parkinson's disease-associated Genes and their Regulation

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Abstract

Parkinson's disease is a highly prevalent neurodegenerative disorder. Several genes have been shown to be associated with familial Parkinson's disease and they usually lead to Parkinson's disease due to the presence of mutations that affect protein function. It has been suggested that variations in the expression of the wild type genes may also lead to Parkinson's disease. The causes of idiopathic Parkinson's disease remain unknown. Several factors may contribute to its onset, including: susceptibility genes, environmental stress and aging.

This study aimed to characterize the influence of oxidative stresses on the regulation of genes associated with Parkinson's disease. The effects of oxidative stress on α-synuclein, parkin and PINK1 were investigated in a cell culture model. Both α-synuclein and parkin were similarly up-regulated when cells were exposed to stresses such as dopamine and 1-methyl-4-phenylpyridinium (MPP+). In constrast, PINK1 levels were up-regulated only by MPP+, and were down-regulated in both dopamine and MG132 treatments. This work confirmed and extended previous reports that oxidative stresses are implicated in Parkinson's disease, and also revealed the complexity of the regulation by these stresses.

A further study into the regulation of α-synuclein showed a novel interaction between the α-synuclein promoter and an Early Growth Response transcription factor family member in oxidative stress conditions. Moreover, this work demonstrated that several other neuronally expressed transcription factors influenced the regulation of α-synuclein, such as the product of the Parkinson's disease associated gene, Nurr1. The decreased expression of this gene increased α-synuclein transcription. This is of interest, as variations in the levels of either of these genes can cause Parkinson's disease and such an interaction was novel. This work further demonstrated that the POU family trancription factor, Brn3a, was involved in this pathway. Brn3a appeared to function antagonistically to Nurr1 in α-synuclein regulation.

In addition to studies of gene regulation, mutational and/or protein analysis were performed on Nurr1 and PINK1. Studies of PINK1 protein established the functional importance of cleavage of precursor PINK1 and also provided a better estimation of the location of the cleavage site. These genes are more recent discoveries compared to α-synuclein and parkin, thus, such studies will give important insights into their Parkinson's disease properties.

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Abbreviations

ANOVA	analysis of variance
AP-1	Activator protein 1
ARJP	autosomal recessive juvenile parkinsonism
ARPD	autosomal recessive Parkinson's disease
bp	base pairs
cDNA	complementary DNA
CHIP	Chromatin Immunoprecipitation
complex I	NADH-ubiquinone reductase
CRE	Calcium/cAMP responsive element
DIG	dioxygenin
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
E.coli	Escherichia coli
EGR	Early Growth Response
EMSA	Electrophoretic mobility shift assay
ERE	EGR Response Element
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
GFP	green fluorescent protein
HBSS	Hank's Balanced Salt Solution
IBR	in-between-RING
Kb	kilobase
kDa	kilodalton
KO	knock-out
LRRK2	leucine rich repeat kinase 2
MG132	Z-Leu-Leu-Leu-CHO
MPP	mitochondrial processing peptidase
mPTP	mitochondrial permeability transition pore

MPP+	methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
NFκB	Nuclear Factor kappa B
Nurr1	NR4A2
PACRG	Parkin co-regulated gene
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	PBS/Tween
PCR	polymerase chain reaction
PINK1	PTEN induced kinase 1
PTEN	phosphatase and tensin homolog gene
RING	really interesting new gene
ROS	reactive oxygen species
RNA	ribonucleic acid
RT PCR	real-time PCR
SD	standard deviation
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of the mean
siRNA	small interference RNA
SP1	Specificity protein 1
SRE	Serum Response Elements
UCH-L1	ubiquitin C-terminal hydrolase 1
TAE	tris-acetate-EDTA buffer
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TMRM	tetramethylrhodamine, methyl ester, perchlorate
Tween 20	polyoxyethylene-sorbitan monolaurate
Ub	ubiquitin
UBL	ubiquitin-like
UPS	ubiquitin proteasome system
wt	wild type
$\Delta\Psi m$	mitochondrial membrane potential (Delta psi)

Declarations

All the work presented in this thesis is the work of Yan Xiang Yang

Publications

Y.X. Yang, M.M.K. Muqit, D.S. Latchman. Induction of parkin expression in the presence of oxidative stress. *Eur J Neurosci.* 2006; 24: 1366-72

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D.G. Healy, M.M.K. Muqit, P.M. Abou-Sleiman, **Y.X. Yang**, J.L. Holton, T.Revesz, N.P. Quinn, K.Bhatia, J.K.J. Diss, A.J. Lees, D.S. Latchman, N.W. Wood. A novel promoter mutation of *Nurr1* reduces *Nurr1* expression in Parkinonson's disease brain *in vivo*. (Submitted) **(Second authorship)**

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"The only way of finding the limits of the possible is by going beyond them into the impossible."

- Arthur C. Clarke

Such sums up the tumultuous journey of my past four golden years, meagerly summarized in this thesis.

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Chapter 1: Introduction

1.1 Parkinson's disease background

1.1.1 History of Parkinson's disease

Parkinson's disease symptomology was initially described as abnormal movement or gait (Goetz et al., 2001; Parkinson, 2002), having been first clinically characterized in 1817 by James Parkinson in his paper entitled 'An essay on the shaking palsy' (Parkinson, 2002). Parkinson's disease was further studied by Jean-Martin Charcot at the Salpetriere hospital in Paris in 1887 (Federoff et al., 2003; Goetz, 1987). He was the first to separate Parkinson's disease into discrete symptoms, e.g. differentiating bradykinesia from rigidity and emphasizing that muscle strength was well preserved in the disease (Federoff et al., 2003; Goetz, 1987). Remarkably, not only was Charcot the first to also recognize and use the feature of Parkinson's disease, micrographia, which involves small, cramped handwriting and/or the progression to continually smaller handwriting, for diagnosis. He also discovered the therapeutic benefits of belladonna alkaloids and rye-based products prior to the recognition that Parkinson's disease was due to dopaminergic/cholinergic imbalance (Federoff et al., 2003; Goetz, 1987).

The most complete pathologic analysis of Parkinson's disease and the clear delineation of the brainstem lesions were performed by Greenfield and Bosanquet at the National Hospital, Queen Square, London (Goetz et al., 2001; Greenfield and Bosanquet, 1953). William Gowers was the first to suggest a possible genetic predisposition to the development of Parkinson's disease, reporting that 15% of patients had a family history of the disease (Gowers, 1886-1888).

1.1.2 Parkinson's disease characteristics

Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease. It is characterized clinically by symptoms like tremor, rigidity, postural instability and bradykinesia (slowness of movement). Other symptoms may also accompany the above abnormalities. These include autonomic dysfunction, depression, and a general slowing of intellectual processes. In some cases, disorders such as constipation (Abbott et al., 2001; Ashraf et al., 1997) and rapid eye movement sleep behavior disorder also emerge as early symptoms of Parkinson's disease (Olson et al., 2000).

Parkinson's disease is also pathologically characterised by the presence of Lewy bodies and neurites in various regions of the nervous system. Lewy bodies are eosinophilic cytoplasmic inclusions which have a fibrillar structure and a dense core, surrounded by a halo. They are often also associated with Lewy neurites, which are just proteinaceous formations found in neurones. They are found in regions such as substantia nigra, cerebral cortex, locus ceruleus, hypothalamus, nucleus basalis of Maynert, cranial motor nuclei, as well as loss of dopaminergic neurons in the substantia nigra pas compacta (Braak et al., 2003a; Braak et al., 2003b). Loss of neuromelanin pigment from the substantia nigra and locus coeruleus and the presence of Lewy bodies in cells from various regions of the brain, especially in the surviving neurons in the substantia nigra pars compacta, are the typical features in Parkinson's disease (Braak et al., 2003a; Braak et al., 2003b).

1.1.3 Epidemiology of Parkinson's disease

Parkinson's disease affects a larger proportion of males compared to females (approx. 1.5:1.0 ratio) (Dluzen and McDermott, 2000). Whether this phenomenon might be due to exposure in the workplace, sex-linked variability or a protective effect of oestrogen is not known. The average age of onset of Parkinson's disease is between 60 and 80 years, and about 1% of the general population above the age of 65 are affected (Tanner, 1992). However, rare familial forms of Parkinson's disease, which account for less than 10% of the Parkinson's disease cases, may also occur, with a more variable age of onset depending on the mode of inheritance, but generally at a younger average age of below 45 (Dawson and Dawson, 2003).

The cause of Parkinson's disease is still unknown, but many factors have been shown to contribute to the development of Parkinson's disease. These factors (Fig. 1.1) include, aging, environmental factors, oxidative stress, and genetic factors (Betarbet et al., 2005). Some factors have also been reported to show protective effects reducing incidence of the disease. These include cigarette smoking, alcohol and caffeine intake (Grandinetti et al., 1994; Mayeux et al., 1994; Schoenberg et al., 1988). However, the evidence for their protective effect is weak and the mechanism is also not clear (Allam et al., 2004). Environmental toxins have been shown to be the most reproducible risk factor for Parkinson's disease (Lai et al., 2002). Many studies have reported a higher risk of Parkinson's disease in those who have lived in rural communities from an early age in Europe or North America (Lai et al., 2002). Possible candidates have included pesticides or herbicides that were used heavily by these communities after the Second World War (Lai et al., 2002). The latest school of thought in the development of late-onset idiopathic Parkinson's disease involves the interaction between multiple

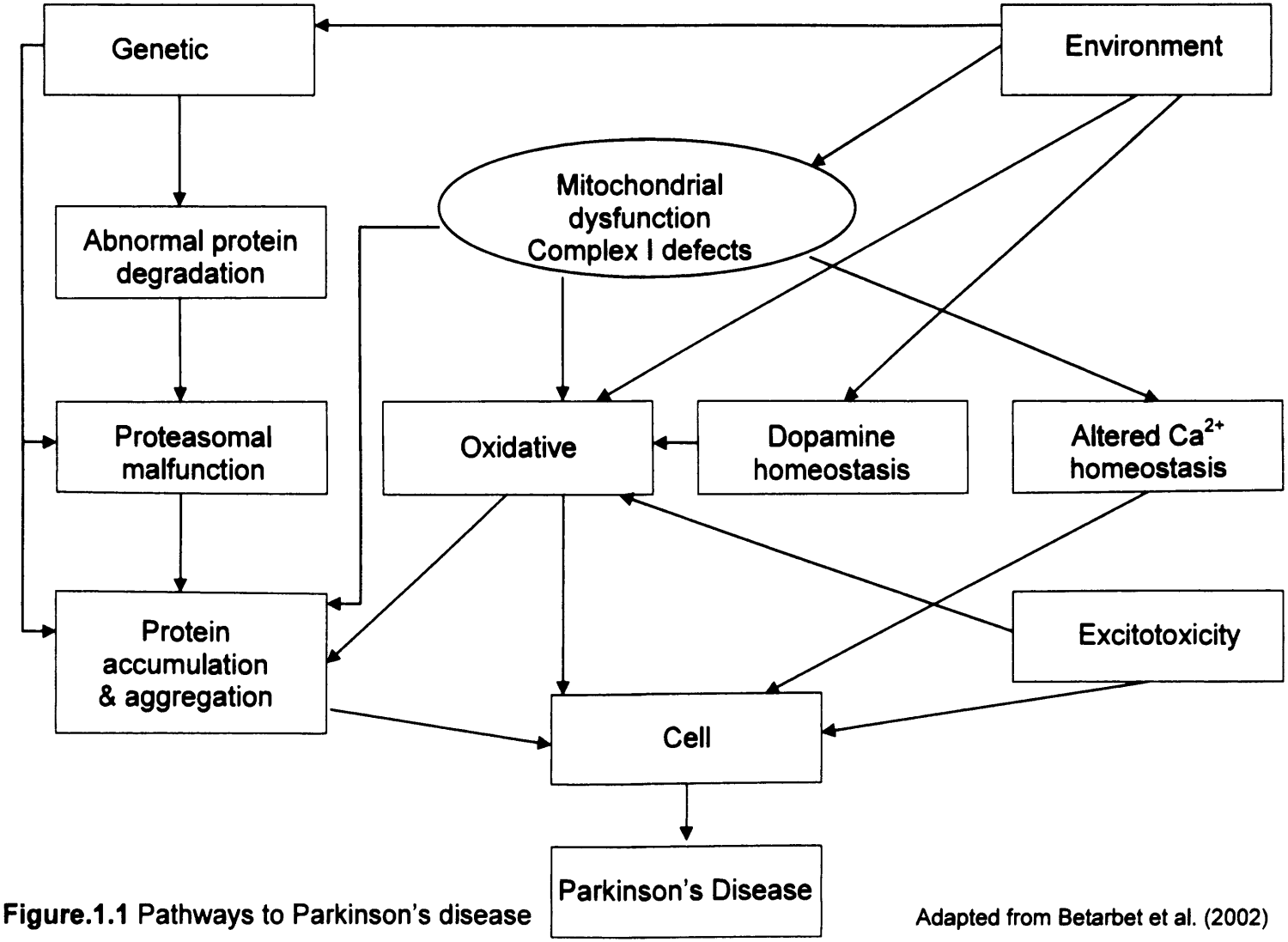


Figure.1.1 Pathways to Parkinson's disease

Adapted from Betarbet et al. (2002)

predisposing genes and environmental factors (Elbaz et al., 2007), with oxidative stresses being of particular importance.

1.1.3.a Oxidative Stress in Parkinson's Disease

The brain has a higher metabolic rate, yet a seemingly lower capacity for regeneration compared to other organs in the body, thus making it especially susceptible to the damaging effects of oxidative stress caused by reactive oxygen species (ROS), which are metabolic by-products of oxygen metabolism (Sayre et al., 2008). In addition, the specific vulnerability of dopaminergic neurons to damage by oxidative stresses in Parkinson's disease may be attributed to their production of dopamine, which are highly prone to auto-oxidation. Furthermore, the metabolism of dopamine produces toxic metabolites (Stokes et al., 1999) and can generate reactive oxygen species (Maguire-Zeiss et al., 2005).

Evidence of oxidative damage has been found within the brain regions where specific neurodegeneration occurs in various neurodegenerative diseases, for example Parkinson's disease, Alzheimer's disease and Amyotrophic lateral sclerosis. For example, α-synuclein molecules with nitrated (protein nitration is a marker of protein oxidation) tyrosine residues have been found to accumulate in Lewy bodies in Parkinson's disease and other synucleopathies, as revealed by postmortem studies (Giasson et al., 2000; Good et al., 1998). However, whether this is a cause of the disease or just a result of other defects is not yet known.

Although environmental stress, in particular oxidative stresses, are strongly believed to contribute to Parkinson's disease, no specific agent has yet been shown to be involved.

Several mitochondrial and proteasomal toxins such as MPTP, rotenone, MG132 and dopamine are known to recreate many features of Parkinson's disease in experimental models. However, the role of such toxins in Parkinson's disease patients continues to be controversial and these toxins remain only as useful experimental models of the disease.

1.2 Mendelian genetics of Parkinson's disease

The possibility of genetic predisposition to the development of Parkinson's disease was first proposed by William Gowers. He reported that 15% of patients had a family history of the disease, and that this suggested genetics as an important factor in the development of Parkinson's disease (Gowers, 1886-1888). However, little attention was given to the genetics of Parkinson's disease until the discovery of Lewy bodies and the role of α-synuclein in the disease a century later, which will be discussed in further detail later in section 1.2.1. One of the reasons for the lack of interest was due to the occurance of post-encephalitic parkinsonism following the influenza pandemic in 1918. The confusion of this syndrome with Parkinson's disease caused many reaserachers to look for a viral aetiology in Parkinson's disease (Lakke et al., 1977). In addition, early twin studies also failed to show increased concordance in identical twins compared to non-identical twins, thus further dismissing the role of genetics in Parkinson's disease (Eldridge and Ince, 1984).

Linkage and positional cloning studies have since led to the identification of six genes and four other genetic loci associated with familial Parkinson's disease. There is strong genetic evidence supporting a causal role for the following genes in familial Parkinson's

disease: α-synuclein (*PARK1; PARK4*) (Polymeropoulos et al., 1997), parkin (*PARK2*) (Kitada et al., 1998), Phosphatase and tensin homologue (PTEN)-Induced Putative Kinase 1 (PINK1; *PARK6*) (Valente et al., 2004b), serine protease Omi/HtrA2 (Strauss et al., 2005), DJ-1 (*PARK7*) (Bonifati et al., 2003), Leucine Rich Repeat Kinase 2 (LRRK2;*PARK8*) (Paisan-Ruiz et al., 2004; Zimprich et al., 2004) and more recently, ATP13A2 (*PARK9*) (Ramirez et al., 2006; Williams et al., 2005) (Table 1.1).

There is also some evidence that mutations in Nuclear Receptor 4A2 (NR4A2/Nurr1) (Le et al., 2003), synphilin-1 (Wakabayashi et al., 2000) and ubiquitin C-terminal hydrolase L1 (UCH-L1;*PARK5*) (Lincoln et al., 1999) may also contribute to Parkinson's disease (Table. 1.1). However, the rarity of disease causing mutations in these genes has raised questions about their importance and relevance to Parkinson's disease. Although familial Parkinson's disease, caused by specific genetic defects, produces only minority of Parkinson's disease cases (<10%), study of these genetic defects may reveal key factors and abnormalities in the protein pathways and gene regulation, which are likely to be involved in the more common sporadic forms of Parkinson's disease.

The discovery of these genes has produced startling insights into the pathogenesis of Parkinson's disease with better understanding of the key factors and abnormalities in the protein pathways that lead to neurodegeneration in Parkinson's disease. Dysfunction in two major pathways has been implicated by the nature of these genes namely: impairment in the ubiquitin-dependent proteasomal pathway and mitochondrial dysfunction and oxidative stress. More recently, increasing evidence suggests that dysfunction in another degradative pathway – autophagy, may contribute to the

pathogenesis of Parkinson's disease. Each of these genes can be categorized into these pathways according to their function; this is described in more detail.

Locus	Location	Gene	Inheritance	Function	Neuropathology	Age of Onset
PARK1/ 4	4q21	α-synuclein	Autosomal dominant	Involved in synaptic function	Lewy bodies, Lewy neurites; variable glial inclusion, tau inclusion and amyloid plaque pathology	Early & late onset
PARK2	6q25-27	parkin	Autosomal recessive	E3 Ligase	Variable Lewy body pathology	Early onset
PARK3	2p13	unknown	Autosomal dominant	Unknown	Lewy body pathology	Late onset
PARK5	4p14	UCH-L1	Autosomal dominant	Ubiquitin hydrolase and ligase	unknown	Early onset
PARK6	1p35-36	PINK1	Autosomal recessive	Mitochondrial kinase	unknown	Early & late onset
PARK7	1p36	DJ-1	Autosomal recessive	Involved in oxidative stress response	unknown	Early onset
PARK8	12p11	LRRK2 (dardarin)	Autosomal dominant	Protein kinase	Variable Lewy body and tau inclusion pathology	Late onset
PARK9	1p36	ATP13A2	Autosomal recessive	Possible ion pump	unknown	Early onset
PARK10	1p32	unknown	Genetic susceptibility locus determined by genome wide linkage study of 51 Icelandic families with 1 or more family members with typical PD			
PARK11	2q	unknown	Genetic susceptibility locus by genome linkage in multiplex US PD families			
	2q22-23	Nurr1/ NR4A2	Autosomal dominant	Transcription factor involved in differentiation & maintenance of dopaminergic neurons	Lewy body pathology	Late onset
	Un-mapped	Htr2A/ Omi	unknown	Serine protease and/or involved in stress response	unknown	Late onset

Table 1.1 Mendelian Parkinson's disease-associated genes

Autosomal Dominant Parkinson's disease

Autosomal dominant Parkinson's disease results from gain of function mutations that lead to the clinical manifestation of Parkinsonism.

1.2.1 α-synuclein

1.2.1.a History

The amino acid sequence of synuclein was first reported in 1988 by (Maroteaux et al., 1988), from the electric lobe of the Pacific electric ray (*Torpedo californica*). It was so named syn(synapse)-nuclein(nucleus) due to its distribution on some regions of the nuclear membrane and high concentrations in presynaptic terminals of the nervous system, but not the peripheral tissue. In 1993, (Ueda et al., 1993) also reported on a similar protein which they then named "non-amyloid-β-component precursor" (NACP) due to the localization of NAC in amyloid plaques from Alzheimer's disease brains. Two homologous proteins were then subsequently identified by Jakes et al. (1994). The first was identical to NACP and was found to be a presynaptic protein with a perinuclear localisation, as such, it was later named as α-synuclein (Iwai et al., 1995; Jakes et al., 1994). The second, being homologous to α-synuclein, was named β-synuclein. A third member of the synuclein family was later identified in a high-throughput direct differential-cDNA-sequencing screen for breast cancer markers and named γ-synuclein (George, 2002). All three synucleins are homologous and are found to be expressed in various regions of the brain. However, only α-synuclein has been

implicated in diseases such as Alzheimers' and Parkinson's disease (George, 2002; Iwai et al., 1995; Lavedan, 1998; Lavedan et al., 1998; Nakajo et al., 1994).

Homo sapiens α-synuclein was mapped onto chromosome 4q21.q23 and was found to consist of seven exons but only five coding regions (exons 2-6). Alternative splicing has been observed for exons 4 and 6, but different protein isoforms have not yet been found.

1.2.1.b α-synuclein in Parkinson's Disease

1.2.1.b.i α-synuclein in Lewy bodies

α-synuclein was found to play an important role in Parkinson's disease when Spillantini discovered full length α-synuclein in Lewy bodies taken from postmortem brain tissue of sporadic Parkinson's disease patients in 1997 (Spillantini et al., 1997). This followed the discovery of α-synuclein mutations in several rare families with inherited Parkinson's disease (Polymeropoulos et al., 1997). This suggested a link between α-synuclein pathology and Parkinson's disease pathogenesis. Despite skepticism, this link was further reinforced when studies revealed that the predominant protein in Lewy bodies and Lewy neurites is fibrillar α-synuclein in both sporadic and familial Parkinson's disease (Baba et al., 1998; Mezey et al., 1998; Spillantini et al., 1997; Wakabayashi et al., 1997). The aggregation of α-synuclein to form these Lewy bodies and neurites is now thought to be the crucial step in the pathogenesis of Parkinson's disease. Such aggregation of α-synuclein is thought to stem from misfolded intermediates which may result when levels of the protein exceed the normal range present (Wood et al., 1999). These misfolded intermediates were found to self-aggregate and form stable β-sheet oligomers or protofibrils (Li et al., 2002; Wood et al., 1999),

which are then capable of acting as a nucleus for α-synuclein aggregation and the formation of Lewy bodies.

1.2.1.b.ii Structure & Cellular role of α-synuclein

The α-synuclein protein consists of 3 domains. The N-terminal repeat domain (residues 1-65), is highly conserved and consists of six copies of imperfect 11aa repeat, which are variations of the KTKEGV consensus sequence (Fig. 1.2). This domain is unordered in solution but may assume a double α-helical conformation, separated by a short break, which resembles lipid binding domains of class A2 apolipoproteins (Chandra et al., 2003; Davidson et al., 1998). The assembly into amphipathic α-helixes occurs when α-synuclein binds to negatively charged phospholipids, suggesting that it might normally be membrane associated (Eliezer et al., 2001; Uversky and Fink, 2002).

The central domain of α-synuclein (residues 66-95) is hydrophobic and is also better known as non-Aβ component of plaque (NAC) found in amyloid plaques in Alzheimer's disease (Ueda et al., 1993). This region is also relatively conserved between species and is required for α-synuclein to undergo a conformational change from random coils to β-sheet as well as form Aβ-like protofibrils and fibrils (el-Agnaf and Irvine, 2002; Giasson et al., 2001).

The carboxyl-terminal domain (residues 96-140) is negatively charged and composed mainly of acidic amino acids (George, 2002). The domain is not conserved between species and is highly variable both in size and sequence (Lavedan, 1998). This acidic domain, between residues 125-140, has been shown via deletion mutants to display a chaperone-like-activity (Kim et al., 2002; Park et al., 2002). Several phosphorylation

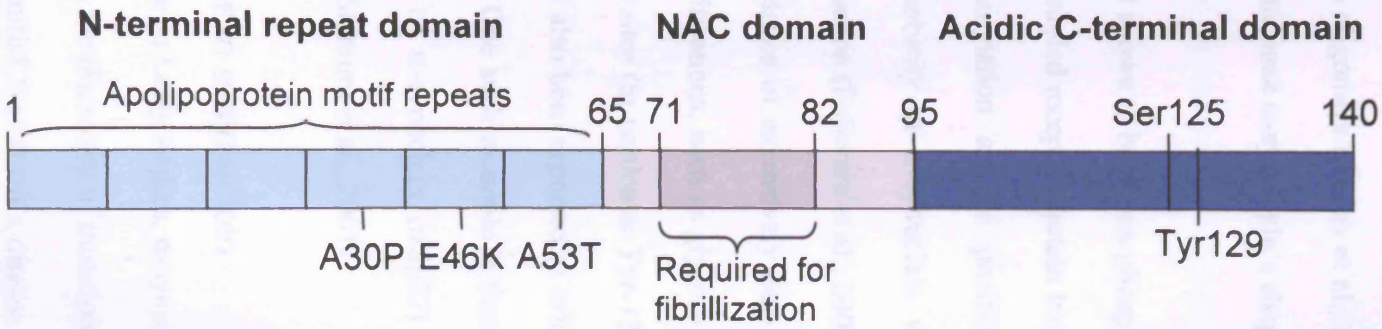


Figure.1.2 Structure of α-synuclein protein

sites have also been identified in the C-terminal domain. These include, Tyr-125, -133, -136 and Ser-129. The phosphorylation of the tyrosine sites by $p72^{syk}$ and other tyrosine kinases, which have similar specificity, have been shown, *in vitro*, to cause α-synuclein to lose its ability to form oligomers (Negro et al., 2002). It is not known, however, if this modification would attenuate α-synuclein's chaperone-like-activity.

α-synuclein has also been shown to be serine-phosphorylated by casein kinase-1, casein kinase-2 and G-protein-coupled receptor protein kinases (Okochi et al., 2000; Pronin et al., 2000). The phosphorylation at this position (Ser-129) changes the charge distribution and hydrophobicity of α-synuclein which then leads to promotion of fibrillation and oligomerisation (Fujiwara et al., 2002; McLean and Hyman, 2002). This can be seen in the abundance of extensively Ser-129 phosphorylated α-synuclein in Lewy Bodies. Other modifications, such as glycosylation and nitration, may also occur to the serine and tyrosine sites (in particular Tyr-125) present (Takahashi et al., 2002). These modifications have also been reported to affect its aggregation and targeting for proteasomal degradation. One such example is that shown by Shimura et al., whereby an O-glycosylated form of α-synuclein (αSp22) was found to be a substrate for ubiquitination by parkin (Shimura et al., 2001).

1.2.1.b.iii Mutations in α-synuclein

Other than for its presence in Lewy bodies, α-synuclein's association with Parkinson's disease was also shown in the discovery of mutations in the α-synuclein gene in several families affected with familial Parkinson's disease. The first mutation to be reported was that from a large Italian-American kindred (the Contursi family) and from three unrelated families of Greek origin (Polymeropoulos et al., 1997). The mutation was

found to be a G-to-A transition at position 157, in exon 4, of the coding region of α-synuclein. This mutation caused the alanine residue at position 53 to change to a threonine (A53T). Despite the mutation causing the families to suffer from an autosomal dominant form of Parkinson's disease, its role was unclear as the threonine at position 53 was naturally found in the rodent homologue, synuclein-1, and the zebra finch homologue, and the frequency of such a mutation was very low (Goedert, 2001; Vaughan et al., 1998).

However, further evidence was soon provided when (Kruger et al., 1998) identified a second mutation in exon 3 of the α-synuclein gene in an unrelated German family. This mutation is a G-to-C transition at position 88 which changes alanine to proline at position 30 (A30P). Unlike the first mutation, this mutation was not found normally in other species examined.

A third mutation was also recently discovered which caused a substitution of glutamate to lysine residue at position 46 (E46K). This mutation was found to cause familial Parkinson's disease and dementia with Lewy bodies in the Spanish kindred (Zarranz et al., 2004)

All the above mentioned point mutations in the α-synuclein gene occurred in the highly conserved N-terminal region of the α-synuclein protein, which displays the characteristic imperfect repeats of consensus sequence KTKEGV, and these mutations are thought to increase the probability of the mutated α-synuclein protein to be in the coiled state, and thus more likely to aggregate to form Lewy bodies.

1.2.1.b.iv Amplification of the α-synuclein gene

Amplification of the α-synuclein gene was recently found to also co-segregate with Parkinson's disease with Lewy bodies. Amplification of the gene includes duplication (Chartier-Harlin et al., 2004; Ibanez et al., 2004a) and triplication (Farrer et al., 2004; Singleton et al., 2003) of the α-synuclein locus. These findings suggested that increased expression of α-synuclein was sufficient, possibly, to cause aggregation and thus lead to Parkinson's disease. Nonetheless, other researchers were quick to point out that overexpression of α-synuclein was extremely rare in Parkinson's disease patients (Gispert et al., 2005; Hofer et al., 2005), and that studies involving the overexpression of α-synuclein in mice also revealed that other mechanisms were needed for aggregation to occur (Martin-Clemente et al., 2004).

1.2.1.b.v Polymorphisms of the α-synuclein promoter

As both mutation and amplification of the α-synuclein gene have been shown to be rare occurrences and have been excluded in the majority of Parkinson's disease cases, other modifications to the α-synuclein gene have also been considered as possible factors contributing to Parkinson's disease. Certain polymorphisms of the α-synuclein 5' non-coding region have been shown to be associated with sporadic Parkinson's disease even in patients who do not express a mutated protein or have increased copy number of the gene (Chiba-Falek and Nussbaum, 2001; Kruger et al., 1999; Mizuta et al., 2002; Ross et al., 2007; Tan et al., 2004a; Tan et al., 2000; Xia et al., 1996). Such allelic variations have been found to occur mostly in the NACP-Rep1 repeat site of basic repeat sequence, $(TC)_x(T)_2(TC)_y(TA)_z(CA)_u$, located ~10kb upstream of α-synuclein gene translational start. The most common variation between Rep 1 alleles was the number of dinucleotide repeats. Even alleles of the same size showed variability in the number of

dinucleotide repeats, $(TC)_{10-11}(T)_2(TC)_{8-11}(TA)_{7-9}(CA)_{10-11}$(the numerical subscript being the possible number of repeats), i.e. the total number of nucleotides remains the same, but variation in repetition may occur for each type of dinucleotide repeat (Farrer et al., 2001b). Non-repetitive sites also displayed variation in the form of single nucleotide polymorphisms (SNPs). These allelic variations were found to affect the levels of α-synuclein expression (Chiba-Falek and Nussbaum, 2001).

Although there are reports claiming otherwise, (Izumi et al., 2001; Khan et al., 2001; Mellick et al., 2005; Parsian et al., 1998; Spadafora et al., 2003; Tan et al., 2003), with an overview of the reports pertaining to studies in oxidative stress, studies on the amplification of the α-synuclein locus as well as polymorphisms on the α-synuclein promoter, we believe it is quite convincing that even subtle changes in α-synuclein expression may lead to Parkinson's disease pathogenesis.

1.2.1.b.vi Oxidative Stress & α-synuclein

Although oxidative stresses had been shown to be important in the genesis of Parkinson's disease, it was not clear how they were involved. Only when evidence, both *in vitro* and *in vivo*, was presented showing that oxidative stress promotes the formation of α-synuclein aggregates and inclusions, did the importance of its interaction with α-synuclein in Parkinson's disease become clearer.

The MPTP (1-methyl-4-phenyl-1.2.3.6-tetrahydropyridine) model has been used by many to decipher the interactions between environmental agents and genetic factors which result in selective neurodegeneration. MPTP is a mitochondrial complex I inhibitor and is selectively taken up into dopaminergic neurons via the dopamine

transporter leading to severe oxidative damage and neuronal degeneration resulting in Parkinsonism in rodents, primates and humans (Javitch et al., 1985; Javitch and Snyder, 1984; Ramsay et al., 1986; Shimura et al., 2001).

In some of the earlier reports on the involvement of MPTP in Parkinson's disease, Vila et al. (2000) and Przedborski et al. (2001) demonstrated that chronic treatment of mice with MPTP selectively upregulated α-synuclein in the midbrain and resulted in tyrosine nitration modification of the protein and subsequently aggregation (Przedborski et al., 2001; Vila et al., 2000). There was also evidence of α-synuclein up-regulation and aggregation in substantia nigra in MPTP treated non-human primate models (Kowall et al., 2000; Purisai et al., 2005). This was further supported by studies which indicated that not only were the disease causing mutant, A30P transgenic mice, especially sensitive to MPTP toxicity (Nieto et al., 2006), α-synuclein null mice also showed resistance to MPTP-induced neurodegeneration (Dauer et al., 2002; Klivenyi et al., 2006; Schluter et al., 2003) when compared to their control littermates of similar genetic background.

Several other toxins were also used to support the hypothesis that environmental factors work in concert with genetic factors to result in Parkinson's disease. Rotenone, a commonly used pesticide, was found to upregulate and aggregate α-synuclein (Betarbet et al., 2006; Sherer et al., 2002; Sherer et al., 2003). Paraquat, another commonly used herbicide, was found to induce fibrillation and aggregation of α-synuclein in mice (Manning-Bog et al., 2002).

Dopamine, in addition, may be the major contributor of oxidative stress since it can be generated endogenously by the substantia nigral neurons (Barzilai et al., 2001; Sherer et al., 2002). The exposure to intra-cellular dopamine may lead to mitochondrial dysfunction and impaired proteolysis via its reactive metabolites, H_2O_2, dihydroxyphenylacetic acid and dopamine-quinones, or directly due to its oxidative nature (Khan et al., 2001; Kruger et al., 1998; Pearson et al., 2001; Stokes et al., 1999).

The main hypothesis involving dopamine dependent oxidative stress revolved around the defective storage of dopamine in synaptic vesicles which then, due to the increase in pH in the cytoplasm, results in its auto-oxidation and generation of reactive oxidative species (ROS) (Barnham et al., 2004). Such leakage of dopamine may be initiated by the perforation of the vesicular membranes by α-synuclein β-sheeted oligomers (Lotharius and Brundin, 2002a; Lotharius and Brundin, 2002b; Volles and Lansbury, 2003), which form when there is an increase in α-synuclein above manageable cell load, or when there is aberrant, misfolded mutated α-synuclein.

There has also been evidence showing that α-synuclein interacts with another presynaptic dopaminergic protein, dopamine transporter (DAT) (Lee et al., 2001). This interaction causes the recruitment of DAT to the membrane, which subsequently results in an accelerated uptake of dopamine. Due to the direct interaction between α-synuclein, DAT and dopamine, any mutation or changes in expression in α-synuclein could lead to an accumulation of dopamine in the cell and thus give rise to intracellular oxidative stress.

Through studies on mutations in α-synuclein and amplifications of the gene locus, both of which lead to familial Parkinson's disease, it is clear that α-synuclein plays an important role in the development of the disease. However, since such abberations are rare, yet the abundance of aggregated α-synuclein in Lewy bodies, present even in sporadic Parkinson's disease patients whom carry neither mutations nor amplifications of the α-synuclein locus, other reasonings have been sought to explain the role of α-synuclein. The most convincing theory to date suggests that minute changes in levels of α-synuclein could, in long term, lead to Parkinson's disease. Such increases in α-synuclein could be due to polymorphisms of the gene, or brought about by external stimuli, for example, environmental toxins or oxidative stresses. The excess α-synuclein would lead to its misfolding and aggregation, which could subsequently lead to cell death due to its toxicity or through the perforation of dopamine containing vesicles, leading to the leakage of oxidative dopamine. In such a case, selective death of dopaminergic neurons would occur resulting in Parkinson's disease.

1.2.2 LRRK2/dardarin (*PARK 8*)

1.2.2.a Structure of LRRK2

LRRK2 (leucine-rich repeat kinase 2) is a large multidomain protein (Fig. 1.3), comprising of residues predicted to adopt the configuration of armadillo repeats and ankyrin repeats (Mata et al., 2006), a leucine-rich repeat (LRR) domain, a Roc (Ras of complex proteins) GTPase domain and C-terminal of Roc (COR) domain (Bosgraaf and Van Haastert, 2003), a kinase domain of the tyrosine kinase-like (TKL) subfamily (Manning et al., 2002) and a C-terminal WD40 domain (Mata et al., 2006).

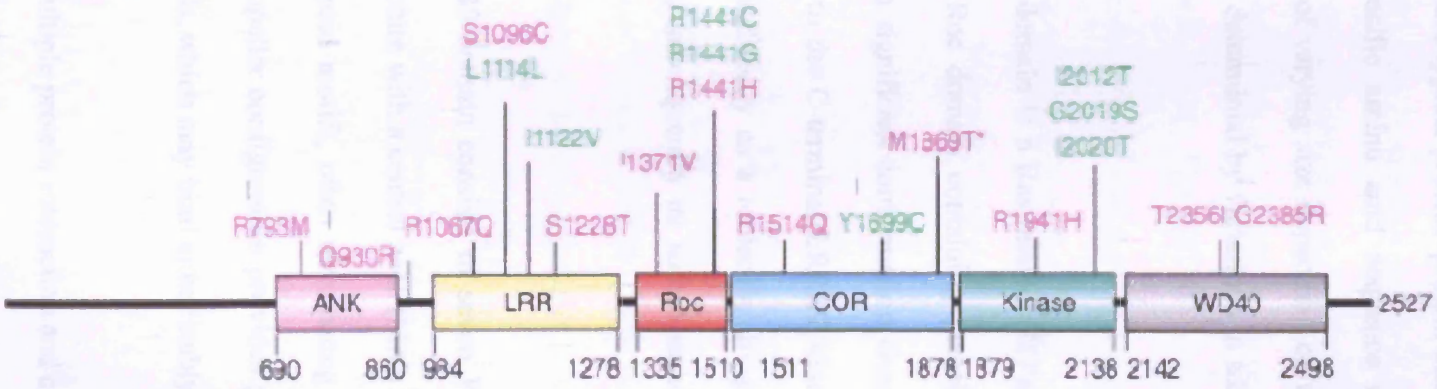


Figure. 1.3 Schematic diagram of LRRK2 domains organisation.
The positions of putative pathogenic amino acid substitutions are in magenta and amino acid substitutions segregating with Parkinson's disease are shown in green. Abbreviations: ANK, armadillo and ankyrin repeats; LRR, leucine-rich repeat domain; Roc, Ras of complex proteins GTPase; COR, C-terminal of Roc domain.

(adapted from Mata et al., 2006)

Armadillo repeats, ankyrin repeats and leucine-rich repeats have been shown, in general, to form the underlying structure of a specific protein-binding interface. In contrast to other more typical protein–protein interaction domains, these repeats do not recognize any specific amino acid sequence or structure. Instead, they form an elongated surface of varying size depending on the number of repeats. Specificity for protein partners is determined by variations in adaptive surface residues (Mosavi et al., 2004).

The LRRK2 Roc domain is a Ras-related GTPase found to be able to form a strong dimer with other Roc domain containing proteins. It is believed that following the binding of GTP, a significant conformation change occurs in the domain which may then be conveyed to the C-terminal LRRK2 kinase domain through the COR domain either directly or indirectly as a molecular hinge, leading to the dimerization of the kinase domain and subsequently its autophosphorylation and activation (Deng et al., 2008).

The LRRK2 WD40 domain consists of seven WD40 repeats which forms a circular propeller-like structure with a central pore (Mata et al., 2006). These WD40 repeats are short ~40 amino acid motifs, often terminating in a Trp-Asp (W-D) dipeptide. The resulting seven-propeller configuration provides potential binding surfaces for proteins and/or small ligands, which may bind either stably or reversibly (Smith et al., 1999b).

The presence of multiple protein interaction and catalytic domains in LRRK2 highlights its cellular importance and postulates its role as a hub for signalling and interaction between various proteins.

1.2.2.b LRRK2 in Parkinson's Disease

1.2.2.b.i Mutations in LRRK2

Mutations in LRRK2 have been found in a large number of Parkinson's disease patients (Fig. 1.3). Most of these mutations are dominant, e.g. G2019, I1122V, R1441C, as only one copy of the mutated gene is sufficient to cause the disease (Abou-Sleiman et al., 2006b; Zimprich et al., 2004). The G2019S mutation in particular was detected in 5-6% of autosomal dominant familial Parkinson's disease and 1-2% of sporadic cases (Abou-Sleiman et al., 2006b). This prevalence was found to be even higher for specific populations such as Ashkenazi Jews and North African Arabs (Abou-Sleiman et al., 2006b).

Pathogenic mutations scatter throughout all functional domains of LRRK2 (Fig. 1.3) (Mata et al., 2005; Taylor et al., 2006). However, the predominance of the G2019S mutation in Parkinson's disease patients has led to it becoming the main focal point of research into LRRK2's role in Parkinson's disease (Gilks et al., 2005). The LRRK2 G2019S mutation is situated in a highly conserved DYG motif of the kinase domain. This mutation results in an increase in the kinase activity of LRRK2, thus implicating a gain of function in its pathogenic mechanism (Thomas and Beal, 2007). Furthermore, the lack of deletions or truncations of LRRK2, as well as the autosomal dominant pattern of inheritance, also point towards a gain-of-function pathology.

Several studies showed that the kinase activity of the mutant LRRK2 was responsible for neuronal toxicity, and that this, in turn, was likely dependent on the activity of its GTPase Roc domain, N-terminal to the kinase domain (Thomas and Beal, 2007). Studies involving mutations in the Roc domain showed that the binding of GTP to this

domain regulates the kinase activity of LRRK2 as well as its phosphorylation by other kinases (Ito et al., 2007). As described earlier in section 1.2.2.a., the binding of GTP to the Roc domain results in a significant conformation change in the domain which may then be conveyed to the kinase domain through the COR domain, leading to the dimerization of the kinase domain and its autophosphorylation and activation. This explains why mutations in the Roc domain such as R1441C and I1371V, led to aberrant kinase activity (Deng et al., 2008). In fact, these mutations resulted in a decrease in GTP hydrolysis, leading to a prolonged GTP-mediated activation of the kinase, further supporting the gain-of-function pathology hypothesis (Deng et al., 2008).

1.2.2.b.ii Cellular role of LRRK2

The cellular function of LRRK2 is still mainly unknown. It has been shown to be essentially a cytoplasmic protein that may associate with intracellular membranes, such as the outer mitochondria membrane, Golgi apparatus and endoplasmic reticulum, and it is capable of interacting with another Parkinson's disease associated gene, parkin (described in detail in section 1.2.5) (Thomas and Beal, 2007). Moreover, parkin appears to promote aggregates enriched with ubiquitinated LRRK2 (Thomas and Beal, 2007). Although LRRK2 is not found to interact with either α-synuclein or tau, the identification of α-synuclein-positive Lewy body pathology or tau-positive neurofibrillary tangle pathology in LRRK2 patients suggests a possible common role of LRRK2 in the processing of these two proteins (Rajput et al., 2006; Ross and Farrer, 2005; Zimprich et al., 2004).

More recently, LRRK2 was shown to phosphorylate actin binding proteins, moesin as well as ezrin and radixin, at a previously known phosphorylation site encompassing Thr^{558} (Jaleel et al., 2007). The phosphorylation of this site regulated the binding of

moesin to actin. The GTPase, COR and kinase domains as well as the WD40 motif and C-terminal tail of LRRK2 were all found to be essential for this phosphorylation (Jaleel et al., 2007). In addition, there is also some speculation that LRRK2 might play a role in vesicular trafficking, as many Rho proteins, which also contain the Roc GTPase domain, have been found to regulate vesicular trafficking, but experimental evidence is still awaited (Abeliovich and Beal, 2006).

1.2.3 Nurr 1 (NR4A2)

1.2.3.a Structure & cellular role of Nurr1

Nurr1 *(NR4A2)* is a transcription factor which belongs to the orphan nuclear receptor superfamily. It was first identified from a mouse brain cDNA library and was localized onto human chromosome 2q22-q23 (Law et al., 1992; Mages et al., 1994). The Nurr1 gene contains 8 exons and 7 introns, and encodes for a 598 amino acid long protein which contains a DNA binding domain, a ligand binding domain and a variable region (Ichinose et al., 1999; Torii et al., 1999; Wang et al., 2003).

The transcriptional activity of Nurr1 is thought to be performed by binding to NGFI-B response element (DNA sequence that is recognised by Nerve Growth Factor induced protein B (NGFI-B) DNA binding domain) on the DNA or by binding to Nurr77 response element (DNA sequence that is recognised by Nurr77 DNA binding domain) in a homodimer conformation. It has been found to regulate the expression of genes such as tyrosine hydroxylase, the dopamine transporter, and vesicular monoamine

transporter-2 and is required for the differentiation and maintenance of nigral dopaminergic neurons (Law et al., 1992; Sacchetti et al., 2001; Sakurada et al., 1999).

1.2.3.b Nurr1 in Parkinson's disease

1.2.3.b.i Oxidative stress & Nurr1

Homozygous Nurr1 knock-out mice have been found to fail to develop midbrain dopaminergic neurons, a perinatal lethal phenotype. Moreover, Nurr1 heterozygote knock-out mice have reduced brain dopamine and suffer from locomotor deficits in response to neurotoxins such as MPTP and normal ageing (Jiang et al., 2005; Zetterstrom et al., 1997). Since a reduction of Nurr1 in the adult brain may increase the vulnerability of dopaminergic neurons to stress - Nurr1 may also play a role in the pathogenesis of Parkinson's disease (Eells et al., 2002; Le et al., 1999).

1.2.3.b.ii Mutations in Nurr1

In 2003, Le et al reported two mutations (–291Tdel and –245T/G) in the 5'-untranslated region (UTR) of Nurr1 in families with autosomal dominant Parkinson's disease. These mutations were shown to decrease Nurr1 expression levels *in vitro* and in lymphocytes of Nurr1 positive Parkinson's disease patients. Since the initial report at least ten follow-up studies in Parkinson's disease have revealed only three additional mutations (-253C/T, -223C/T, and Ser125Cys) indicating that Nurr1 mutations are rare (Grimes et al., 2006; Hering et al., 2004; Ibanez et al., 2004b; Karamohamed et al., 2005; Levecque et al., 2004; Nichols et al., 2004; Rawal et al., 2002; Tan et al., 2004b; Wellenbrock et al., 2003; Zimprich et al., 2003). The rarity of disease causing Nurr1 mutations together with little *in vivo* functional data for Nurr1 pathogenicity has raised questions about the importance and relevance of Nurr1 to Parkinson's disease.

1.2.4 UCH-L1

1.2.4.a Cellular role of UCH-L1

Ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1) is a de-ubiquitinating enzyme involved in the ubiquitin proteasome system (UPS) that hydrolyses the C-terminal of ubiquitin to generate ubiquitin monomers that can be reutilized to target proteins for clearance by the proteasome (Mouradian, 2002).

1.2.4.b UCH-L1 in Parkinson's disease

Leroy et al. (Leroy et al., 1998) identified, in a sequencing project involving 72 families with Parkinson's disease, a single missense mutation (Ile93Met) in the UCH-L1 gene in two German siblings. In both patients the clinical syndrome was typical for Parkinson's disease. The significance of these finding is however uncertain, as no other families with mutations in this gene have been found to date (Harhangi et al., 1999; Lincoln et al., 1999). A common coding polymorphism (Ser18Tyr) in the UCH-L1 gene has been identified. This variant was reported to be inversely associated with Parkinson's disease in a dose-dependent manner (Maraganore et al., 1999). This is supported by a recent meta-analysis of 11 published studies (Maraganore et al., 2004).

UCH-L1 is one of the most abundant proteins in the brain and immunofluorescence studies of Lewy bodies are positive for UCH-L1 protein, which implicates it either directly or indirectly with the development of Parkinson's disease (Lowe et al., 1990). With UCH-L1 also functionally involved in the ubiquitin-dependent proteolytic

pathway; it could potentially be a good candidate gene for Parkinson's disease (Gosal et al., 2006).

Autosomal Recessive Parkinson's disease

Autosomal recessive Parkinson's disease occurs when both alleles of a gene are mutated resulting in the clinical manifestation of Parkinsonism. DJ-1, parkin, PINK1 and ATP13A2 are all associated with early onset autosomal recessive Parkinson's disease and, interestingly, are also all linked to mitochondrial function.

1.2.5 Parkin (PARK 2)

1.2.5.a Cellular role of Parkin

Parkin is a 465 amino acid long protein containing two really interesting new gene (RING) finger domains separated by an IBR (in-between-ring) domain at the carboxyl terminal and an UBL (ubiquitin-like) domain at the amino terminal (Sakata et al., 2003; Shimura et al., 2000). It has been identified as an E3 ubiquitin-protein ligase, and plays a role in the ubiquitin proteasome system (UPS), by recruiting E2 components e.g. synphilin and facilitating the transfer of ubiquitin to damaged or misfolded target substrates (Fig. 1.4). These polyubiquitylated subtrates are then targeted to the 26S proteasome complex where they are degraded (Shimura et al., 2001; Upadhya and Hegde, 2003; Yang et al., 2006).

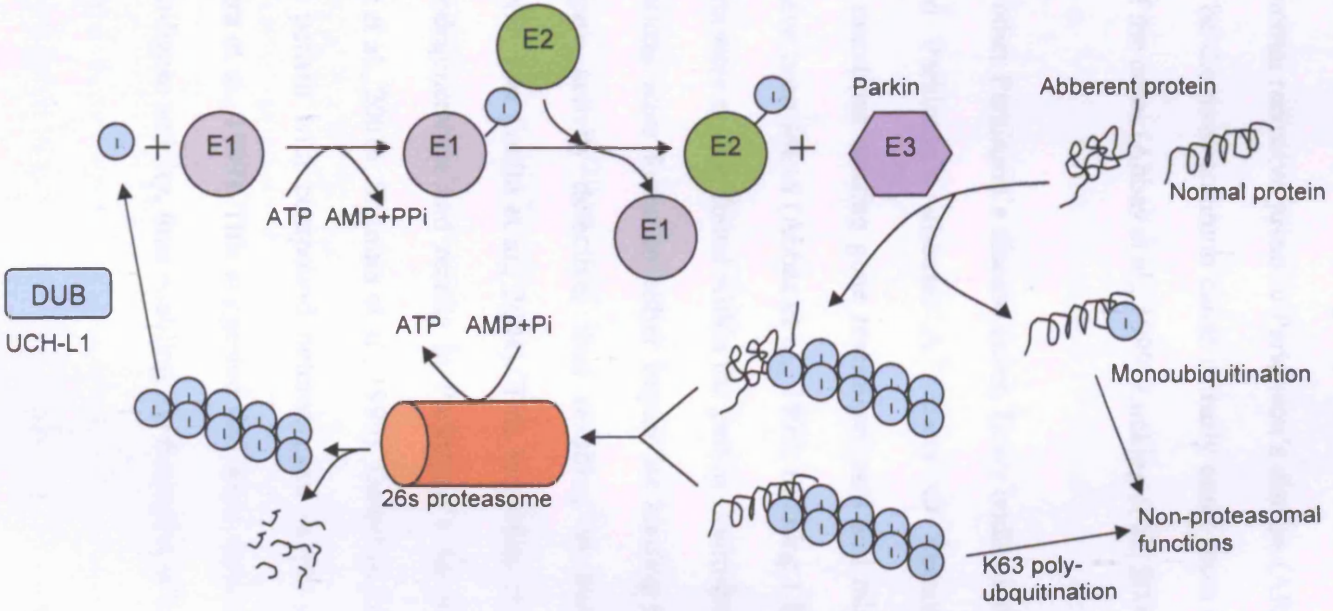


Figure. 1.4 Role of Parkin in ubiquitin proteasomal system

1.2.5.b Parkin in Parkinson's Disease

1.2.5.b.i Mutations in parkin

(Kitada et al., 1998) were the first group to find mutations in the parkin gene in Japanese autosomal recessive juvenile Parkinson's disease (ARJP) families. Parkin was then found to be the most common cause of early onset cases of ARJP, accounting for almost 50% of the cases (Abbas et al., 1999; Lucking et al., 2000).

In contrast to other Parkinson's disease cases, Lewy bodies are generally not present in parkin related Parkinson's disease. A variety of homozygous and compound heterozygous mutations causing gene rearrangements and mis-sense mutations in the parkin gene have been found (Abbas et al., 1999; Lucking CB, 2000). Of these, mis-sense mutations were mainly found within the parkin C-terminus, RING1 domain. Most of these mutations were found to either impair its binding to putative substrates or render its ligase activity defective, thus resulting in loss-of-function (Imai and Takahashi, 2004; von Coelln et al., 2004). This loss-of-function mechanism normally leads to neurodegeneration and results in Parkinson's disease with a lack of Lewy bodies (Farrer et al., 2001a; Shimura et al., 1999). However, Lewy body pathology was found in one patient with compound heterozygous parkin mutations (Farrer et al., 2001b; Shimura et al., 1999). This suggested that some parkin mutants may still retain some ubiquitin-ligase activity, thus enabling the formation of Lewy bodies (Hattori and Mizuno, 2004).

1.2.5.b.ii Oxidative stress & parkin

There is accumulating evidence that parkin may play a role in maintaining mitochondrial function and preventing oxidative stress (Shen and Cookson, 2004).

Parkin knock-out mice develop mitochondrial deficits (Palacino et al., 2004) and parkin knock-down in cell lines renders cells more vulnerable to oxidative stress (MacCormac et al., 2004). Parkin's ubiquitin ligase activity is also modified by nitric oxide mediated oxidative stress. Various groups have reported that the reactive metabolite of dopamine, dopamine quinone may decrease the solubility of endogenous parkin by covalently binding to cysteine residues of parkin (LaVoie et al., 2005; Tan et al., 2004b; Yao et al., 2004). This results in the loss of parkin's activity. Furthermore, they found increased insoluble parkin in the caudate nucleus of Parkinson's disease patients (LaVoie et al., 2005). Our group have also previously shown that endogenous parkin localizes to aggregates following exposure to dopamine in neuroblastoma cells (Valente et al., 2004a).

The protective function of parkin and its additional role in the mitochondria, apart from being involved in the ubiquitin proteasomal system, was further established when it was found to function in the same pathway, but downstream, of another Parkinson's disease associated gene, PINK1 (PTEN-induced kinase 1). PINK1 is also a protective protein which resides mainly in the mitochondria. This is discussed in further detail below.

1.2.6 PINK1 (PTEN-induced kinase 1; *PARK 6*)

1.2.6.a Structure of PINK1

PINK1 (PTEN-induced kinase 1) was first identified in cancer cell expression profile experiments and was shown to be transcriptionally activated by PTEN, thus its name PTEN-induced kinase 1 (Abou-Sleiman et al., 2006b). PINK1 is an 8 exon gene which

encodes for a 581 amino acid long protein. Via sequence comparisons with other proteins, it was found to consist of an N-terminal mitochondrial targeting motif, a C-terminal autoregulatory domain and a highly-conserved serine/threonine kinase domain (Abou-Sleiman et al., 2006b; Valente et al., 2004a). Recently, this has been corroborated using a baculovirus-infected insect cell system showing that PINK1 preferentially phosphorylates serine/threonine residues on basic substrates compared to acidic substrates, and does not target tyrosine residues (Sim et al., 2006). This phosphorylation was further shown to be auto-regulated by PINK1 C-terminal domain (Sim et al., 2006).

1.2.6.b Mutations in PINK1

Mutations in PINK1 were first discovered in three large consanguineous families with autosomal recessive juvenile parkinsonism (ARJP) – one Spanish, two Italian (Valente et al., 2004a). More mutations of the PINK1 gene have subsequently been found (Abou-Sleiman et al., 2006b). These mutations are recessive as only homozygous mutation, i.e. mutation on both copies of the gene, would lead to the disease.

The discovery that PINK1 mutations are mostly distributed around the kinase domain (Abou-Sleiman et al., 2006b; Kubo et al., 2006; Valente et al., 2004a), has led to assumptions that the disruption of kinase activity is the most probable disease mechanism. Some studies, using the measure of mitochondrial membrane potentials, have been performed which suggest that the loss of PINK1 function adversely affects mitochondrial function and thereby also increases cellular susceptibility to stress (Abou-Sleiman et al., 2006a; Valente et al., 2004a). However, more definitive studies have not yet been performed to support this hypothesis.

1.2.6.c PINK1 substrates & interactions

Over-expression of PINK1 has been shown to protect neuronal cells against mitochondrial and oxidative stresses such as MPTP, staurosporine and rotenone (Deng et al., 2005; Haque et al., 2008; Petit et al., 2005). TRAP1 (TNF receptor-associated protein 1, also known as heat shock protein 75) was identified as a substrate of PINK1. Omi/HtrA2 was also found to interact with PINK1. TRAP1 and Omi/HtrA2 are both associated with the protection of mitochondria against cell death via apoptosis (Plun-Favreau et al., 2007; Pridgeon et al., 2007). This supports the cytoprotective role of PINK1.

1.2.6.c.i TRAP1 substrate of PINK1

TRAP1 is a mitochondrial molecular chaperone associated with an anti-apoptotic function preventing oxidative-stress-induced mitochondrial cytochrome c release. This protective function was found to be dependent on TRAP1's phosphorylation by PINK1 (Pridgeon et al., 2007). Pathogenic PINK1 mutations such as G309D, L347P and W437X were shown to have an impaired ability to phosphorylate TRAP1 (Pridgeon et al., 2007).

1.2.6.c.ii Omi/HtrA2 substrate of PINK1

Omi/HtrA2 is a serine protease from the GLGF (glycine-leucine-glycine-phenylalanine; also known as PDZ or DHR, Dlg homologous region) domain containing family (Walsh et al., 2003). The GLGF domain is a common structural domain of 80-90 amino-acids found in the signaling proteins and helps anchor transmembrane proteins to the cytoskeleton and hold together signaling complexes (Ponting, 1997). It also contains a N-terminal mitochondrial-targeting domain as well as a reaper-like motif (Walsh et al., 2003). The reaper-like motif is a N-terminal motif first identified in the drosophila

protein, *Reaper*. This motif have been shown to disrupt inhibitor of apoptosis (IAP)-caspase complexes, displacing active caspases (Martins, 2002).

Omi-HtrA2 was thought to reside normally in the mitochondrial intermembrane space and when released to the cytosol during apoptosis, relieved caspase inhibition by binding to inhibitor of apoptosis proteins (IAPs), thereby stimulating apoptosis (Strauss et al., 2005). Omi/HtrA2 was first linked with Parkinson's disease when knockout mice showed parkinsonian phenotypes (Martins et al., 2004). Following this, a heterozygous mutation at Gly399 was found in four sporadic Parkinson's disease German patients and an Ala141 polymorphism which showed increased risk to Parkinson's disease further reinforcing its association with Parkinson's disease (Strauss et al., 2005).

Contrary to previous evidence of Omi/HtrA2 being pro-apoptotic, these loss-of-function mutations indicated that the deregulation of the protease activity of Omi/HtrA2 increased the susceptibility of cells to stress, most probably through mitochondria dysfunction. Although genetic proof of a role for this gene in Parkinson's disease has not been unequivocally established, it was shown that phosphorylation of Omi/HtrA2 during stress, modulated its proteolytic activity and that this phosphorylation was dependent upon PINK1 (Plun-Favreau et al., 2007).

Omi/HtrA2 was found to be phosphorylated by the p38 pathway during stress, in the presence of PINK1. In support of this relationship, brains of Parkinson's disease patients with PINK1 mutations were found to have less phosphorylated Omi/HtrA2 compared to idiopathic patients (Plun-Favreau et al., 2007). The p38 pathway involves p38 mitogen-activated protein kinases (MAPK). These kinases are responsive to various

stress stimuli such as inflammatory cytokines, ultraviolet irradiation and osmotic shock, and are involved in cell differentiation and apoptosis. Following activation by phosphorylation, p38 MAPK in turn phosphorylates and activates other proteins including various transcription factors such as ATF-2, Mac and MEF2 (Pearson et al., 2001).

It was postulated that upon p38 pathway activation due to stress, PINK1 was involved in the phosphorylation of Omi/HtrA2, which then increased its protease activity and thus provided mitochondrial protection. The direct interaction between PINK1 and Omi/HtrA2, however, was not shown (Plun-Favreau et al., 2007).

It is still unclear whether Omi/HtrA2 is indeed anti-apoptotic or not. Although Omi-HtrA2 contains the reaper-like motif and should promote apoptosis by lifting caspase inhibition, all molecular studies of Omi-HtrA2 done in the context of Parkinson's disease have proven otherwise. More research would be required before the cellular role of Omi-HtrA2 can be unequivocally established.

1.2.6.c.iii Parkin is downstream of PINK1

The association of PINK1 and its interacting proteins with the mitochondria strengthened the hypothesis that mitochondrial dysfunction may play a role in Parkinson's disease pathogenesis. This hypothesis was supported by postmortem studies showing mitochondrial impairment and oxidative damage (Beal, 2003). Nonetheless, despite the evidence, the mitochondrial dysfunction hypothesis has, until recently, been overshadowed by another theory based upon α-synuclein and parkin, which focused on protein aggregation and proteasomal dysfunction (Jenner and Olanow, 1998).

More recently, it was found that the phenotypes in PINK1 loss-of-function mutations in *Drosophila* could be rescued by using transgenic expression of parkin. This implied that parkin and PINK1 may act in a common pathway, showing that the two theories described above might, in fact, act together in the pathogenesis of Parkinson's disease (Tan and Dawson, 2006; Yang et al., 2006).

Both PINK1 and parkin mutant *Drosophila* models have similar phenotypes – male sterility, muscle and dopaminergic neuronal degeneration and increased sensitivity to oxidative stresses. Furthermore, double knockouts of both genes resulted in an identical phenotype to the single mutants suggesting a linear pathway between them (Tan and Dawson, 2006; Yang et al., 2006). Parkin has been suggested to act downstream from PINK1 in this pathway as parkin could rescue PINK1 knock-out phenotypes but not vice versa (as mentioned earlier). Interestingly, over-expressing parkin could restore normal mitochondrial morphology, DNA and protein content, but did not alleviate the PINK1 mutant *Drosophila* from its sensitivity to stressors, suggesting that the pathway involving PINK1 and parkin may only be involved in the maintenance of mitochondrial integrity (Yang et al., 2006).

The relevance of the observations in the *Drosophila* model to Parkinson's disease was much debated, especially when PINK1 knock-out mice failed to display any nigrostriatal neurodegeneration (Kitada et al., 2007). Nonetheless, Exner et al. subsequently demonstrated, using human cell lines including primary cell lines from patients with two different PINK1 mutations, that PINK1 knockout or mutation resulted in mitochondrial morphology abnormalities that could only be reverted by enhanced expression of parkin or the re-introduction of wild type PINK1 (Exner et al., 2007).

Following this discovery that PINK1 and parkin act through the same pathway, it would be especially interesting to identify substrates that, when phosphorylated by PINK1 in the mitochondria, lead to the recruitment or activation of parkin, which predominantly resides in the cytoplasm.

1.2.7 DJ-1 (PARK 7)

DJ-1 is a member of the THiJ/PfpI/DJ1 superfamily, a diverse family of proteins involved in various functions such as RNA-protein interaction regulation, chaperone activity, thiamine biosynthesis, Ras-related signal transduction and protease activity (Quigley et al., 2004; Wilson et al., 2004). They share the THiJ domain, which constitutes an overall α/β structure, the function of which is unclear (Lee et al., 2003).

Parkinson's disease causing DJ-1 mutations are rare and account for only about 1-2% of early onset autosomal recessive Parkinson's disease cases. Its cellular and subcellular localization is unclear, but it has been shown to be enriched in the brain and peripheral tissues and is primarily cytoplasmically localized, with a small pool associated with the mitochondria (Zhang et al., 2005).

DJ-1 is thought to be involved in the oxidative stress response by acting as a redox-dependent chaperone (Zhou et al., 2006). It is capable of such a role due to the presence of several cysteine residues which undergo an acidic shift in pI-value when exposed to reactive oxidative species (ROS), thus potentially mopping them up (Abou-Sleiman et al., 2006b; Kubo et al., 2006). However, as the quenching ability of DJ-1 is modest, it is suspected that other pathways are involved, of which much evidence points towards the

involvement of regulation of apoptosis via the phosphoinositide-3 kinase (PI3-K) – AKT pathway (Abou-Sleiman et al., 2006b; Wood-Kaczmar et al., 2006). This hypothesis is supported by evidence showing that RNAi knockdown of DJ-1α in *Drosophila* increased its sensitivity to oxidative stress which was reduced with increased PI3K/Akt signaling capacity (Wood-Kaczmar et al., 2006). However, the mechanism of interaction between DJ-1 and the PI3K/Akt pathway remains unknown.

1.2.8 ATP13A2 *(PARK 9)*

ATP13A2 encodes for a member of the P_5-ATPases of the P-type ATPase superfamily. P-type ATPases generally makes use of ATP to maintain an ion gradient across the cell membrane. As each step in this process is reversible, they in turn use the membrane potential to produce ATP (Kuhlbrandt, 2004). The substrate specificities and functions of the P_5-ATPases are still relatively unknown, but due to their close homology to P_1- and P_4-ATPases, they are most probably ion pumps. It has been shown that ATP13A2 is expressed in most tissues in mice, but is especially abundant in the brain (Schultheis et al., 2004).

Mutations in ATP13A2 have been identified recently as the underlying cause of an autosomal recessive form of early onset parkinsonism with pyramidal degeneration and dementia, Kufor-Rakeb syndrome (KRS) (Ramirez et al., 2006; Williams et al., 2005). Little is known about how these loss-of-function mutations lead to the genesis of this parkinsonian syndrome and whether there is any interaction between this protein and the other Parkinson's disease associated genes. However, *in vitro* models have shown that, while wild-type ATP13A2 is localized to the lysosomes, the mutated proteins were retained in the endoplasmic reticulum and degraded by the proteasome (Ramirez et al.,

2006), it is suspected that, an overload of such retained proteins may thus lead to neurodegeneration as seen in KRS due to proteasomal dysfunction.

Another theory is that, such loss of functional ATP13A2 from the lysosomes may lead to lysosomal dysfunction which may, in turn, impair cellular autophagy, which is the process of degradation of intracellular components via the lysosome. There is, currently, increasing evidence in the importance of autophagy impairment in Parkinson's disease (Rubinsztein et al., 2005).

In view of the ample evidence supporting the importance of a genetic role in Parkinson's disease, in particular the hereditary form of the disease, it is plausible that the genes described above may still be important in the idiopathic form of Parkinson's disease. This may occur through the deregulation of the normal, wild-type gene. This deregulation may result due to various reasons, including exposure to toxins. Nevertheless, a major part of this process is carried out via gene regulation by transcription factors. As such, we will discuss, in the following sections, the regulation of genes and possible transcription factors involved.

1.3 Regulation of Genes

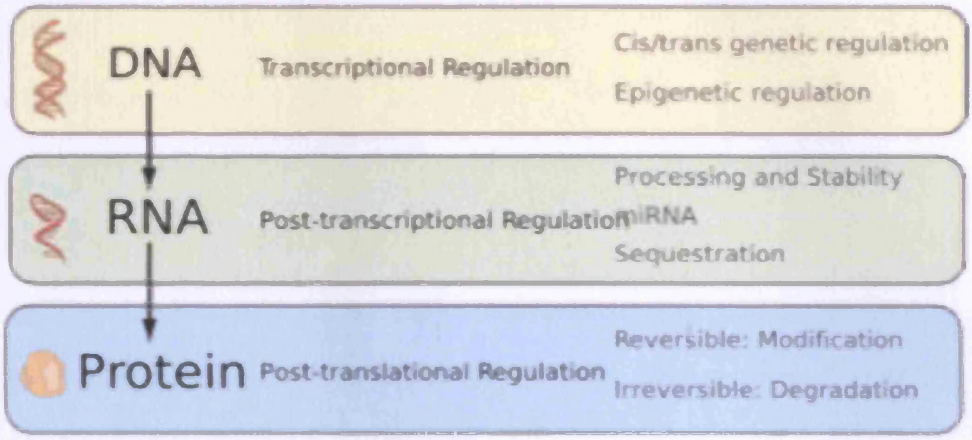


Figure. 1.5 Central dogma of molecular biology

Adapted from http://en.wikipedia.org/

Gene expression is the process by which inheritable information from a gene, such as the DNA sequence, is made into a functional gene product, such as protein or RNA. Several steps in the gene expression process may be modulated, including the transcription step and the post-translational modification of a protein. Gene regulation gives the cell control over structure and function, and is the basis for cellular differentiation, morphogenesis and the versatility and adaptability of any organism. Gene regulation may also serve as a substrate for evolutionary change, since control of the timing, location, and amount of gene expression can have a profound effect on the functions (actions) of the gene in the organism (Alberts et al., 2008; Karp, 2007).

Any step of gene expression may be regulated, from the DNA-RNA transcription step to post-translational modification of a protein. The following is a list of stages where gene expression is regulated:

- Epigenome (Chemical and structural modification of DNA or chromatin)
- Transcription

- Post-transcriptional modification e.g. splicing
- RNA transport (nuclear sequestration, nuclear export, sequestration in processing bodies)
- mRNA degradation
- Translation
- Post-translational modifications

Although potentially every step, as shown above, involved in the expression of a gene may be regulated, for most genes, the initiation of RNA transcription is the most important point of control. This is because, only transcriptional regulation ensures that the cell will not synthesize superfluous intermediates (Alberts et al., 2008; Karp, 2007).

1.3.1 Transcriptional Regulation

Transcription involves the synthesis of RNA from a DNA template by RNA polymerase (RNA pol). In eukaryotic transcription, there are three classes of RNA polymerases: I, II and III, catalyzing the transcription of genes encoding different classes of RNA. RNA pol I transcribes pre-rRNA 45S, which matures into 28S, 18S and 5.8S rRNAs. RNA pol III synthesizes tRNAs, 5S rRNA and other small RNAs found in the nucleus and cytosol. Only RNA pol II is involved in the transcription of protein coding genes and hence functions in the production of mRNA. As RNA pol II is not able to bind the DNA directly, other proteins bind to the DNA to form the pre-initiation complex prior to RNA pol II recruitment. Transcription then initiates with RNA pol II directing 3'-hydroxylation of the RNA chain on a ribonucleoside triphosphate, leading to extension of the chain in a 5'→3'direction (Alberts et al., 2008; Karp, 2007).

Transcription is regulated by the binding of proteins (transcription factors) to cis-regulatory DNA response elements (Ptashne, 1988). Several types of cis-acting elements have been defined including promoters, enhancers, upstream promoter elements and response elements. Transcription factors can be divided into two main groups, namely general transcription factors that form the pre-initiation complex and gene specific transcription factors which act to either stimulate or repress the assembly of the pre-initiation complex.

1.3.1.a Regulation by Transcription Factors

General transcription factors are sufficient for low levels of accurate transcription initiated by RNA pol II from core promoters, many of which contain the TATA box or initiator sequence, Py_2CAPy_5 (Roeder, 1996). However, general transcription factors are common to all RNA pol II transcribed genes, independent of cell type. As such, gene specific transcription factors are essential for the activation and repression of specific genes to ensure a tightly controlled temporal and/or tissue specific expression.

Transcription factors bind to promoter elements or enhancers to influence the initiation of transcription by recruiting chromatin modifiers and/or members of the pre-initiation complex. For example, assembly of general transcription factor TFIID into the pre-initiation complex is a rate limiting step and is stimulated by the presence of transcriptional activators that bind to other general transcription factors such as TATA box binding protein (TBP) or specific TBP associated factor (TAF) subunits. SP1 is one such transcriptional activator. It interacts with dTAF110 and facilitates the pre-initiation complex assembly (Roeder, 1996). Alternatively, some activators may recruit factors

that modulate chromatin structure to make the promoter and enhancer elements more accessible.

Conversely, transcriptional repressors may act to prevent activators from binding to their DNA binding sites via competition or formation of non-DNA binding protein-protein complexes with activators. In addition, inhibitors may interact with activators and block their activation domain in a phenomenon known as quenching. Direct inhibition may also occur by transcriptional repressors binding to their DNA elements in promoters and making contacts with the general transcription factors that hinders the formation of the pre-initiation complex (Latchman, 2008). One such example is the Eve (Drosophila even skipped) protein which functions by preventing the association of TFIID with the promoter TATA box element (McKay et al., 1999).

Additionally, some transcription factors bind co-activators or co-repressors that in turn interact with the basal apparatus. These co-activators and co-repressors themselves do not bind DNA but their specificity is conferred by their ability to associate with DNA binding transcription factors.

1.3.1.b Regulation of Transcription Factors

To allow for precise temporal and spatial regulation of transcription, the activity of gene specific transcription factors has to be regulated. This may be achieved in several ways. Some transcription factors may have restricted distribution and are only synthesized in certain tissues or at a specific time. This is typical of factors involved in the regulation of development (Alberts et al., 2008). Other transcription factors may require the

presence of tissue-specific co-factors before they are able to regulate transcription (Alberts et al., 2008).

Alternatively, some transcription factors may be activated under certain conditions, such as an extracellular stimulus, by protein modifications in the form of phosphorylation, acetylation and/or ubiquitination. For example, NFκB is sequestered in the cytoplasm by binding to IκB. Extracellular stimuli such as stress, cytokines and free radicals cause the phosphorylation and ubiquitination of IκB. This releases NFκB and allows it to localise to the nucleus and active target genes (Karin and Ben-Neriah, 2000). Some factors may also be regulated by binding to a ligand. For example, steroid and thyroid hormones enter cells and combine with intracellular receptors to form active transcription factors (Beato and Klug, 2000).

The activation or synthesis of gene specific transcription factors in response to various spatial or temporal stimuli as well as the combinatorial control by various transcription factors allows the establishment of a vastly complex pattern of gene expression required in every organism for normal development and function.

1.3.1.c Types of Transcription Factors

In general, transcription factors bind to DNA as homodimers or heterodimers and recognize DNA through one of the more common structural motifs: zinc finger, leucine zipper, helix-loop-helix and helix-turn-helix.

1.3.1.c.i Zinc finger motif

The zinc finger motif is present in the largest class of mammalian transcription factors. Two common zinc finger motifs exist. The first type (Cys_2His_2) involves a zinc ion which holds an α-helix and a β-sheet together, coordinated through two histidines and two cysteines residues respectively (Alberts et al., 2008). Proteins that contain this motif share extensive homology (90% identical or conserved residues) throughout each zinc finger DNA-binding domain (Luscombe et al., 2000).

The second type of zinc finger motif (multicysteine: C_4 or C_6) is similar to helix-turn-helix motif, in which two α-helices are held together by zinc ions (Alberts et al., 2008). Although the two types of zinc finger motifs are structurally different, they both require zinc for part of their structure and they both use an α-helix to recognise and bind to DNA. Both EGR (contain Cys_2His_2 zinc finger motif) and Nuclear Receptor proteins (contain multicysteine, C_4, zinc finger motif), to be described later, belong to this group of transcription factors.

1.3.1.c.ii Leucine zipper motif

The leucine zipper motif gets its name because leucine residues occur every seventh amino acid along a stretch of α-helix. As an α-helix turns every 3.5 residues, this places all the leucine residues on one side of the α-helix. Unlike the zinc finger proteins, the leucine zipper proteins can only bind DNA in dimers. One α-helix from each monomer is held together by hydrophobic interactions between the leucine residues to form a short coiled-coil conformation. Just beyond the dimerization interface, the two α-helices separate to form the DNA binding domain (Alberts et al., 2008).

1.3.1.c.iii Helix-loop-helix motif

The helix-loop-helix motif is characterized by a shorter α-helix separated to a longer one by an intervening loop. Transcription factors with this motif, like those with leucine zipper motif, can only bind DNA when dimerised. However, transcription factors with the helix-loop-helix motif may bind to DNA as homodimers or heterodimers. This allows for a higher diversity in regulatory factors present. In addition, some helix-loop-helix transcription factors lack the α-helical extension required for DNA binding. Nonetheless, they can still dimerize with another full-length helix-loop-helix transcription factor. This heterodimer is unable to bind DNA tightly as only half of the necessary contacts for DNA binding are present (Karp, 2007).

1.3.1.c.iv Helix-turn-helix motif

Finally, another very common motif, the helix-turn-helix is composed of two α-helices connected by a short extended chain of amino acids. The two helices are held at a fixed angle through interactions between the two helices. The C-terminal helix recognises and binds to consensus sequences on the DNA. Outside the helix-turn-helix motif, the structure varies drastically between different transcription factors. The POU-domain transcription factors which will be described later belong to this class of transcription factors (Alberts et al., 2008).

To further illustrate the importance of gene regulation in general and in Parkinson's disease, the following sections will discuss in detail a few transcription factors, from different families, which are of relevance to this thesis.

1.3.2 EGR sub-family of Transcription factors

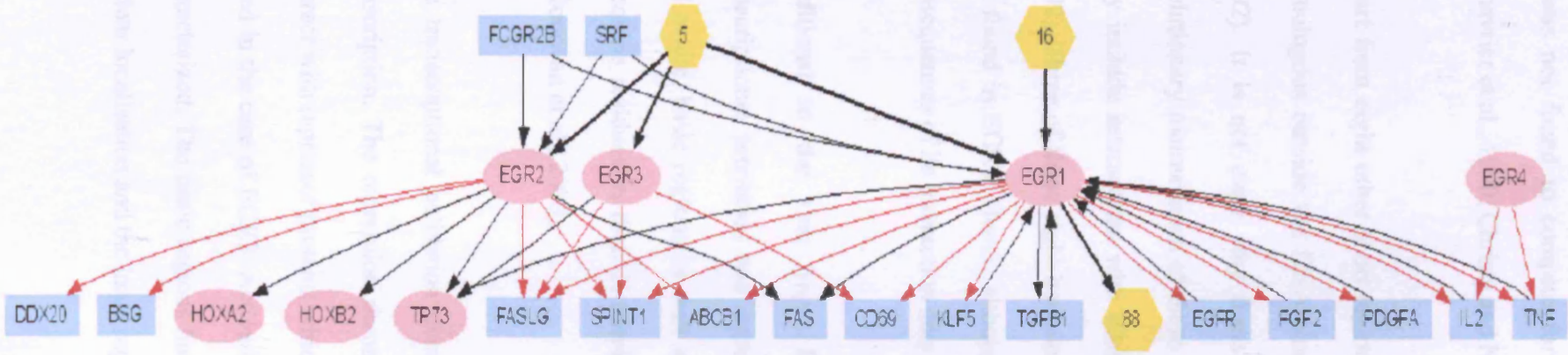
Immediate early genes are the first gene targets activated by the diverse intracellular signaling systems relaying events and stimuli at the cell surface to the nucleus. This is essential to enable coupling of short-term, cell-surface events to the long-term, coordinated changes in gene expression that give rise to the required alteration in cell function (Beckmann and Wilce, 1997). The Early growth response (EGR) family of genes consists of inducible transcription factors belonging to a subclass of the immediate early genes. They respond rapidly to various stimuli and neuro-transmission by mediating a cascade of altered gene expression (Fig 1.6) (Beckmann and Wilce, 1997).

1.3.2.a EGR protein structures & domains

The EGR family of transcription factors include EGR1 (also known as NGFI-A, krox-24, zif268 and TIS8), EGR2 (also known as krox-20), EGR3 (also known as PILOT) and EGR4 (also known as NGFI-C and pAT133) (Beckmann and Wilce, 1997). EGR transcription factors belong to the Cys_2His_2 class of zinc finger motif-containing proteins and contain three zinc fingers each. The three zinc fingers recognize a nine base-pair sequence of DNA, with each finger spanning three nucleotides (O'Donovan et al., 1999).

All four members of the EGR family recognize the same consensus sequence GCG(G/T)GGGCG (Cao et al., 1990; Chavrier et al., 1988; Christy and Nathans, 1989a; Crosby et al., 1991; Lemaire et al., 1990; Patwardhan et al., 1991). Although the consensus binding motif of Sp1 is related to, and may overlap, the EGR consensus site,

Figure. 1.6 Gene Regulatory Network of TF family EGR in human



Note:
Ellipses are 'transcription factors (TFs)' Boxes are genes.
Hexagons are the clustered genes. The number of the genes is shown inside.
Red lines indicate the protein-DNA binding is known.
Only official gene symbols are used in the network.

Listing of the gene clusters (TFs are underlined):
16→EGR1: POU4F1, CSF2, CSF3, IL1B, IL12A, NAB1, NAB2, NGFB, OSM, MAPK1, MAPK3, MAPK4, MAPK8, MAPK11, CXCL12, CUL5.
5→EGR1 + EGR3 + EGR2: CHRM1, CHRM2, CHRM3, CHRM4, CHRM5.

EGR1→88: CEBPB, ELK1, AR, JUN, MYB, NFKB1, PPARG, BCL2, RELA, TP53, WT1, CDK5, CDKN2A, CHGA, CHRNA7, COL1A2, COL2A1, CRABP2, ZFP90, CTNNB1, ACE, DUSP4, EIF4EBP1, ABCA2, F3, FCER2, FGFR3, FLT1, FLT3, FN1, ALOX5, GCG, ANKRD1, HGF, HSD11B2, APOA1, IFNG, IGF2, IL2RB, IL3, IMPDH2, LHB, LTB, MAOB, ACHE, MMP14, ATP2A2, SERPINE1, PCSK2, PDGFB, PECAM1, PLAU, POR, PDGFC, PSEN2, PTEN, BAX, RB1, CCND1, RET, SLC1A4, SLC5A5, SOD1, SOD2, SRY, VAMP2, SYN1, SYN2, TGFBR2, THBS1, TIMP1, TIMP2, TIMP3, TPO, VEGF, VEGFB, PTP4A1, TFPI2, FOSL1, EPX, CAT, CCKBR, CD9, CD19, CD28, GDF15, PTGES, CD44.

Figure courtesy of http://rulai.cshl.edu/TRED/GRN/EGR.htm

it was not found to compete for binding to DNA with EGR1 or EGR2 proteins (Chavrier et al., 1990; Christy and Nathans, 1989a).

Apart from eight other short regions of homology, the different EGR proteins are not homologous outside the DNA-binding zinc finger domain (Fig. 1.7) (Crosby et al., 1992). It is not clear why these eight homologous regions are present, however, evolutionary maintenance of these regions suggests they have critical functions which may include interactions with other proteins involved in transcription (Crosby et al., 1992). Three of these eight homologous regions found in EGR1, EGR2, and EGR3 are not found in EGR4. Hence, although EGR4 may bind to the same consensus site, the consequences of its interaction may be unique (Crosby et al., 1992).

Additional to the zinc finger DNA-binding domain, EGR proteins also have transcriptional activation and repression domains, nuclear localisation signal domain and acidic, basic regions as well as large regions rich in proline, alanine, serine and threonine residues in their N-terminal region (Fig 1.7) (Beckmann and Wilce, 1997; O'Donovan et al., 2000).

The transcriptional activation domains allow EGR proteins to activate target gene transcription. The repression domains, on the other hand, have only been found to interact with repressor proteins which repress EGR transcriptional activity. This was not noted in the case of EGR3. Activation and repression domains of EGR4 have yet to be characterized. The basic regions have been shown to aid in EGR transcriptional factor nuclear localisation and the large regions rich in proline, alanine, serine and threonine

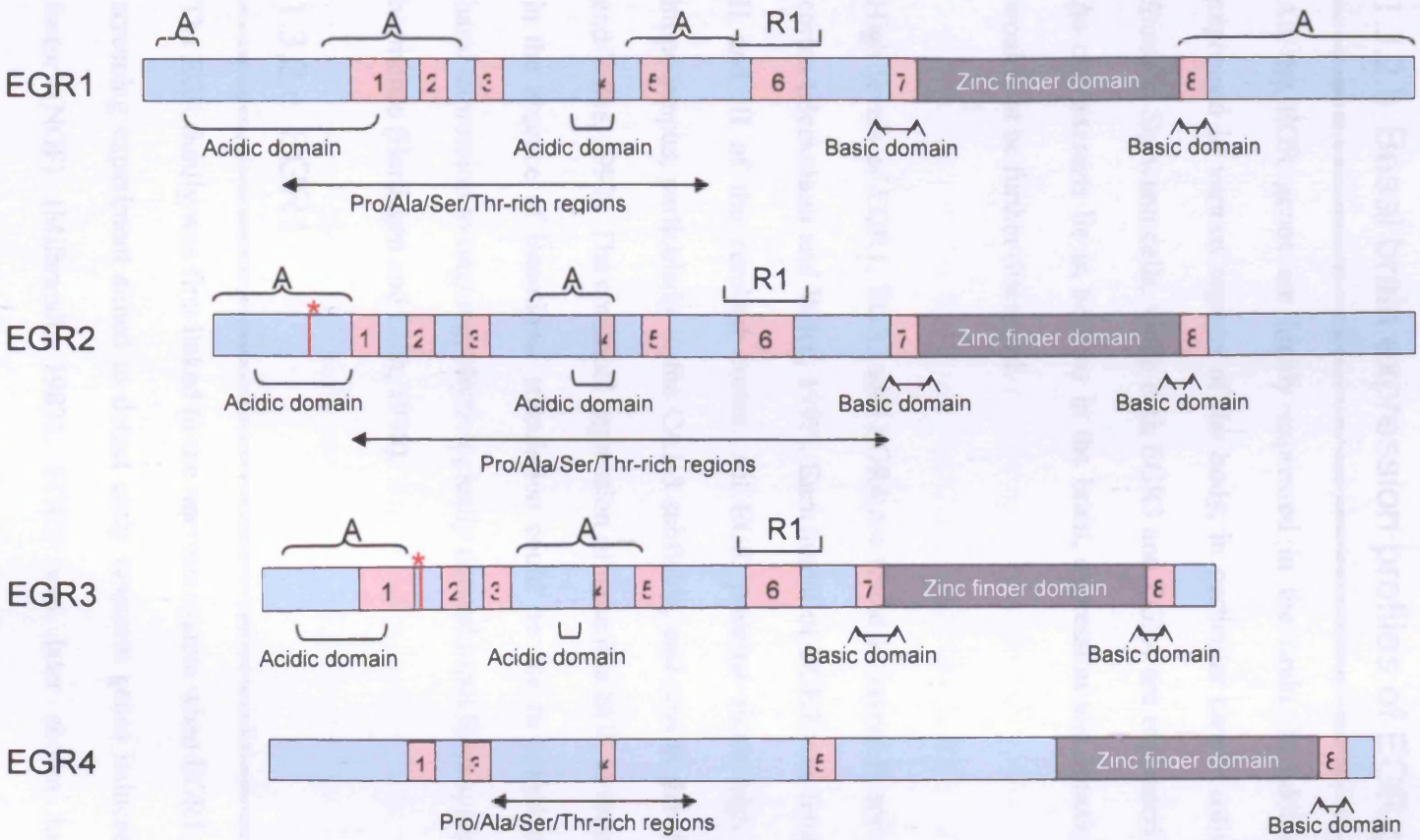


Figure. 1.7 Schematic diagram of structural and functional regions of EGR transcription factors. The pink boxes represent the 8 regions of homology between the different EGR proteins. EGR4 only contain 5 of the 8 homology regions. 'A' represent activation domains while 'R1' represent the region whereby NAB1 or 2 interact. The red asterisk and line represent the location of splicing observed in EGR2 and 3.

residues may serve for post-translational modifications to occur (Knapska and Kaczmarek, 2004).

1.3.2.b Basal brain expression profiles of EGR genes

All the EGR genes are highly expressed in the brain. In addition, EGR1 is also expressed in various regions of the body, in particular cancer cells; EGR2 is mainly found in Schwann cells, while both EGR3 and EGR4 are expressed in T-lymphocytes. As our interests lie in neurons in the brain, expression and function in other regions would not be further discussed.

High levels of EGR1, EGR3 and EGR4 are found in layers II and IV of the cerebral cortex (Beckmann and Wilce, 1997). Enrichment of EGR2 was found mainly in layers II and III of the cerebral cortex. All EGR proteins show high expression in the hippocampus, particularly in the CA1-3 subfields, and also in the striatum (Beckmann and Wilce, 1997). The continual expression of inducible EGR in these areas of the brain in the absence of intentional stimulation could be due to constitutive expression, or basal expression to ongoing, physiologically normal input from synapses or neuroactive hormones (Herdegen and Leah, 1998).

1.3.2.c EGR1

The EGR family was first linked to the nervous system when EGR1 was identified in a screening experiment aimed to detect early response genes induced by nerve growth factor (NGF) (Milbrandt, 1987). EGR1 was later shown to be induced by neurotransmitters or depolarization, thus indicating that it is not just involved in differentiating neurons but mature neurons as well (Sheng and Greenberg, 1990). Since

then, EGR1 has also shown to be induced, in various regions of the brain, by different pharmalogical stimulations (shown in detail in Table 1.2).

EGR1 is mainly implicated in neuronal plasticity. Numerous studies have been performed to examine the function of EGR1 in the sensory cortex, in response to visual and sensory stimulations (Kaczmarek and Chaudhuri, 1997; Knapska and Kaczmarek, 2004); in the hippocampus, in response to various seizure stimuli (Gall et al., 1990); in long-term potentiation (Worley et al., 1993) and especially in learning (Knapska and Kaczmarek, 2004). EGR1 knock-out mice have been observed to have impaired long-term potentiation following tetanic stimulation (Jones et al., 2001; Wei et al., 2000) and learning studies showed impaired acquisition and severe deficits in long-term spatial memory (Jones et al., 2001). Of particular relevance to Parkinson's disease, EGR1 was shown to be induced when D1 dopamine receptors were stimulated in the striatum, where excitatory amino acid afferents from cerebral cortex and dopamine afferents from the substantia nigra synapse on common projection neurons (Keefe and Gerfen, 1996).

Spatial expression patterns of EGR1 mRNA correspond tightly with its protein level, indicating that the basal level of EGR1 expression is likely to be regulated mainly at the level of transcription (Beckmann and Wilce, 1997). Several response elements have been found in the EGR1 promoter, these include serum response elements, Sp1 sites, AP-1 (activator protein 1) sites, CRE (calcium/cAMP response elements), NFκB sites and ERE (EGR response elements) (Aicher et al., 1999; Sakamoto et al., 1991; Schwachtgen et al., 2000). Of these elements, the serum response elements (SRE) appear to play the main role in the coordinated induction of EGR1 as they are required for the induction of EGR1 by serum, platelet-derived growth factor, phorbol 12-

Table 1.2 Summary of EGR induction after pharmalogical stimulations in the brain and/or cultured neurons

Stimulus	EGR mRNA/protein induced	Brain region/cell type
Glutamate	EGR1 mRNA	Cortico-striatal monolayers
NMDA	EGR1 mRNA & protein	Cerebral cortex, CA1-3, dentate gyrus, inferior colliculus, cerebellum, hypothalamus, olfactory bulb
Quinolinic acid	EGR1 mRNA	Cortex, CA1-3, dentate gyrus
Kainic acid	EGR1 mRNA & protein	Neocortex, CA1-3, dentate gyrus, striatum, pyriform, cingulated & primary olfactory cortices, amygdale, lateral fornix
	EGR2 protein	Somatosensory & pyriform cortices, olfactory tubercle, CA1-3, dentate gyrus, amygdale, striatum, retrosplenal cortex
Kainic acid (cold)	EGR1 mRNA & protein	Sensory, pyriform & entorhinal cortices, CA1-3, dentate gyrus
AMPA/Quis agonists	EGR1 mRNA	Cortico-striatal monolayers
Dizocilpine	EGR1 protein	Retrospenal & cingulated cortices, paraventricular & dorsomedial thalamic nuclei
	EGR2 protein	Retrospenal & cingulated cortices, paraventricular & dorsomedial thalamic nuclei
Pentylenetetrazole	EGR1 mRNA & protein	Neocortex, pyriform & cingulated cortices, CA1-3, dentate gyrus
	EGR4 mRNA	Dentate gyrus
Picotoxin	EGR1 mRNA	Neocortex, pyriform & cingulated cortices, CA1-3, dentate gyrus
Bicuculline	EGR1 mRNA	Neocortex, pyriform & cingulated cortices, CA1-3, dentate gyrus, entorhinal & retrospenal cortices, amygdaloid & habenular nuclei
	EGR2 protein	Somatosensory pyriform, cingulated & retrospenal cortices, fornix, amygdale
Dopamine (D_1) agonist	EGR1 mRNA	Striatal neurons, cerebral cortical monolayers, caudate putamen, olfactory tubercle, cortex
	EGR2 mRNA	caudate putamen, olfactory tubercle, cortex
Dopamine (D_2) agonist	EGR1 mRNA & protein	Caudate putamen, nucleus accumbens
	EGR3 mRNA	Striatum
Amphetamine (D_1)	EGR1 mRNA	Caudate putamen, nucleus accumbens, olfactory tubercle
Cocaine (D_1)	EGR1 mRNA	Caudate putamen, nucleus accumbens
	EGR3 mRNA	Striatum
Cocaine seizure	EGR1 mRNA	Medial amygdale, striatum, ventral medial thalamus, pyriform & entorhinal cortices, CA1-3, dentate gyrus
α_1 adrenoceptor antagonist	EGR1 mRNA	Cerebral cortex
Muscarinc antagonist	EGR1 mRNA & protein	Striatum
Caffeine (adenosine A_1 & A_2 antagonist)	EGR1 mRNA	Striatum
Morphine withdrawal	EGR1 mRNA	Caudate putamen, nucleus accumbens, anterior cingulated cortex
	EGR1 protein	Cerebral cortex, CA1-3, dentate gyrus, cerebellum, brain stem
	EGR2 mRNA	Caudate putamen, nucleus accumbens, anterior cingulated cortex
Vasointestinal peptide	EGR1 mRNA	Cortical neurons
Ethanol withdrawal	EGR1 mRNA & protein	Cerebral cortex, olfactory bulb, CA1-3, dentate gyrus, inferior colliculus, cerebellum, brain stem

(Adapted from Beckmann and Wilce, 1997)

myristate 13-acetate (PMA), nerve growth factor and following antigen receptor cross-linking in B lymphocytes (Christy and Nathans, 1989b; DeFranco et al., 1993; McMahon and Monroe, 1995). Binding to the other elements on EGR1 promoter seems to only modulate the induction conferred by binding to SRE (Knapska and Kaczmarek, 2004). Brn3a (which will be described later) has also been shown to regulate EGR1 expression (Smith et al., 1999a). However, whether this interaction involves direct DNA binding of Brn3a was not confirmed.

The transcriptional activating function of EGR1 depends on its modifications and binding partners. EGR1 has been found to be phosphorylated on serine residues and glycosylated (Cao et al., 1990; Lemaire et al., 1990). The activation potential of EGR1, which may be through phosphorylation, is distributed over the extensive serine/threonine rich regions as shown previously in Fig. 1.7 (Gashler et al., 1993). In addition to protein modifications, interaction with other transcription factors plays a major role in modulating EGR1 transcription regulation activity.

Several binding partners have been found for EGR1, these include NR4A1 from the nuclear receptor family, NF-κB, c/EBPβ, JunD, c-Fos, cAMP responsive element binding protein binding protein (CBP/p300), NGFI-A binding protein (NAB1) and NAB2 (Knapska and Kaczmarek, 2004). NAB1 and NAB2 were of special notice as they were the only proteins that showed repression of EGR1 activity during interaction (Russo et al., 1995; Svaren et al., 1996). While NAB1 is an active repressor which works by a direct mechanism and its repression is not specific to any particular activators (Swirnoff et al., 1998), NAB2, which shares two large regions of homology with NAB1, is regulated by stimuli that also induce EGR1 expression (Svaren et al.,

1996). This suggested that NAB2 could act as a negative-feedback mechanism for EGR1 (Knapska and Kaczmarek, 2004). The diversity of potential interactions emphasizes the versatility and sensitivity of EGR1 mediated regulation.

1.3.2.d EGR2

EGR2 was first isolated from serum treated human 303 cells using a low stringency hybridization with EGR1 cDNA probe (Joseph et al., 1988). This newly identified protein was found to be co-regulated with EGR1 by fibroblast and lymphocyte mitogens (Joseph et al., 1988). However, differences in induction of EGR1 and EGR2 by different stimuli were also shown (Joseph et al., 1988). In addition different pharmalogical stimuli also induced different responses from EGR1 and EGR2 in different regions of the brain (Table 1.2).

The EGR2 promoter contains two SRE-like elements, with a CArG-box as an inner core element (Beckmann and Wilce, 1997). CArG-1 was found to be responsible for the serum and PMA responsiveness of EGR2 via protein kinase C-dependent and independent pathways respectively (Rangnekar et al., 1990). EGR2 transcription could be repressed by other inducible transcription factors like c-Fos and Fra-1 and this suppression was also found to occur through the CArG-box. Although AP-1-like elements are present in the EGR2 promoter, EGR2 is not inducible by NGF (Joseph et al., 1988).

The two acidic regions at the EGR2 N-terminal are responsible for the trans-activation properties of EGR2 (Chavrier et al., 1990). Modifications in the C-terminal of EGR2 did not appear to affect its trans-activation abilities (Herdegen and Leah, 1998). Similar

to EGR1, interactions with NAB1 or NAB2 were also found to block EGR2 trans-activation (Russo et al., 1995; Svaren et al., 1996).

1.3.2.e EGR3

EGR3 was later isolated from human 303 cells using a similar strategy as that used to identify EGR2 (Patwardhan et al., 1991). The promoter region of EGR3 has not been fully characterized.

EGR3 has been found to be induced by a variety of stimuli including mitogens that also induced EGR1 and 2 (Patwardhan et al., 1991). Brain derived neurotrophic factor (BDNF) has also been found to be the endogenous signal that induces EGR3 expression, via a PKC/MAPK-dependent pathway, in dentate granule cells of the hippocampus (Roberts et al., 2006). EGR3, in turn, was found to regulate type A GABA receptor alpha 4 subunits in these neurons (Roberts et al., 2006). The regulation of these receptors is associated with temporal lobe epilepsy (Roberts et al., 2006). EGR3 has also been linked to learning, memory, stress and novelty in mice (Gallitano-Mendel et al., 2007; Li et al., 2007).

EGR3 is believed to have several translation start sites and exists in several isoforms in the brain (O'Donovan and Baraban, 1999; O'Donovan et al., 2000). These different isoforms were observed to vary in their trans-activation abilities, as the smaller isoforms lack the first of two activation domains present at the N-terminal of EGR3 (O'Donovan and Baraban, 1999). NF-κB was shown to interact with EGR3 in T-cells to transactivate genes encoding inflammatory cytokines (Wieland et al., 2005). Similar to both EGR1 not known whether EGR3 could also be similarly repressed by NAB2.

1.3.2.f EGR4

In contrast to the other EGR proteins, EGR4 has not been studied in such considerable detail. EGR4 was isolated from mitogen stimulated human T lymphocytes and identified as the human homologue of the rat *Egr-4* clone, which itself was isolated via homology to the zinc finger region of *Egr-1* in NGF-stimulated rats (Crosby et al., 1991; Muller et al., 1991). Most of the studies on EGR4 were concentrated in T-lymphocytes and spermatogenesis (Skerka et al., 1997; Tourtellotte et al., 2000).

The EGR4 promoter was shown to contain two calcium response elements, four sites for Nur77 binding, a Sp1 site and an EGR DNA-binding motif (Crosby et al., 1992). EGR4 has been shown to auto-regulate its own expression through binding to the GC-rich EGR binding motif on the EGR4 promoter (Zipfel et al., 1997). This auto-regulation appeared to occur in a dose-dependent manner and could suggest a negative feedback mechanism (Zipfel et al., 1997). SREs were not found in the promoter despite EGR4 being shown to be strongly induced by serum stimulation (Crosby et al., 1992). As mentioned earlier, similarly to EGR1, EGR4 was also found to be induced by NGF in rat PC12 cells (Crosby et al., 1991).

The activation or repression domains of EGR4 have not been clearly characterized. Nonetheless, as with EGR3, NF-κB was also shown to interact with EGR4 in T-cells to transactivate genes encoding inflammatory cytokines (Wieland et al., 2005). In addition, EGR4 was also shown to interact with NFAT (nuclear factors of activated T cells) to form heterodimers and regulate pro-inflammatory cytokines gene expression following antigenic stimulation (Decker et al., 2003). EGR4 activity has not been shown to be repressed by either NAB1 or NAB2.

Although none of the EGR members have been associated with Parkinson's disease, their inducible nature via various stimuli, including dopamine, and their strong expression in the striatum makes them good candidates for studies into the regulation of Parkinson's disease associated genes in oxidative stress.

1.3.3 Orphan nuclear receptor family of Transcription factors

Nur77 (NR4A1/NGFI-B), Nurr1 (NR4A2) and Nor1 (NR4A3) are highly homologous receptors that form a sub-group in the nuclear receptor superfamily of ligand-activated transcription factors (Law et al., 1992; Mangelsdorf et al., 1995). The nuclear receptor family includes receptors for steroid hormones, retinoic acid, thyroid hormone, vitamin D, and several other small, lipophilic signaling molecules (Mangelsdorf et al., 1995). Nurr1, Nor1, Nur77 and a large number of members in the family lack identified ligands. Therefore, they are referred to as orphan receptors. Nuclear receptors have a common structural organization with a variable N-terminal, a conserved zinc finger DNA binding domain of the multicysteine C_4 class, and a less conserved ligand binding domain.

Nurr1, Nor1 and Nur77 transcription factors are products of immediate-early genes and their expression and activity are regulated in a cell-specific manner by a variety of extracellular mitogenic, apoptotic and differentiation stimuli (Jankovic et al., 2005). As mentioned earlier (section 1.2.3.b), Nurr1 is one of the genes associated with Parkinson's disease and mutations in the Nurr1 gene have led to the development of Parkinson's disease.

Other than the importance of Nurr1 in the development and survival of mesencephalic dopaminergic neurons, Nurr1 also appears to regulate the expression of tyrosine hydroxylase (TH), dopamine transporter (DAT), vesicular monoamine transporter 2 (VMAT2), and l-aromatic amino acid decarboxylase (AADC), all of which are important in the synthesis and storage of dopamine (Jankovic et al., 2005). Nurr1 is essential to the development and maintenance of dopaminergic neurons through its interaction with factors such as Pitx-3, Lmx1b, GDNF receptor, Wnt family of glycoproteins and p57kip2. These factors are vital to the various stages in the development and maintenance of dopaminergic neurons (Jankovic et al., 2005).

In view of the involvement of Nurr1 in Parkinson's disease, dopaminergic neuron survival and functions, it is included in our studies into transcription factors that regulate α-synuclein.

1.3.4 Brn3 sub-family of Transcription factors

The POU family of transcription factors was originally defined on the basis of a conserved region identified in the Pit-1, Oct-1, Oct-2 and Unc-86 regulatory proteins, which play a critical role in regulating gene expression in specific cell types (Latchman, 1996; Verrijzer and Van der Vliet, 1993; Wegner et al., 1993). The original Brn3 factor was first isolated as a novel POU protein by He et al. (He et al., 1989) using a degenerate PCR approach to isolate novel POU domain transcription factors specific to neuronal cells. The Brn3 factor was later discovered to be, in fact, three distinct factors encoded by different genes which are expressed in specific subsets of neuronal cells (Gerrero et al., 1993; Theil et al., 1993; Theil et al., 1994; Turner et al., 1994). These

factors are Brn3a (Gerrero et al., 1993; Lillycrop et al., 1992), Brn3b (Lillycrop et al., 1992; Turner et al., 1994) and Brn3c (Gerrero et al., 1993; Ninkina et al., 1993). Although encoded by different genes, these three factors are closely related to one another and exhibit very strong homology to the nematode regulatory factor, *unc*-86 (Latchman, 1996). Like unc-86, they also play a critical role in regulating neuronal specific gene regulation (Latchman, 1996).

Brn3a is important for the differentiation and survival of sensory as well as motor neurons. Knock-out Brn3a mice suffer from severe neuronal developmental defects and are usually non-viable (McEvilly et al., 1996). Brn3b and Brn3c are involved in the differentiation and maintenance of retinal and vestibular neurons of the inner ear respectively. Unlike Brn3a knock-out mice, both Brn3b and Brn3c mice are viable but suffer from blindness and deafness respectively (Erkman et al., 1996). Due to the importance of Brn3a in the development and maintenance of motor neurons, it was the main focus in our experiments and will be described in further detail below. As Brn3b has been shown to interact with Brn3a and display possible antagonistic effects on Brn3a activity, it will also be described below (Latchman, 1999). Brn3c will not be discussed further.

1.3.4.a Brn3a

Brn3a is the most widely expressed Brn3 protein in the nervous system. It is expressed both in the peripheral nervous system as well as in the central nervous system. In the central nervous system, Brn3a expression is only observed in post-mitotic neurons (Fedtsova and Turner, 1995; Gerrero et al., 1993; Turner et al., 1994; Xiang et al., 1995). High levels of Brn3a are observed in the developing midbrain, hindbrain and in

the spinal cord (Fedtsova and Turner, 1995; Ninkina et al., 1993). Expression in adult brain is restricted to specific regions. These regions include specific areas in the hindbrain such as the medial habenula, the nucleus ambiguous and the inferior olivary complex; regions in the midbrain such as the red nucleus, the mesencephalic nucleus and the superior colliculus (Turner et al., 1994).

The Brn3a protein is encoded by a single gene, but two transcriptional promoters are present. Transcription from the upstream promoter followed by splicing to remove an intron between the first and second exons results in the long form of Brn3a (Fig. 1.8) (Latchman, 1998). On the other hand, transcription from the second promoter, located within the intron downstream of the first exon, results in the production of an unspliced RNA which encodes the short form of Brn3a lacking the first 84 amino acids, which contains a POU IV box (Theil et al., 1993). Both isoforms of Brn3a contain the POU domain, as such, they are able to activate genes mainly involved in neuronal differentiation. In addition, Brn3a-long is also able to activate genes such as α-internexin and anti-apoptotic genes such as Bcl-2 via its additional POU IV box activation domain in the longer N-terminal (Smith et al., 1997a; Smith et al., 1997b).

The two forms of Brn3a are produced in different proportions by different neuronal cells (Liu et al., 1996) and have different functional properties. The presence of alternative promoters to produce the two different isoforms allows this differential regulation (Frass et al., 2002). In addition, two clusters of 1 and 4 copies of Brn3

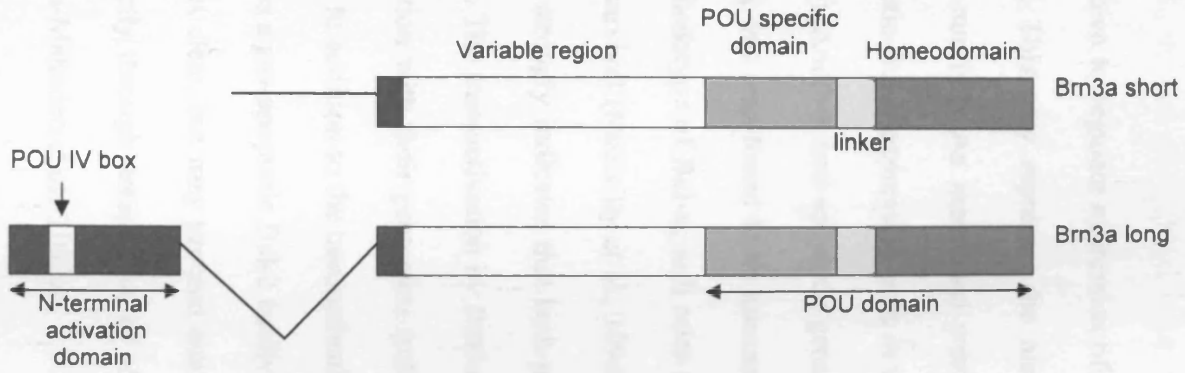


Fig 1.8 Schematic representation of Brn3a isoforms
The long isoform contain and extra N-terminal domain that contains a highly conserved region, the POU IV box. The POU domain is found on both the long and short isoforms of Brn3a.
(Adapted from PhD thesis 'Transcriptional regulation by Brn-3a POU domain containing transcription factors' Dr. J. Dennis, 1999)

consensus binding sequences were found within the Brn3a promoter approximately 10 and 5kb upstream of the transcription start site respectively (Trieu et al., 1999). These sites offer a possible autoregulation mechanism to regulate Brn3a expression in the different neurons (Trieu et al., 1999).

Brn3a has been shown to regulate expression of both anti-apoptotic and pro-apoptotic factors in neurons. This may represent the main mechanism through which Brn3a promotes neuronal survival. As mentioned previously, Brn3a was found to strongly activate anti-apoptotic Bcl-2 expression both *in vivo* and *in vitro* (Smith et al., 1998a; Smith et al., 1998b). Another anti-apoptotic gene, Bcl-x_L, which shares a high level of homology to Bcl-2, was also found to be transactivated by Brn3a (Smith et al., 2001). Interestingly, the phenotype of Bcl-x_L null mice is similar to Brn3a knock-out mice in terms of neuronal survival (McEvilly et al., 1996; Motoyama et al., 1995; Xiang et al., 1996). Hence, this strongly indicates that both proteins may exist in the same neuro-protective pathway. The transactivation by Brn3a of Bcl-2 and Bcl-x_L was found to be via a direct interaction with their promoters (refer to Table 1.3) (Smith et al., 1998b; Smith et al., 2001). In addition to the transactivation of anti-apoptotic genes, Brn3a was also found to repress a pro-apoptotic Bcl-2 family member, Bax. The mechanism of this repression is not yet clear, but may proceed either through a direct repression on Bax promoter or indirectly through antagonism of p53, which was found to activate Bax expression (Budram-Mahadeo et al., 2002).

Although Brn3a has never been implicated in any disease states, it is important in survival and maintenance of neurons (McEvilly et al., 1996), possesses neural-protective properties (Latchman, 1998) and is expressed in mid-brain neurons (Gerrero

et al., 1993). These factors suggest that it would be interesting to study whether Brn3a could be involved in the development of Parkinson's disease.

1.3.4.b Brn3b

Brn3b expression in the developing and adult brain show considerable overlap with Brn3a. It is found in particularly high levels in the optical, intermediate and deep gray areas of the superior colliculus, the dorsal column of the mesencephalic, pontine central gray, and the lateral interpeduncular nucleus in the midbrain of adult rat brains. Brn3b was also found in retina, trigeminal ganglia and dorsal root ganglia neurons.

In spite of its presence in many regions of the central nervous system, Brn3b knock-out mice only show defects in their retinal ganglion organization, resulting in their blindness. In fact, a loss of approximately 70% of retinal ganglion cells was reported by Gan et al. and the knock-out mice show disorganized neurites which subsequently fail to fasciculate efficiently, thus leading to apoptosis (Erkman et al., 2000; Gan et al., 1999; Gan et al., 1996). No other apparent defects were observed in regions expressing Brn3b in knock-out mice thus suggesting that functional redundancy may occur through the presence of other related proteins.

Similarly to Brn3a, Brn3b also exists in two isoforms, a longer spliced form of Brn3b consisting of both a POU IV box in the N-terminal and the POU domain, and a shorter unspliced form lacking the POU IV box (Fig. 1.9). The functional significance of the POU IV box in Brn3b is yet to be determined.

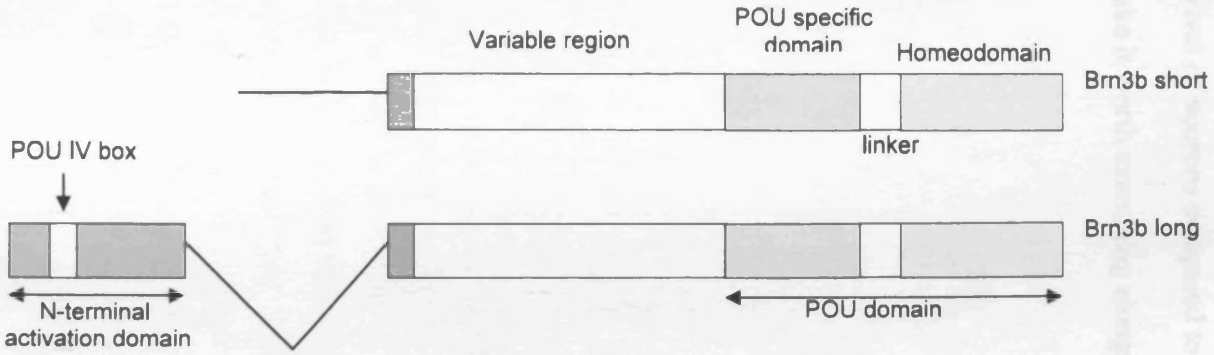


Fig 1.9 Schematic representation of Brn3b isoforms
The long isoform contain and extra N-terminal domain that contains a highly conserved region, the POU IV box. The POU domain is found on both the long and short isoforms of Brn3b.
(Adapted from PhD thesis 'Transcriptional regulation by Brn-3a POU domain containing transcription factors' Dr. J. Dennis, 1999)

Brn3b have been found to be antagonistic to Brn3a, and has been shown to repress the basal activity of many promoters activated by Brn3a (Latchman, 1999). Table 1.3 gives a summary of the various gene targets regulated by Brn3a and Brn3b as well as the antagonistic effects of Brn3b on Brn3a. Although Brn3b appears to be less likely to be involved in the survival of neurons compared to Brn3a, its interactions and antagonistic effects on Brn3a make it worth examining alongside Brn3a.

Promoter	Brn3a	Brn3b	Domain
Snap-25	+	-*	N-term
α-internexin	+	-*	POU
Synaptotagmin 1	+	-	POU
Synapsin 1	+	+	POU
NFH	+	-*	POU
NFM	+	-*	POU
NFL	+	-*	POU
Bcl-2	+	0	N-term
Bcl-x_L	+	0	Upstream promoter requires N-term, the downstream promoter requires sequences located between the POU and N-term domain
Bax	-	N.T	POU and sequences located between the POU and N-term domain
$p21^{Waf1/Cip1}$	+	N.T	POU
CDK4	N.T	+	N.T
Plakoglobin	N.T	-	N.T
BRCA 1	+	-	N.T
HSP-27	+	+	N.T
Vitellogenein	0	+	POU

Table 1.3 Promoter regulation by Brn3a and Brn3b transcription factors
Activation of the promoter is indicated by +, repression by -, no effect by 0, effect not tested by N.T and * indicates that Brn3b not only represses the promoter, but also negates activation by Brn3a. The domain involved in the Brn3 mediated transcription on each promoter is also listed.
(Adapted from PhD thesis 'Transcriptional regulation by Brn-3a POU domain containing transcription factors' Dr. J. Dennis, 1999)

1.4 Thesis Aims

Environmental stresses and toxins have long been associated with Parkinson's disease. This view was bolstered following the discovery of MPTP, a toxin found to cause an acute Parkinsonian syndrome. Subsequently, various other chemicals such as pesticides and herbicides were also associated with the onset of idiopathic Parkinson's disease. These toxins and stresses were mainly oxidative and caused neuronal cell death by the production of free reactive oxygen species or caused mitochondrial dysfunction by interfering with electron transport chain. Many of these toxins and stresses were used to create animal and cell models for Parkinson's disease research. The linkage between environmental stresses and genetics of Parkinson's disease was then conceived when protein abnormalities such as increased levels or aggregation were detected in these models.

Since oxidative stresses and genetic factors are important in the development of Parkinson's disease, the principal aim of this thesis was to profile the regulatory mechanisms and upstream pathways mediating Parkinson's disease associated genes in normal conditions and in the event of oxidative stress. The main focus was to investigate expression changes of these genes in various conditions and to examine the transcription factors involved in the regulation.

In addition, mutational and/or protein analysis were also performed on Nurr1 and PINK1. These genes are more recent discoveries compared to α-synuclein and parkin, thus, such studies will give important insights into their Parkinson's disease properties.

Chapter 2: Materials & Methods

2.1 General Reagents & Equipment

2.1.1 General Suppliers

General laboratory chemicals were of analytical or molecular biology grade 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 Plasmid vectors, DNA constructs & siRNA

The α-synuclein promoter plasmids used were '1.46', '1.9', '3.4', '3.8', '4.1' and PGL3 basic, a promoterless luciferase construct (gifts from Dr Robert L Nussbaum: NHGRI, Bethesda, USA).

EGR4 cloned into pSG5 vector was a gift from Dr. C. Skerka (Hans Knöll Institute, Netherlands). EGR4 21nt siRNA was obtained from MWG together with a non-specific 21nt siRNA as control.

Wild type Nurr1 and mutant construct -245T>G Nurr1 cloned into the pCMX vector were kind gifts from Dr Weidong Le (Houston, USA), with permission from Dr Hiroshi Ichinose (Tokyo, Japan) who isolated the genomic Nurr1 clone. The -309C>T Nurr1 mutation was introduced by site-directed mutagenesis using the QuikChange site-

directed mutagenesis kit (Stratagene) according to manufacturer's instructions (performed by Dr. M.Muqit, Institute of Child health, London, UK). The empty pCMX plasmid and the NuRE-POMC-Luc plasmid were kind gifts from Dr John Achermann (London, UK) and Dr Jacques Drouin (Montreal, Canada) respectively.

The Brn3 and pLTR poly constructs were gifts from Dr. T. Moroy (Phillips University, Marburg, Germany). The Brn3a-short, a-long, b-short, b-long constructs were obtained by amplification from cDNA and cloning into the pLTR poly construct downstream of the eukaryotic expression MoMuLV promoter.

The parkin plasmids used were '4500', '363', '282', '140', Control (all gifts from Dr Andrew West: UCLA, Los Angeles, USA) and PGL3 basic.

Full-length PINK1 cDNAs were amplified using the primers hPINK1 (5'-ggcggatccatggcggtgcgacaggcg-3') and hPINK2 (5'-ctcgaattcgggacatcacagggctgc-3') and cloned into the *Bam*HI and *Eco*RI sites of the vector pRK5myc so that a 9E10 (myc) tag is attached to the N-terminus. Mutations E476K, R501P, L102Q, R98A, S73A, R71A, F70Y, F70Q, R68P and R68G were introduced into pRK5mycPINK1 using the Quikchange site directed mutagenesis kit (Stratagene) (Dr. Robert Harvey, London School of Pharmacology, UK).

21nt siRNA used to knock-down specific genes were from MWG BIOTECH, Germany:

siRNA	**Sequence**
Nurr1	5'-ACGUGUGUUUAGCAAAUAA(dTdT)-3'
EGR4	5'-AGAUUGAGGACUUGCUGUC(dTdT)-3'
Non-specific control	5'-AGGUAGUGUAAUCGCCUUG(dTdT)-3'

2.1.3 Laboratory Equipment

Trans-BlotTM 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 Bacterial cell culture

2.2.1 Reagents

Bacto®-Tryptone, Bacto®-Yeast extract, and Bacto® micro-agar bacterial growth medium were obtained from Duchefa (Harlem, Netherlands). DH5α strain of E. coli was obtained from Invitrogen Ltd. (Paisley, UK). QIAprep Mini and Maxi DNA prep kits were from Qiagen Ltd. (Crawley, UK).

2.2.2 Bacterial Competent cells

Reagents required: MR buffer – 10mM RbCl, 10mM MOPS, pH7

[100µl RbCl (2.4g/10ml)

200µl MOPS 1M, pH7

Make up to 20ml with H_20]

MRC buffer – 10mM RbCl, 50mM $CaCl_2$, 100mM MOPS,

pH6.5

[100µl RbCl

400µl $CaCl_2$ (3.7g/10ml)

2ml MOPS 1M, pH6.5

Make up to 20ml with H_20]

A colony of DH5α *E.coli* untransfected cells was picked from a plate or 5µl of glycerol cell stock was obtained and grown in 10ml of LB (without antibiotics) in a 50ml falcon tube overnight (~16hrs) in 37°C shaker.

1ml of cultured cells was added to 99ml of LB and grown til an absorbance of 0.3-0.4 OD at 600nm was reached (~3hrs). The culture was then aliquoted into 50ml falcon tubes and centrifuged at 1500g for 5mins at 4°C to pellet the cells. The LB was then discarded. 10mls of MR buffer was then used to resuspend each of the pellets gently by swirling. The cells were then centrifuged again at 1500g for 5mins at 4°C, and the supernatant discarded. 8mls of MRC buffer was used to resuspend each of the pellets. This mixture was then incubated on ice for 30mins before centrifuging at 1500g, 5mins, 4°C. The supernatant is again discarded. The pellets of cells were again resuspended in 2ml of MRC buffer.

For storage of the competent cells, 1ml of 80% (v/v) glycerol was added to the 4ml of mixture and the final mixture was aliquoted into 100µl aliquots and snap-freezed using methanol and dry ice. These were then stored at -80°C.

2.2.3 Transformation of bacterial cells & retrieval of amplified DNA

Materials required:

LB media (1L) - 10g tryptone

5g yeast extract

10g NaCl

LB Agar (1L) - LB media

15g microagar

pour onto plates and left to set o/n then store at 4°C

Ampicillin aliquots (50mg/ml) - 1g ampicillin

20ml Millipore H_2O(autoclaved)

filter through 0.22μm Millex filter

1ml aliquots (store in -20°C)

Xμl of 5mg/ml ampicillin was added to Xml of LB media/agar (The agar was then poured onto plates and allowed to set overnight (~16hrs))

The required number of aliquots of competent cells (2 transformations per aliquot) were retrieved from -80°C freezer and thawed in ice. 50μl of the thawed cells were put into each labeled eppendorf tube. 1μl of 10-50ng/μl DNA sample was added to each respective tube and shook gently to mix. This was then incubated on ice for 20mins before being subjected to a heat shock of 42°C for 45secs. They were then incubated again on ice for 2-3mins before adding 500-800μl of LB (without antibiotics) was added to each tube and incubated for 1hr on 37°C shaker. 50μl of each culture was plated onto agar plates with selection (e.g. ampicillin) and incubated overnight (~16hrs) in 37°C. Leftover culture can be stored up to a week in 4°C.

1 colony from each plate was picked and inoculated in 200-300ml LB (ampicillin positive) and incubated overnight (~16hrs)/until confluent in 37°C shaker. For smaller cultures, each picked colony could also be inoculated in 5ml LB (ampicillin positive) in 50ml falcon tubes. The plates were sealed using nesco film and stored in 4°C.

The transformed cells were then stored by aliquoting into 500µl aliquots. 500µl of glycerol was added to each aliquot and mixed quickly and thoroughly by vortexing and then stored in -80°C

To retrieve amplified DNA, the cultures were then poured into centrifuge barrels and balanced with LB media. They are then centrifuged at 10000g for 15mins in Sorvall RC26 plus centrifuge in SLA-3000 rotor at 4°C. DNA was subsequently retrieved and purified by using the Qiagen Maxi/mini prep according to the manufacturer's protocol.

2.3 Mammalian cell culture

2.3.1 SH-SY5Y neuroblastoma cells

SH-SY5Y cells are human dopaminergic neuroblastoma cell lines. The cell line was obtained from the European Collection for Animal Cell Culture (ECACC) in the 13th passage.

Cells were grown and maintained in 85cm^2 flasks in the following medium:
F12 Hams/1X MEM with Earles Salts (1:1) (Gibco® Invitrogen, Paisley, UK); 15% (v/v) Foetal calf serum (PAA Laboratories); 1% (v/v) Non-essential amino acids; 1% (v/v) Penicillin/streptomycin antibiotic; and 1% (v/v) 2mM L-Glutamine (all from

Gibco® Invitrogen). Cells were incubated in a humidified 5% (v/v) CO2 atmosphere at 37°C.

For storage, cells were frozen long-term in liquid nitrogen in the following medium: 90% (v/v) Foetal Calf Serum + 10% (v/v) dimethylsulphoxide (DMSO) (Sigma).

Tissue culture media and media supplements were obtained from Invitrogen Ltd. (Paisley, UK). All tissue culture plastic-ware was obtained from Nunc (Roskilde, Denmark). Sterile 0.2μm filters were obtained from Millipore (Watford, UK). Lipofectamine 2000R reagent was obtained from Invitrogen Ltd. (Paisley, UK). Disposable sterile 0.22 μM filters were obtained from Millipore, Watford, UK.

2.3.2 Thawing of cells

5ml of warm media (suitable for the cell type being thawed) was put into each 50ml falcon tubes required (1 for each aliquot of cells). Aliquots of the required cells were retrieved from liquid nitrogen and 1ml of media from each falcon tube was pipetted into each tube and mixed using the pipette to quickly thaw the cells. This mixture was then pipetted back into each falcon tube (with remaining 4ml of media), and centrifuged at 120g for 1min at 20°C. The supernatant including the DMSO initially present with the cell aliquots was discarded. Each pellet was resuspended with fresh warm media and added into an $85cm^2$ flask. The total volume in the flask was made up to 5ml using media. The flasks were incubated in a humidified 5% (v/v) CO2 atmosphere at 37°C. The media was changed after 2-3 days if there was significant level of cell death.

2.3.3 Maintanence of cell cultures

When the cells have reached 80-90% confluency in the flask, they would be split. Media was retrieved from the confluent flasks and HBSS (Gibco® Invitrogen, Paisley, UK) was used to wash the cells.

1ml of trypsin-EDTA (Gibco® Invitrogen, Paisley, UK) was put into each flask and the flask was shaken to ensure that all the cells have come into contact with the trypsin. The trypsin was then retrieved and the flask of cells was incubated in the hood for 10mins for the cells to dislodge from the flask wall. Fresh media was then added to suspend the cells and split 1 in 8, 85cm^2 flasks, to a total volume of 5ml per flask. (SH-SY5Y cells are always grown in 85cm^2 flasks)

2.3.4 Freezing down of cells

Media was retrieved from the confluent flasks and HBSS was used to wash the cells. The cells were dislodged from the flask walls as described above (maintenance of cell cultures) and the suspensions were centrifuged at 120g for 1min at 20°C. The supernatant was discarded and freeze media was used to resuspend each pellet (1 aliquot - 1.5ml; 1 aliquot/ 85cm^2 flask) and transferred to cryotubes for freezing in -80°C overnight (~16hrs). They were then transferred to liquid nitrogen to be stored.

2.3.5 Transfection of mammalian cells

Cells were plated 1day before transfections. SH-SY5Y cells were plated at 2 x 10^5 cells per well in 6 well plates, 3.5 x 10^4 cells per well in 24 well plates and 1, 95% confluent, 85cm^2 flask per 10cm plate.

SH-SY5Y cells (passage 21-27) were plated onto 24 well plates at $3.5x10^4$cells per well and incubated overnight (~16hrs) to 90% confluency. For 1 plated well of the 24 well plate, DNA used for transfection was added at 3μg in 200ml OPTIMEM (Gibco® Invitrogen, Paisley, UK), this mixture was then added to 2μl Lipofectamine 2000 in 200ml OPTIMEM and incubated for 15mins. During this time, the plated wells were washed with HBSS to get rid of serum in the wells, which would otherwise affect the transfection efficiency. The DNA-Lipofectamine-OPTIMEM mixture was then added dropwise onto the cells in the wells at 400ml/well. Full culture media was replaced 4hrs later.

2.3.6 Cellular stress studies

2.3.6.a Dopamine stress

Cells were incubated for 24 hours in full growth media containing 0.0625mM, 0.125mM, 0.2mM, 0.25mM dopamine (3-hyroxytyramine hydrochloride; Sigma) or vehicle (media) prior to harvesting.

2.3.6.b MPP+ stress

Cells were incubated for 6 or 12 hours in full growth media containing 1mM, 5mM or 10mM MPP+ iodide (Sigma) or vehicle (media) prior to harvesting.

2.3.6.c MG-132 stress

Cells were incubated for 24 hours in full growth media containing 5mM, 10mM or 15mM MG-132 (Z-Leu-Leu-Leu-H; Sigma) or vehicle (DMSO) prior to harvesting.

2.4 *In vivo* mice model

2.4.1 Genomic DNA isolation from mouse tissue

Approximately 5-10mm of tail tips from breeding or postnatal day1 mice were removed for genotyping analysis. The mouse tissue was placed in 1.5ml centrifuge tube containing 700µl of tail tip buffer (50mM Tris pH 8.0, 100mM EDTA pH 8.0, 100mM NaCl and 1% (w/v) SDS). 35µl of 10mg/ml proteinase K (Roche) was added and the tubes were incubated at 55^{0}C overnight (~16hrs) with occasional agitation. Protein was extracted by mixing with an equal volume of phenol, centrifuging to separate phases and removal of the aqueous phase to a fresh tube. A second phenol extraction was performed followed by a chloroform:iso-amyl alcohol extraction. The DNA was precipitated by adding an equal volume of isopropanol to the final aqueous phase. The DNA was then pelleted by centrifugation, washed in 70% (v/v) ethanol, dried and resuspended in water and stored at 4°C until the samples were analysed by PCR.

2.4.2 Genotyping

The Brn3a-KO F (forward)/R (reverse), Brn3b-KO F/R and Neo F/R primers (Table 2.1) were used to check the genotypes of Brn3a and Brn3b mice to ensure mating of heterozygous males and females. The Brn3a KO, Brn3a heterozygous, wildtype, Brn3b KO, Brn3b heterozygous and wildtype postnatal day1 mice obtained from these matings were dissected to retrieve brain samples and were also genotyped. As the size of the products differed, but the conditions for the reaction were the same, both amplifications, Brn3 and Neo, were performed in the same tube. Together with the template (50ng of genomic DNA isolated from mouse tails) each PCR contained 1 unit of Taq DNA

polymerase (Promega) in a final volume of 25μl (1X Mg-free buffer, 2.5mM $MgCl_2$, 1mM dNTPmix, 100ng of each primer). The cycling parameter is shown below:

Step	Temperature	Time	Process
1	95 °C	5 mins	Denaturation
2	95 °C	45 secs	Denaturation
3	64 °C	45 secs	Annealing
4	72 °C	45 secs	Extension
	Repeat steps 1-4 29 times		
5	72 °C	5 mins	Final extension
6	4 °C	∞	

The products were visualized on a 1.5% (w/v) agarose gel.

Table 2.1

Primer	Primer sequence
Brn3a KO F	5'-GGCGCGCAGCGTGAGAAAATGAA-3'
Brn3a KO R	5'-GTCTCACACCCTCCTCAGTAACT-3'
Brn3b KO F	5'- CACATGGGCTGCATGAGCGACGT-3'
Brn3b KO R	5'- CGGCTTAGTGAGCTTCTCGCGGT-3'
Neo F	5'- TGATGCCGCCGTGTTCCGGCTGT-3'
Neo R	5'- TCGCCGCCAAGCTCTTCAGCAAT-3'

2.5 DNA analysis

2.5.1 Determination of DNA concentration

DNA concentration was determined by measuring the UV absorbance of samples at 260nm using NanoDrop ND1000 spectrophotometer (Thermo Scientific). Concentrations were calculated on the basis that 1 absorbance unit at 260nm corresponds to approximately 50μg/ml DNA. Absorbance at 280nm was also measured and the ratio between the absorbances at 260nm and 280nm were used to provide an estimate of the purity of the DNA sample.

2.5.2 Restriction enzyme digestion

Restriction enzymes and DNA modifying enzymes with their respective buffers were obtained from Promega (Southampton, UK) or New England Biolabs (Hitchin, UK). DNA sequencing of constructs was performed by MWG-Biotech AG (Ebserberg, Germany).

DNA analysis was carried out by restriction digests. These digests were carried out in 20µl reactions with 1µg of DNA. Restriction enzymes(s) (5units) were added and the buffer recommended by the manufacturer was used at a 1X concentration. Digests were incubated at the appropriate temperature for 2 hours. The digested DNA was electrophoresed on an agarose gel of 1% (w/v). An undigested sample was run in parallel, as a reference for the enzyme cut samples.

2.5.3 Agarose gel electrophoresis

Depending on the expected sizes of the DNA fragments, appropriate percentage gels in 1X TAE were cast, e.g. 1% (w/v) gel was cast for fragments larger than 1kb, 1.5% (w/v) for general purposes and 2% (w/v) to resolve DNA fragments less than 1kb. Ethidium bromide was added to a final concentration of 0.5µl/ml. Approximately 0.1 volume of 10X loading buffer (1X TAE, 50% (v/v) glycerol, 0.025% (v/v) bromophenolblue) was added to the DNA samples prior to loading. For size reference, the 1kb ladder, 100bp ladder or λDNA/HindIII marker, all from Promega, was used. Electrophoresis of the DNA was carried out at 70-120V for 0.5-1.5hours allowing for good resolution of bands. The bands were visualized on a long wave UV transilluminator and photographed using the Syngene doc system.

2.6 RNA analysis

2.6.1 RNA extraction

Cells were harvested post transfection and/or treatment using TRIZOL® Reagent (Invitrogen) and retrieval of RNA was carried out according to the manufacturer's protocol. Each experiment was repeated 3 times. Each mouse brain tissue was immersed in 500µl cold TRIZOL® Reagent and homogenised. Retrieval of RNA was then also carried out according to the manufacturer's protocol.

All RNA extracted was subjected to a DNAse step to remove all traces of DNA present. This was carried out using DNase I (Amersham-Pharmacia).

Sample reaction mixture: 20µg RNA

10µl 10x buffer

1.5µl DNAse I

Made up to 100µl with H_2O

The RNA was then purified using phenol/chloroform extraction, precipitated using ethanol and dissolved with H_2O to a concentration of approximately 0.5µg/µl. RNA concentration was determined by measuring the UV absorbance of samples at 260nm using NanoDrop ND1000 spectrophotometer (Thermo Scientific). Concentrations were calculated on the basis that 1 absorbance unit at 260nm corresponds to approximately 40µg/ml RNA. Absorbance at 280nm was also measured and the ratio between the absorbances at 260nm and 280nm were used to provide an estimate of the purity of the RNA sample.

2.6.2 cDNA production

cDNA was produced, with purified RNA as template, using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's protocol, primed by random hexamer primer mix.

Samples of 5μg RNA mixed with 250ng random hexamer primers and the sample volumes made up to 11μl are incubated at 70°C for 10mins and cooled rapidly in ice. 4μl of superscript II 5x buffer, 2μl of 5% (w/v) DTT, 1μl of 120ng dNTP and 1μl of RNase OUT (Invitrogen) is subsequently added to each sample mixture and incubated at 25°C for 2mins prior to the final addition of 1μl of Superscript II reverse transcriptase enzyme. The samples were incubated for 10mins at 25°C, then 1hr at 42°C. Enzyme activity is attenuated by incubated for 10mins at 70 °C.

2.6.3 Real Time PCR

Real-time PCR was performed on the DNA Engine Opticon system (MJ Research) using SYBR I Green technology (Platinum® SYBR® Green qPCR Supermix-UDG (Invitrogen)), according to the manufacturer's instructions, with an initial denaturation step at 95°C for 15 minutes (activating the Platinium® Taq DNA polymerase) and subsequently three-step cycling of 95°C for 30 seconds, 58-67°C (depending on the primer pair) for 30 seconds and 72°C for 30 seconds. Fluorescence plate measurements were then taken after each cycle at 72°C. A final melt curve was then carried out after the program, from 65-95°C (in 0.3°C steps). Each cDNA sample was amplified in triplicate reactions simultaneously and a standard calibration curve for each target

primer set was also produced using six appropriate serial dilutions of a cDNA template sample. To control for cross- contamination from other sources, in each PCR run, blank reactions in which no cDNA were added, were included. The Opticon software on the DNA Engine Opticon system was used to determine the threshold amplification cycle (CNt), which represents the first discernable PCR cycle at which product fluorescence increases to just above background noise, for each reaction.

Control primers - βactin, β-microglobulin or CB5B reductase were subsequently used for the normalization of the target's expression relative to its expression, to control for variations such as RNA quality, reverse transcription efficiency, and sample-to-sample RNA input differences. The sequences of the normalizing gene primer pairs, as well as other primers used, are shown in Table 2.2. All the primers span introns, such to prevent amplification from genomic DNA. (apart from α-synuclein primers, whereby for each sample run, a corresponding negative sample will also be tested to show if contaminating genomic DNA levels were at least 5 CNt lower than the other sample. This would show that the contamination was low enough not to affect the results. The negative samples refer to a portion of each corresponding sample to be tested which would not have Superscript II reverse transcriptase (Invitrogen) added when going through cDNA production step, and thus will not have any cDNA made from RNA. Any DNA present would be contaminating genomic DNA.

Table 2.2

Primer	DNA primer sequence
α-synuclein F	5'- CAGTGGCTGAGAAGACCAAA -3'
α-synuclein R	5'- TGTCTTCTGAGCGACTGCTG -3'
EGR1 F	5'- CAGCACCTTCAACCCTCAG-3'
EGR1 R	5'- AGCGGCCAGTATAGGTGATG -3'
EGR2 F	5'- TTGACCAGATGAACGGAGTG -3'
EGR2 R	5'- ACCAGGGTACTGAGGGTCAA -3'
EGR3 F	5'- CAATCTGTACCCCGAGGAGA -3'
EGR3 R	5'- GGAAGGAGCCGGAGTAAGAG -3'
EGR4 F	5'-AACCCAGCGCTGAATTGC-3'
EGR4 R	5'-GAGTCGGCTAAGTCCCCACT-3'
WT1 F	5'-CAAATGACATCCCAGCTTGA-3'
WT1 R	5'-GATGCCGACCGTACAAGACT-3'
Nurr1 F	5'- GTTTAAAAGGCCGGAGAGGT -3'
Nurr1 R	5'- TGCTGGGTGTCATCTCCACT -3'
Brn3a F	5'-GGCGCGCAGCGTGAGAAAATGAA-3'
Brn3a R	5'-GTCTCACACCCTCCTCAGTAAGT-3'
Parkin F	5'- CCAGTGACCATGATAGTGTT –3'
Parkin R	5'- TGATGTTCCGACTATTTGTTG –3'
PINK1 F	5'-TTGAAAGCCGCAGCTACCAA-3'
PINK1 R	5'-ACCCCAGAGGCTTAGATGAA-3'
Housekeeping control genes:	
βActin F	5'- AGCCTCGCCTTTGCCGA –3'
βActin R	5'- CTGGTGCCTGGGGCG –3'
β2microglobulin F	5'- TGCTGTCTCCATGTTTGATGTATCT –3'
β2microglobulin R	5'- TCTCTGCTCCCCACCTCTAAGT –3'
GAPDH F	5'-AGCCACATCGCTCAGACAC-3'
GAPDH R	5'-GAGGCATTGCTGATGATCTTG-3'
Cytochrome b5 reductase F	5' – TATACACCCATCTCCAGCGA - 3'
Cytochrome b5 reductase R	5' – CATCTCCTCATTCACGAAGC - 3'

2.6.4 Data Analysis

The data obtained from real-time PCR were manipulated by the 2-ΔΔCt method (Livak et al. 2001). To ensure that the PCR efficiency of the primers were comparable such that the calculations for ΔΔCt were valid, a dilution curve comprising of data from a five serial dilutions of a sample had to be plotted, which should have a slope close to 0 and thus proving the assumption.

The variation in activation and expression levels in the experiments for each construct, determined by luciferase assays and real-time PCR, were assessed by one-way analysis of variance (ANOVA) with a Bonferroni post-hoc test.

2.7 Promoter analysis

2.7.1 Dual luciferase assay

Cells were washed with PBS and harvested following transfection and treatment using 1x passive lysis buffer (Promega) and subsequently assayed for firefly and renilla luciferase levels using the Dual-Luciferase® Reporter Assay System according to the manufacturer's protocol. Firefly luciferase is first measured from each sample in the luminometer by adding an equal volume of substrate dissolved in buffer I. The firefly luciferase is then quenched and renilla luciferase activated and measured by adding another equal volume of buffer II with 10% stop and glo substrate. Each experiment was repeated 3 times.

2.8 DNA/protein interaction analysis

2.8.1 Nuclear protein extraction

Reagents required:	Hypotonic Buffer -	20mM Hepes, pH7.9
		0.5mM DTT
		Complete mini protease inhibitors (add according to manufacturer's protocol)
	EB-1 Buffer -	20mM Tris-HCl, pH7.5
		1% (v/v) Triton X-100
		150mM NaCl
		5mM EDTA
		50mM NaF
		Complete mini protease inhibitors (add according to manufacturer's protocol)

SH-SY5Y cells were plated, at 1 90% confluent 85cm^2 flask per 10cm plate, for extraction 1 day before stress treatment. Dopamine hydrochloride and MPP+ iodide were added to each plate at a concentration of 0μM, 200□μM, and 0mM, 8mM respectively. The plates were then incubated for 24hrs and 12hrs respectively.

The media was removed from each plate and the plates were washed with cold PBS. 3ml of cold PBS was added to each plate and the cells were scrapped with a scrapper and collected in 1.5ml aliquots and placed in ice. The aliquots were then centrifuged at 1500g for 5min at 4°C. Supernatant was removed and 500μl Hypotonic Buffer was used

to resuspend the cells. This suspension was incubated for 15min on ice before adding 20µl of 10% (v/v) Igepal and vortexed for 10 secs. The mixture was centrifuged at 900g for 1min to pellet the nucleus. Supernatant may be collected and stored at -20°C (cytosolic extract). The remaining pellet was washed using 500µl of Hypotonic Buffer and 140µl of 10% (v/v) Igepal and spun down at 900g for 1min. The pelleted nucleus was then washed by only 500µl of Hypotonic Buffer. A sample of this mixture may be used for viewing under the microscope to check for intact nucleus. The nucleus were pelleted at 900g for 1min. 50µl of ice-cold EB-1 Buffer is used to resuspend the pelleted nucleus and placed on ice for 30mins. The nuclear debris was then pelleted and discarded by spinning at 900g for 1min. The supernatant was then used for EMSA experiments or stored at -80°C.

2.8.2 Electrophoretic Mobility Gel Shift Assay

1 EMSA gel: 5ml 10x TBE (108g Tris base, 55g Boric acid, 40ml 0.5M EDTA, pH 8.0)

23ml Acrylamide

100µl Temed

240µl 10% (w/v) APS

72ml H_20

DIG Gel Shift Kit, 2nd Generation ROCHE

Cat no. 03 353 591 910

According to Roche manufacturer's protocol

Sample reaction mixes

	Control (μl)	Stressed (μl)	Specific competitor (μl)	Non specific competitor (μl)
5x buffer	4	4	4	4
Poly [d(I-C)] (1μg/μl)	1	1	1	1
Poly L-lysine (0.1μg/μl)	1	1	1	1
Labeled oligonucleotide (0.4ng/μl)	2	2	2	2
H_20	10	10	8	8
Nuclear extract (from section 2.8.1)	2	2	2	2
Unlabelled oligonucleotide (0.1μg/μl)	-	-	2	-
Non-specific competitor oligonucleotide (0.1μg/μl)	-	-	-	2

The mixture was incubated for 30mins in room temperature (~21°C) before adding loading buffer and loading onto a 10% (v/v) non-denaturing acrylamide gel. The gel was run at 40mA in 4°C and transferred onto nylon membranes via semi-dry passive transfer. The DNA was then cross-linked onto the membrane using UV 1.2J and then blocked using blocking buffer provided for 2hrs and probed using anti-DIG AP antibodies also provided in the kit and developed using CSPD.

2.8.3 Affinity isolation & silver staining

Biotinylated oligonucleotides of specified sequences were obtained from MWG Biotech. These oligonucleotides were then affinity bound onto magnetic Dynabeads® M-280 streptavidin beads (Dynal Invitrtogen) according to manufacturer's protocol. The bead-oligonucleotide complex were then mixed into a reaction mixture as shown below and incubated for 30mins at room temperature (~21°C) to allow protein/oligonucleotide interaction. A sample reaction mixture is shown below:

	Control (μl)	Stressed (μl)	Specific competitor (μl)	Non specific competitor (μl)
5x buffer	4	4	4	4
Poly [d(I-C)] (1μg/μl)	1	1	1	1
Poly L-lysine (0.1μg/μl)	1	1	1	1
Bead/oligo	4	4	4	4
H_20	6	6	-	-
Nuclear extract (from section 2.8.1)	4	4	4	4
Unlabelled oligonucleotide (0.1μg/μl)	-	-	6	-
Non-specific competitor oligonucleotide (0.1μg/μl)	-	-	-	6

The beads were then washed 3 times with 1x buffer used in the reaction mix. The mixtures were then subjected to 5mins at 100°C to denature the proteins and release interaction bonds. The denatured mixtures were loaded and run in a 10% (v/v) SDS-PAGE. The electrophoresed gel was then silver stained in the following procedure to visualize the proteins in the gel.

The proteins in the gel are first fixed onto the gel by incubating for 30mins in 400ml methanol, 100ml acetic acid glacial and 500ml water. The gel is then sensitized to silver-staining by incubation for 30mins in 300ml ethanol, 40ml of 5% (w/v) sodium thiosulphate, 68g of sodium acetate and made up to 1L with water. The gel is subsequently washed three times for 5mins each with water. A solution made up of 0.625g of silver nitrate, 0.1ml formaldehyde and made up to 250ml with water is added to the gel and incubated for 20mins. The gel is washed twice, 1min each, with water and developing solution of 25g sodium carbonate, 0.2ml formaldehyde and made up to 1L with water is added and incubated for 10-15mins (until bands show up). The reaction is

then stopped by adding acetic acid for 10mins and the resulting gel is washed for three times with water for 5mins each time.

The target bands were cut out and sent for sequencing.

2.9 Protein analysis

2.9.1 Reagents

Polyacrylamide gels were made using Acrylamide/bisacrylamide (30% w/v) solution obtained from Amresco Ltd. (Ohio, USA). Ammonium persulphate, N,N'-methylenebis- acrylamide, N,N,N'N'-tetramethylethylene-diamine (TEMED) were obtained from Sigma (St Louis, MI, USA). Protein molecular weight RainbowTM marker, HybondTM-C nitrocellulose membranes, Enhanced Chemiluminescence system (ECL) and Kodak XOMAT imaging photographic film were purchased from Amersham Pharmacia Biotech. (Little Chalfont, Bucks., UK). Pre-stained protein markers were obtained from NEBS Biolabs. 3MM chromatography paper was obtained from Whatman International Ltd. (Maidstone, Kent, UK). Photographic developing and fixing chemicals were obtained from X-OGRAPH Ltd. (Tetbury, UK).

2.9.2 Antibodies

Rabbit polyclonal anti-parkin (C-terminal) antibody was purchased from Cell Signalling Technologies; mouse anti-β-actin antibody was purchased from Santa Cruz Biotechnology. PINK1-49 antibodies were gifts from Dr. Sonia Gandhi (Institute of Neurology, UCL, London, UK) and were produced by immunizing rabbits with a mix

of the following synthetic peptide, CKSKPGPDPLDTRRLQ corresponding to amino acid residues 135 - 149 of human PINK1. The peptides were coupled to keyhole limpet haemocyanin prior to immunization. Anti-serum was affinity purified on columns against each of the synthetic peptides (Eurogentec, Liege, Belgium).

Secondary anti-rabbit and -mouse horseradish peroxidase (hrp) -linked antibodies used for western blotting were all from DAKO Ltd., Glostrup, Denmark.

2.9.3 SDS-PAGE

Standard SDS-polyacrylamide gels were prepared with the composition for the stacking and resolving gels as described by Sambrook and colleagues (Sambrook et al, 1989). Acrylamide gels are defined by their overall acrylamide concentration and percentage cross-linker. Protein samples were heated to 95^0C for 5 minutes. 50-100 μg of protein from each sample and 10 μl of protein marker were prepared and run in a vertical gel electrophoresis system (Sambrook et al, 1989). The gels were run at a constant voltage of 100 V in 1 X running buffer (25 mM Tris, 250 mM glycine and 0.15 (w/v) SDS, pH 8.3). Gels were run until the protein of interest was sufficiently resolved as determined by the migration of the molecular weight marker.

2.9.4 Western Blot

RIPA buffer (for soluble protein extract):	50mM Tris-HCl, pH 8.0
	150mM NaCl
	1.0% (v/v) Igepal CA-630
	0.5% (w/v) sodium deoxycholate
	0.1% (w/v) SDS

5x Laemmli buffer (for total protein extract):250mM Tris-HCl, pH 6.8

50% (v/v) Glycerol

500mM DTT

10% (w/v) SDS

10x TBS: 876.6 g NaCl (FW 58.44),

121.1 g Tris,

40 ml HCl

pH 8.0

The cells were lysed and harvested post transfection and/or treatment using RIPA buffer or Laemmli buffer to extract soluble or total protein respectively. Each experiment was performed 3 times. Lysates were run on 10% (w/v) SDS-PAGE gels, and transferred onto nitrocellulose membranes. The membranes were then blocked with 5% (w/v) milk powder in 0.1% (v/v) TBS-Tween and probed overnight (~16hrs) at 4°C using primary antibody in 5% (v/v) BSA in 0.1% (v/v) TBS-Tween. Membranes were washed and probed using appropriate HRP-conjugated secondary antibodies and developed using enhanced chemiluminescence (ECL, Amersham Biosciences).

2.10 Molecular Biology analysis

2.10.1 Annexin V apoptosis assay

To determine the level of apoptosis, a FACS based assay was used. Cells were transfected and stressed with vehicle or MG-132 as described above. Cells were then harvested and washed in PBS and pelleted. Pellets were resuspended in 1X Annexin V binding buffer (BD Biosciences, Oxford, UK) and then incubated with Annexin V conjugated with phycoerythrin (Annexin V-PE) for 15mins at room temperature

(~21°C) before being analyzed immediately on an Epics XL flow cytometer. For each sample, 20,000 cells were analyzed and Annexin V-PE fluorescence was determined in the FL2 channel gated for GFP positive events. Experiments were performed in triplicate

2.10.2 Mitochondrial membrane potential assay

Tetramethylrhodamine, methyl ester, perchlorate (T-668) (TMRM) was obtained from Molecular Probes (Eugene, USA). TMRM was solubilised in methanol (200 mM stock concentration, kept in -20°C) and used at final concentration of 100 nM.

To measure mitochondrial membrane potential ($\Delta\psi m$), a fluorescence-activated cell sorting (FACS)-based assay was used. Cells were treated with vehicle or 15μM MG-132 for 24 hours, then incubated with 10μM verapamil (Sigma, Paisley, UK) and 100nM of the $\Delta\psi m$-sensitive dye tetramethylrhodamine methyl ester (Molecular Probes, Leiden, Netherlands) in a humidified 5% (v/v) CO2 atmosphere at 37°C for 45 min. Cells were then harvested with trypsin, pelleted and resuspended in phosphate buffered saline (PBS) on ice. For each sample, 20,000 cells (events) were analyzed on an Epics XL flow cytometer with a 488-nm argon laser. The TMRM signal was analyzed in the FL2 channel gated for GFP positive events. The channel was equipped with a band-pass filter at 580±30 nm; the photomultiplier value of the detector was 631V. Data were acquired on a logarithmic scale. Arithmetic mean values of the median fluorescence intensities (MFI) were generated for graphic representation. Experiments were performed in triplicate.

2.10.3 Immunofluorescence & microscopy

Goat serum was obtained from Invitrogen Ltd. (Paisley, UK). Coverslips and slides were obtained from BDH. Fluorescent mounting medium was obtained from Dako Ltd. (High Wycombe, Bucks., UK). Mouse monoclonal anti-c-myc (clone 9E10) (Sigma); Alexa Fluor 488 goat anti-mouse IgG conjugates (Molecular Probes).

Cells were fixed and prepared for immunofluorescence microscopy. Cells were washed three times in PBS and fixed in 4% (w/v) paraformaldehyde for 10 min. Cells were washed three times with PBS and permeabilised for 5 min in 0.1% (v/v) Triton X-100 in TBS (TBST). Coverslips were then incubated in block buffer (20% (v/v) goat serum, 0.1% (v/v) TBST) for 20 min. Cells were incubated overnight (~16hrs) at 4°C with the appropriate primary antibody in antibody diluting buffer (1% (v/v) goat serum, 0.1% (v/v) TBST). Coverslips were washed three times in 0.1% (v/v) TBST and incubated for 30 min at room temperature (~21°C) with the appropriate secondary antibody in antibody diluting buffer. For nuclear staining Hoechst 33258 (2μg/ml) was added to the secondary antibodies in buffer. For double labelling studies coverslips were incubated with primary antibodies sequentially at 4°C for 12 hours each and after washes were incubated with a mixture of both secondaries. Coverslips were washed three times with TBS and mounted on glass slides (Fisher) with antifade mounting medium (Dako). Standard immunofluorescence was performed using a Zeiss Axioskop 2 plus microscope and digital images were captured using a Zeiss Axiocam camera. Negative controls omitting each primary antibody separately or in combination were performed in each case and no significant staining was seen.

Chapter 3: Regulation of α-synuclein in oxidative stress

3.1 Introduction

3.1.1 Regulation of α-synuclein in Parkinson's disease

As established in chapter 1, the dysregulation of α-synuclein has a strong causative role in Parkinson's disease. Not only do mutations in the protein cause disease, but also the over-expression of α-synuclein was found to be sufficient to cause toxicity to neuronal cells. For instance, the amplification of the α-synuclein locus caused an increase in the expression of α-synuclein, leading to Parkinson's disease (Chartier-Harlin et al., 2004; Farrer et al., 2004; Ibanez et al., 2004a; Singleton et al., 2003). However, the presence of such amplifications are rare and do not account for the majority of Parkinson's disease cases, which are mostly sporadic rather than familial. As such, it is important to examine putative mechanisms that may lead to α-synuclein dysregulation associated with sporadic Parkinson's disease.

3.1.1 Oxidative stress, α-synuclein & Parkinson's disease

As mentioned earlier, genetic predisposition, is only one of several factors that lead to Parkinson's disease. Others include aging, head trauma and environmental factors (Elbaz et al., 2007) and they usually lead to sporadic Parkinson's disease. As aging and head trauma mainly lead to non-specific neuronal death, the attention of this study focuses on environmental factors, in particular, oxidative stress. Oxidative stresses have been

previously linked to α-synuclein up-regulation and increased aggregation (Betarbet et al., 2006; Manning-Bog et al., 2002; Przedborski et al., 2001; Sherer et al., 2002; Sherer et al., 2003; Vila et al., 2000). However, there have not been any studies as yet, on whether oxidative stress may directly regulate α-synuclein at the transcriptional level. To further examine the importance and possible role of oxidative stress in the development of Parkinson's disease, we sought to investigate the influence of oxidative stress on the regulation of the expression of a key gene, α-synuclein.

3.1.2 Hypothesis & Aims

As mentioned earlier, several factors contribute to the development of Parkinson's disease. Of these factors, strong evidence associate genetic predisposition and environmental factors to the disease. In view of the importance of investigating the causes of idiopathic Parkinson's disease, which account for the majority of cases, this work aimed to extend current findings which show the linkage of oxidative stresses with Parkinson's disease and α-synuclein. We propose that one of the ways oxidative stresses may act to increase susceptibility to Parkinson's disease is via altering the regulation of α-synuclein expression.

Aims of project:

1. To determine if selected oxidative stresses alter the expression of α-synuclein.

2. If the expression of α-synuclein mRNA is altered, to confirm if this event is transcriptional.

3. To investigate the mechanism of α-synuclein regulation by oxidative stress

3.2 Results

3.2.1 Oxidative stresses can regulate α-synuclein expression

Previous studies have shown that certain stresses and toxins can alter the levels of intracellular α-synuclein as well as increase its aggregation (Betarbet et al., 2006; Manning-Bog et al., 2002; Przedborski et al., 2001; Sherer et al., 2002; Sherer et al., 2003; Vila et al., 2000). To confirm such regulation, oxidative stresses such as dopamine were used to investigate its effect on the expression of α-synuclein.

Cellular activities are regulated at several levels, namely: transcriptional, post-transcriptional, translational, post-translational modifications and protein interaction. In this section, α-synuclein gene expression was monitored at the pre-translational level since the copy number of a specific mRNA can be used to quantify, not only, changes in the regulation of the gene, but also RNA stability.

Traditionally, the amount of a particular mRNA produced, and thus the expression level of a gene has been estimated by a technique known as northern blotting, whereby RNA is separated by agarose gel electrophoresis, transferred to a filter and then probed with a specific DNA probe that is complementary to the gene of interest (Alwine et al., 1977). Although this technique is still used to assess gene expression, it requires relatively large amounts of RNA and thus cannot be performed when RNA amounts are limited.

In order to detect gene expression at minute levels from single or small numbers of cells, amplification of the gene transcript is necessary. Reverse transcription followed by polymerase chain reaction (PCR) is a common method for amplifying the RNA signal (Bartlett and Stirling, 2003).

Development of PCR technology using fluorophores permits measurement and quantification of DNA amplification in real time (Nolan et al., 2006). In quantitative real-time PCR, the amplified product is measured at the end of each PCR cycle. The data obtained can be analysed by computer software to calculate relative gene expression between several samples, or mRNA copy number based on a standard curve. This method was used to quantify α-synuclein mRNA during exposure to oxidative stress.

α-synuclein mRNA levels in SHSY5Y cells incubated with 0uM, 62.5μM, 125μM and 250μM dopamine for 24hrs were quantified using real-time Polymerase Chain Reaction (RT PCR). The quality of RNA isolated from the cells was verified prior to production of cDNA for RT PCR (Fig. 3.1). Results were normalised to control house-keeping genes, β2microglobulin, β-actin and NADH cytochrome b6-reductase mRNA in the cells. The normalisation to control for housekeeping genes eliminates variation caused by differences in total RNA used in each sample. Two or more control genes are used for the normalisations, and results compared, to ensure that expressions of these control genes are not also altered by variables used. The identity and specificity of the product of each RT PCR was confirmed after the experiment (Fig 3.2).

RT PCR results demonstrated that dopamine caused a dose-dependent increase in endogenous α-synuclein mRNA (Fig. 3.3). The expression increase reached an average of 1.5 fold from normal levels when 250μM dopamine was used. These results indicated that dopamine affects α-synuclein expression.

To find out if general oxidative stress, rather than an isolated dopamine-mediated effect, may cause this regulation of α-synuclein, another oxidative stress, MPP+, was used to carry out a similar quantitative analysis. This time, the cells were incubated with 0mM, 1mM, 5mM and 10mM MPP+ for 6 and 12hrs. An increase in α-synuclein mRNA was also observed (Fig, 3.4; results for one normalisation are shown; similar results for the different normalisations were obtained). There was a two fold higher increase in α-synuclein mRNA when cells were incubated with MPP+ for 12hrs compared to 6hrs. The increase in expression for both incubation periods of 6hrs and 12hrs was maximal at 5mM MPP+ with 2 and 4 fold increases from normal respectively. α-synuclein mRNA decreased at 10mM MPP+ due to cell death caused by toxicity. The higher expression achieved when cells were stressed by MPP+ should not be compared to when dopamine was used as the extent of toxicity caused by both stresses were not examined.

The RT PCR results show that oxidative stress increases α-synuclein expression, however, this did not indicate if the increase in α-synuclein mRNA was due to transcriptional or post-transcriptional regulation.

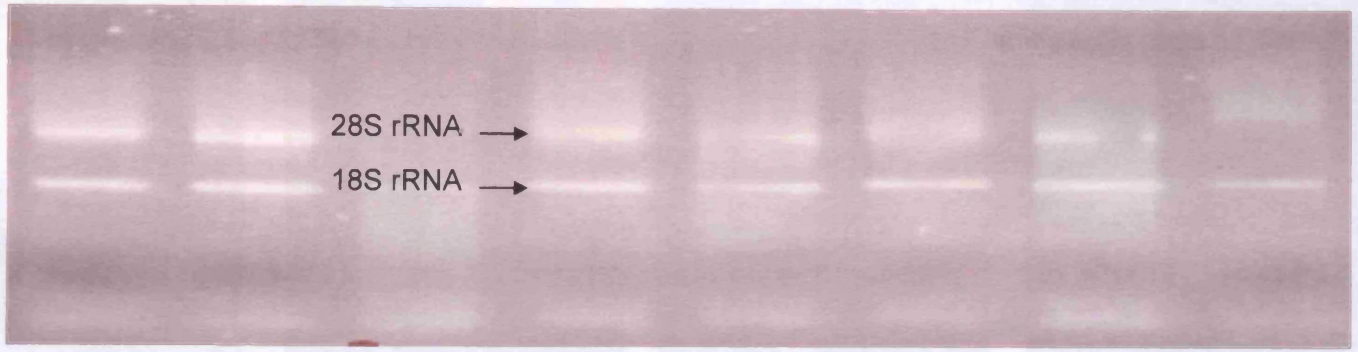


Figure 3.1 RNA quality agarose gel check
1.5% (w/v) agarose gel check on RNA quality of RNA extractions from SH-SY5Y cells which were then used for reverse-transcriptions into cDNA and subsequently for real-time PCR analysis. This gel photo shows a random set of extracted RNA samples. Lanes 3 and 8 shows degradation of RNA extracted while the other lanes show undegraded RNA.

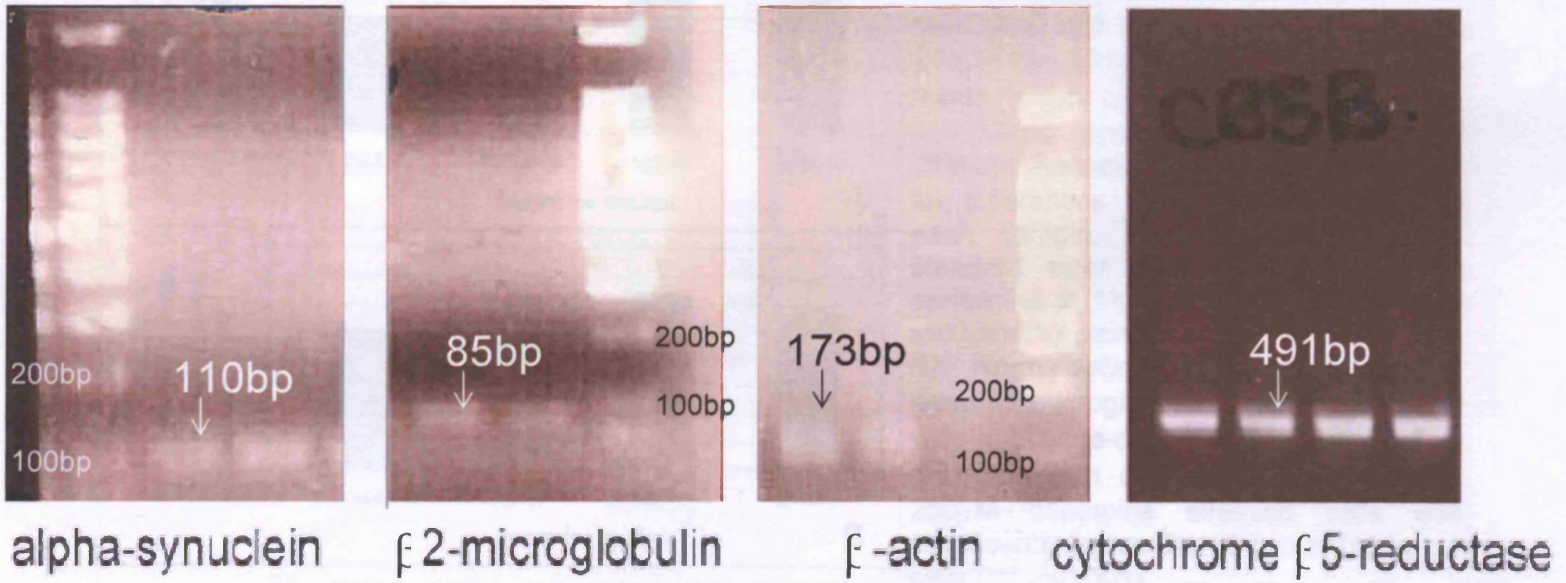


Figure 3.2 RT PCR α-synuclein, β2-microglobulin, β-actin and cytochrome β5-reductase product agarose gel check

Agarose gel check on RT PCR products to confirm product identity and PCR specificity. Specific product of the correct size was obtained for each primer set used for each gene in RT PCR. DNA products in bands were cut out, purified and sequenced to ensure correct DNA amplification by primers were obtained.

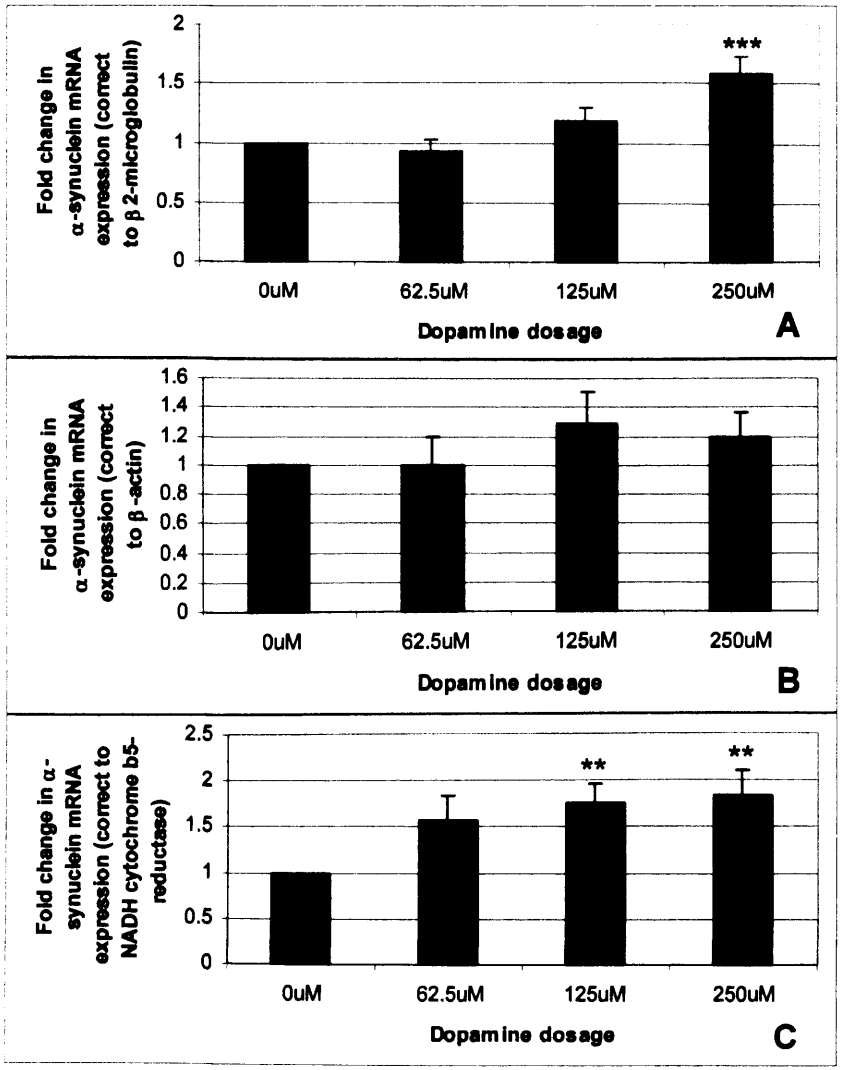


Figure 3.3 Effects of dopamine on α-synuclein mRNA expression

Real-time PCR on cDNA reverse-transcribed from mRNA extracted from cells incubated for 24hrs in 0μM, 62.5μM, 125μM and 250μM dopamine in full culture media. α-synuclein RT PCR results were normalised onto RT PCR results of different housekeeping genes to correct for differences in total mRNA levels in each sample. Error bars indicate the standard error of 3 experiments each performed in triplicates. Differences were analysed by paired T-test.

A) Normalisation using housekeeping gene β2-microglobulin showed that there was a dosage-dependent increase of α-synuclein with dopamine. α-synuclein in 250μM dopamine stressed cells was significantly higher than those in untreated cells (*** $p<0.001$).

B) Normalisation using housekeeping gene β-actin also showed increases in α-synuclein with dopamine treatment.

C) Normalisation using housekeeping gene NADH cytochrome b6-reductase similarly showed a dosage-dependent increase of a-synuclein with dopamine. α-synuclein in 125 and 250μM dopamine stressed cells were significantly higher than those in untreated cells (** $p<0.01$).

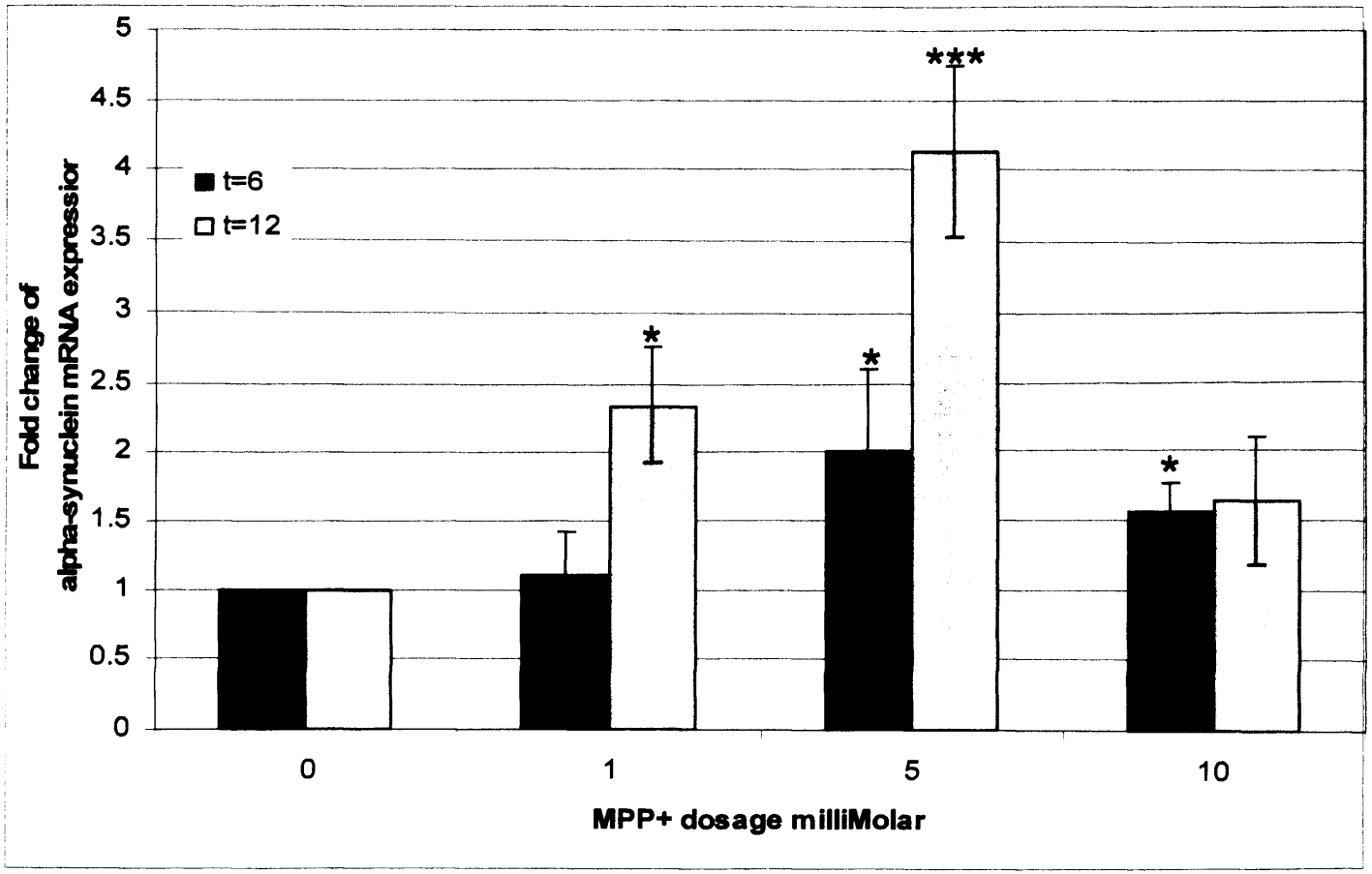


Figure 3.4 Effects of MPP+ on α-synuclein mRNA expression
Cells were incubated for 6hrs and 12hrs in 0mM, 1mM, 5mM and 10mM MPP+ in full media culture media before mRNA retrieval. The error bars indicate the standard error of the mean of 3 experiments. Differences were analysed by paired T-test. There were significant dose-dependent increases in α-synuclein mRNA for cells stressed for both timings (*$p<0.05$; ***$p<0.001$).

3.2.2 Oxidative stress regulation of α-synuclein promoter activity

To further investigate if the increase in α-synuclein mRNA obtained previously was due to transcriptional or post-transcriptional regulation, dual luciferase reporter assays were used.

Certain genes are chosen as reporters because expression of their corresponding protein is easily detected and quantified. Reporter genes can be linked to the promoters of specific genes to examine aspects of regulation.

Dual luciferase reporter assay using the luminescence of the firefly (Photinus pyralis) and sea pansy (Renilla reniformis) luciferase proteins is used in this section. The assay exploits the differing biochemical requirements for each luminescence, thus allowing for the sequential quantitative measurement of both luciferase activities in a single protein extract (Sherf et al., 1996).

A 5'deletion series of the α-synuclein promoter cloned upstream of the luciferase gene (Fig. 3.5) were used to measure α-synuclein promoter transactivation. These included 1.46, which contains approximately 1.46kb of the 5'UTR region between the translational and transcriptional start sites. 1.9 construct contains ~1.9kb of sequence which includes both the 5'UTR and ~0.44kb of promoter. 3.4, 3.8 and 4.1 constructs contain sequences from 1.9 construct and a further 0.5kb, 0.9kb and 1.2kb upstream respectively. These constructs

were transfected into SH-SY5Y cells and luciferase activity assayed for their basal levels and in response to varying concentrations of dopamine and MPP+.

Under basal conditions (Fig. 3.6), all the constructs displayed different activity levels except 3.8 and 4.1 whose activities were similar to each other. 1.9 showed the highest promoter activity compared to the other constructs which indicated the presence of essential promoter sequences (core promoter) and possible enhancer domains of the α-synuclein promoter. Although 1.46 did not contain any sequence upstream of the transcriptional start site, some activity above the empty PGL3 basic vector was still observed. 3.4 activity was the lowest, indicating possible repressor sites upstream of the core promoter whilst the increase in activity in 3.8 and 4.1 indicated presence of enhancer sites further upstream.

Administration of dopamine resulted in significant increases in luciferase activity in all the α-synuclein promoter constructs for both 125μM and 250μM dopamine concentrations used (Fig. 3.7). A higher increase in luciferase activity was generally seen in cells treated with 250μM dopamine. The biggest increase in activity was observed for construct 1.9 with 1.5 and 2 fold increase for 125μM and 250μM dopamine used respectively. This suggested that the increase in α-synuclein mRNA observed in earlier experiments was due to transcriptional activation.

Similar dual luciferase assays were also carried out for MPP+ stress to find out if the similar results shown in the real-time for both dopamine and MPP+ would also be shown here, and thus show that the regulation of α-synuclein was not just an isolated dopamine mediated effect but occured during exposure to other oxidative stresses as well (Fig. 3.8).

MPP+ treatment also resulted in an activation of α-synuclein promoter activity similar to that mediated by dopamine. 1.9 construct showed increase in activity of 5.5 fold in 5mM MPP+ compared to control conditions. The other constructs showed an increase of an average of 3 fold in MPP+. Surprisingly, the minimal promoter activity detected with the 1.46 construct, which did not contain any of the α-synuclein core promoter, could also be up-regulated when subjected to both stresses. This was shown not to be an artificial effect as the empty vector did not show any increase in activity in response to the stresses.

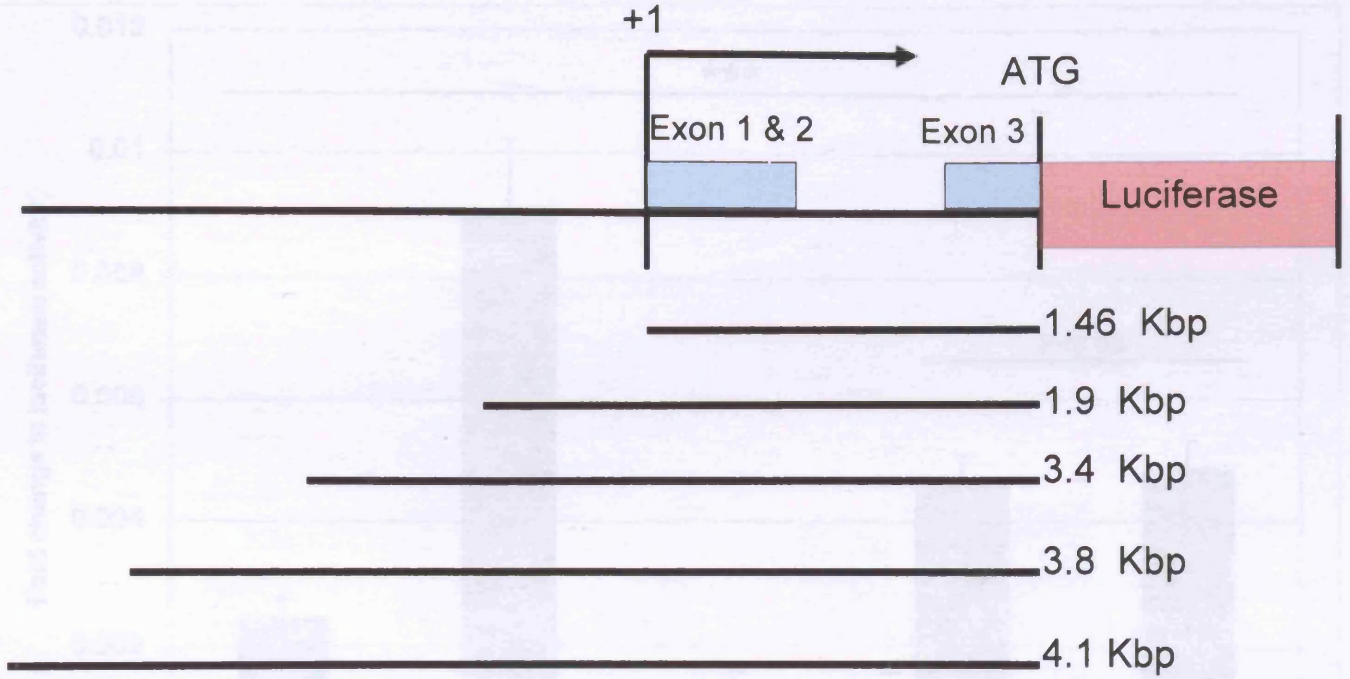


Figure 3.5 α-synuclein promoter luciferase constructs
Constructs used for dual luciferase assays. Each of the inserts was cloned in pGL-basic plasmid upstream of the firefly luciferase reporter gene. In all the transfections, they were co-transfected with pRL-SV40, which produces *Renilla* luciferase and thus corrects for transfection efficiency when measuring for the overall luciferase activity. 4.1 encodes for approximately 4kb upstream of α-synuclein translational start site and thus contains the core α-synuclein promoter and possible additional enhancer/repressor sites; 3.8 encodes for about 3.8kb upstream of α-synuclein translational start site; 3.4 contains about 3.4kb of the α-synuclein promoter; 1.9 encodes for approximately 1.9kb upstream of translational start site and contains the core promoter; 1.46 contains about 1.46kb and encodes for the 3' untranslated region.

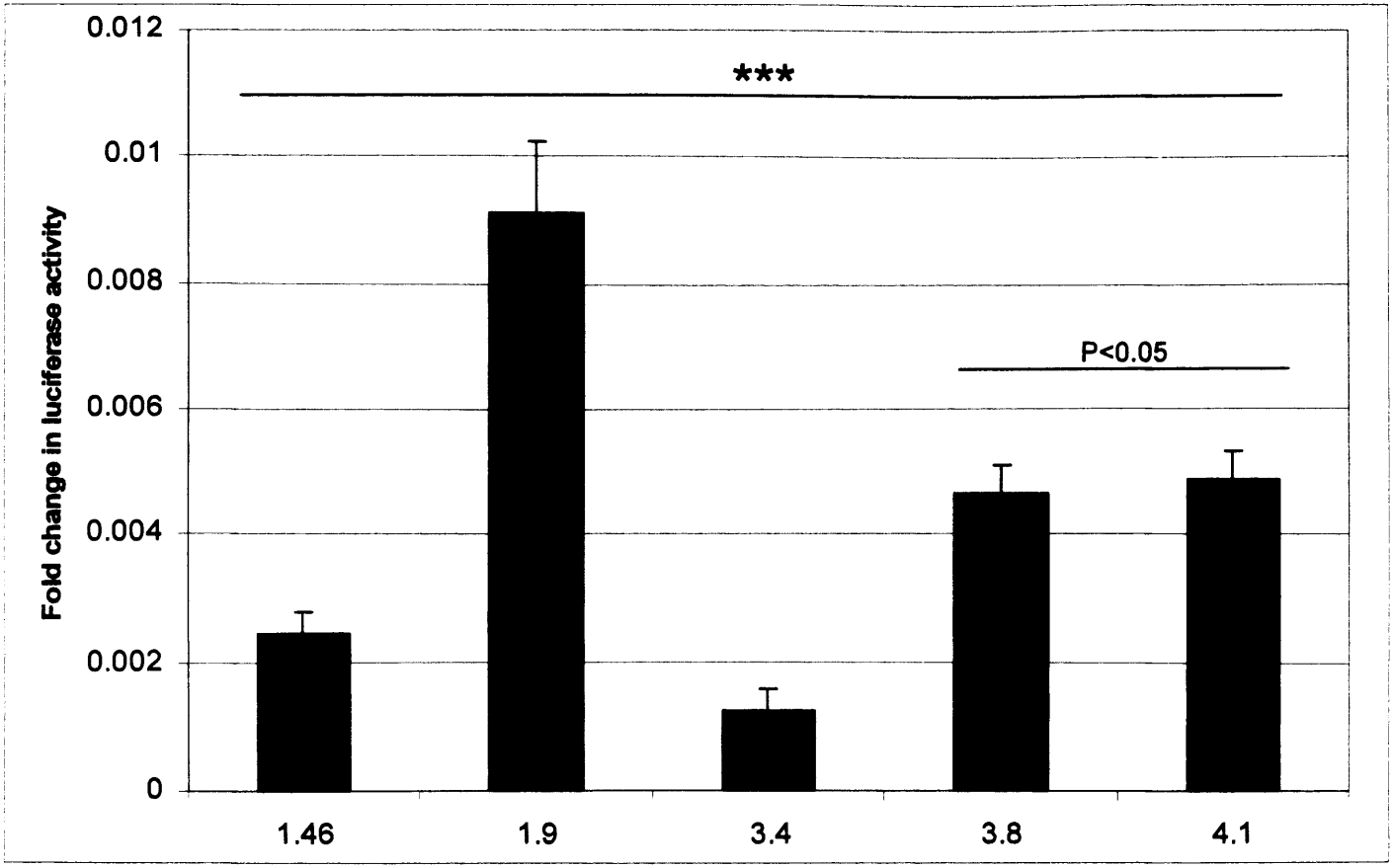


Figure 3.6 Basal α-synuclein promoter activity
SH-SY5Y cells were co-transfected with different α-synuclein promoter luciferase constructs and SV40 promoter renilla luciferase construct. Cells were subsequently incubated in full culture media for 24hrs. Dual luciferase assay analysis comparing the different luciferase activities was performed. Data was analysed by paired T-test. All the contructs expressed luciferase activity that were significantly different from each other (***$p<0.001$) except for between constructs 3.8 and 4.1.

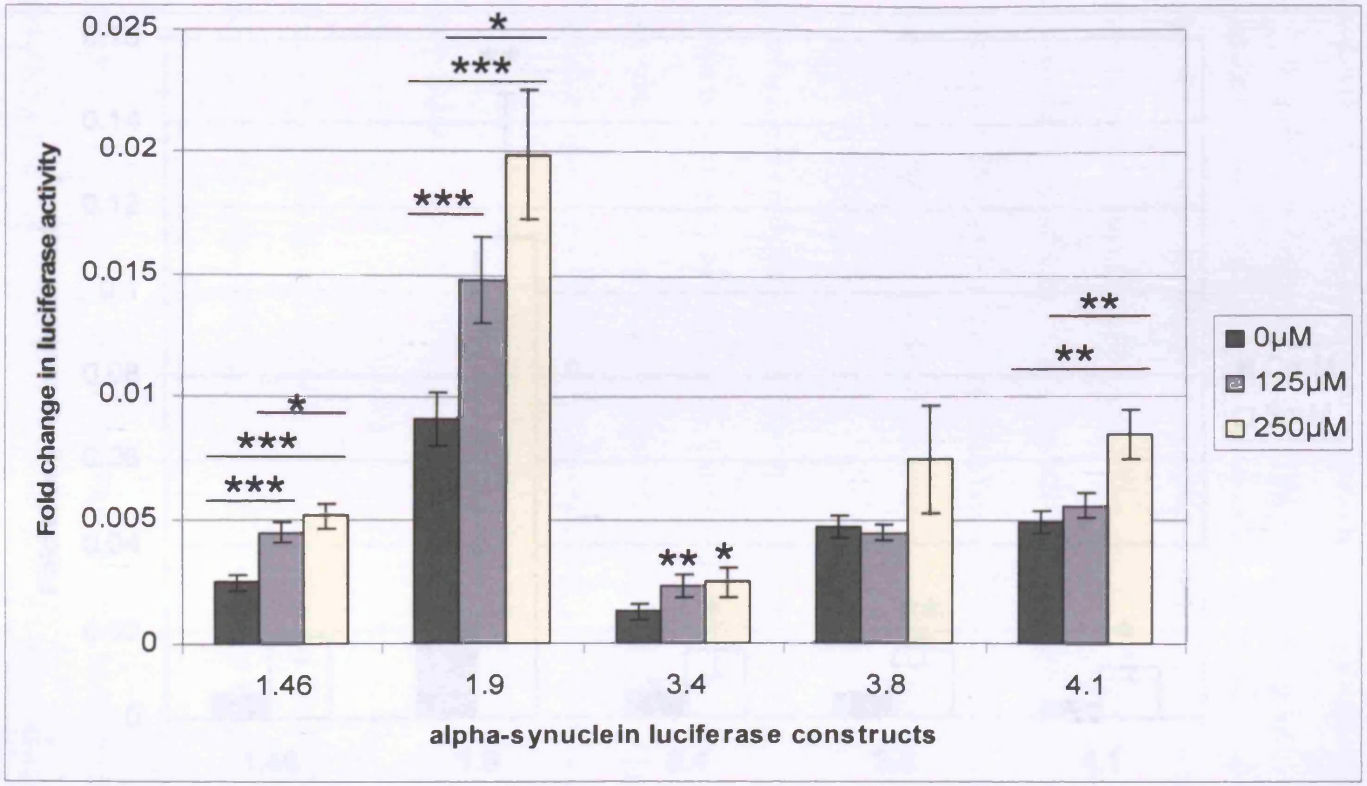


Figure 3.7 Effects of dopamine on α-synuclein promoter activity
Cells were transfected with the different a-synuclein constructs and incubated for 24hrs in 0mM, 125mM and 250mM dopamine in full culture media. Constructs 1.46 and 1.9 in treated cells were significantly different from untreated cells (***p<0.001). Those stressed with 125mM dopamine was also different (*p<0.05) from those stressed with 250mM. For construct 3.4, both stresses resulted in significant increases in luciferase activity (**p<0.01). In 4.1, only 250mM dopamine stressed cells were different. The error bars indicate the standard error of the mean of 3 experiments, each performed in triplicates.

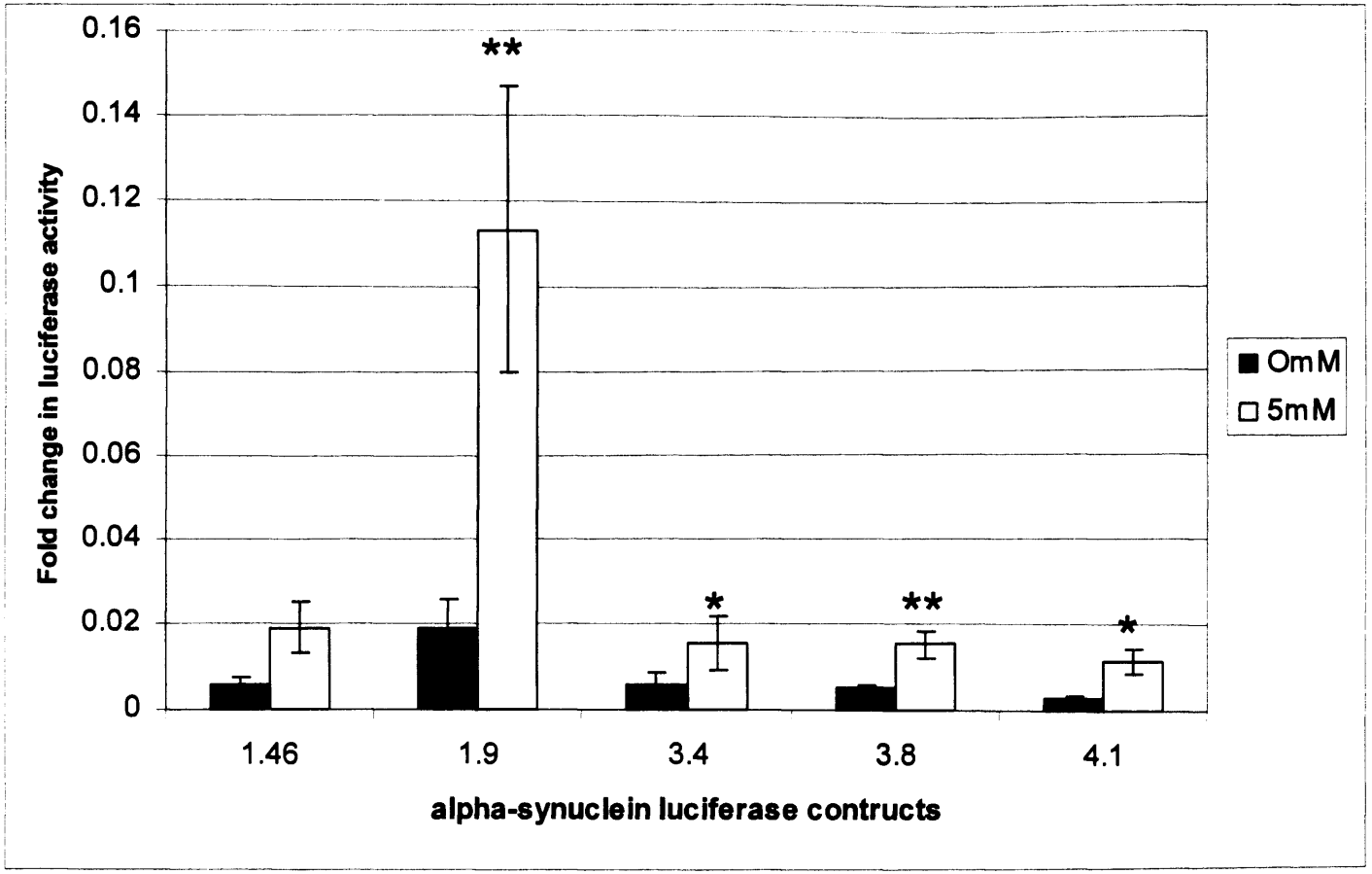


Figure 3.8 Effects of MPP+ on α-synuclein promoter activity
Transfected cells were incubated for 6hrs in 0mM & 5mM MPP+ in full culture media. There were significant increases in luciferase activity for all the constructs tested in 5mM MPP+ compared to 0mM (**p<0.01, *p<0.05) except for 1.46. The error bars indicate the standard error of the mean of 3 experiments, each performed in triplicates.

3.2.3 Promoter Mapping

3.2.3.a Electrophoretic Mobility Shift Assay

From the results above, largest increase in promoter activity between control and oxidative stress conditions was detected using 1.9 promoter construct. This indicated that activating transcription factors bind to this promoter region during the stress conditions, inducing transcription. In view of this, the α-synuclein promoter region contained in this construct was subsequently examined for transcription factor interaction during stress.

Electrophoretic Mobility Shift Assay (EMSA) was used to detect protein:DNA interactions on the designated promoter region. The EMSA technique is a sensitive method, used to determine if a protein or mixture of proteins is capable of binding to a given DNA or RNA sequence, and is based on the observation that protein:DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis (Garner and Revzin, 1981).

The EMSAs were performed using 110base pairs (bp) long, 20bp overlapping region on either side, oligonucleotides that span the 1.9kb sequence upstream of the translational start site (Fig. 3.9). The oligonucleotides were incubated with control, dopamine or MPP+ stressed nuclear extracts, and the mixture was separated by electrophoresis on a non-denaturing gel. This was done to identify possible transcription factors that bind to the promoter region during stressed conditions. Oligonucleotides with bound protein would be

retarded when running down the gel. The retarded oligonucleotide-protein complexes would then be detected as exposed bands above the signal for free labelled oligonucleotides. By adding specific non-labelled oligonucleotides in excess (150x) to the labelled ones in the reaction would effectively compete away the specific binding proteins, hence no/lowered signal would be detected.

In Fig. 3.10a, a retarded band was obtained for the oligonucleotide representing 112bp immediately downstream of the transcriptional start site, when using nuclear extract from dopamine treated cells, which was not present when incubated with control nuclear extract and was, competed away using specific competitors and not with non-specific competitors. This indicated that transcription factors bind to this particular region during dopamine stress.

The efficiency of the EMSA was checked using a labelled oligonucleotide with known Oct2A binding sites present (Fig. 3.10b). Oct2A is a transcription factor that binds strongly to a known consensus sequence. As Oct2A levels are not known to be altered during oxidative stresses, the Oct2A binding oligonucleotide was used with nuclear extracts from control and dopamine treated cells. A stronger exposed band was obtained with the extract from control cells compared to the extracts from treated cells (Fig. 3.10c), thus indicating that the band obtained with extract from treated cells but not extract from control cells in Fig. 3.10a was not due to a lower protein concentration in control compared to dopamine extracts.

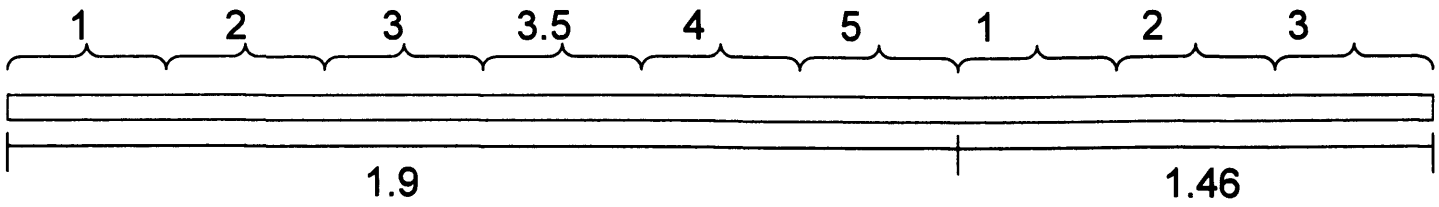


Figure 3.9 Schematic diagram of EMSA oligonucleotides location on α-synuclein promoter
110bp oligonucleotides used in EMSAs both as probe or competitors, to detect the location of possible binding by the protein or protein complex

EMSA oligo	Sequence
1.9(1)	gtcgaccctc aggccctcgg ctctcccagg gcgactctga cgaggggtag ggggtggtcc ccgggaggac ccagaggaaa ggcggggaca agaagggagg
1.9(2)	ggaaggggaa agaggaagag gcatcatccc tagcccaacc gctcccgatc tccacaagag tgctcgtgac cctaaactta acgtgaggcg
1.9(3)	caaaagcgcc cccactttcc cgccttgcgc ggccaggcag gcggctggag ttgatggctc accccgcgcc ccctgcccca tccccatccg
1.9(3.5)	agatagggac gaggagcacg ctgcagggaa agcagcgagc gccgggagag gggcgggcag aagcgctgac aaatcagcgg
1.9(4)	gagccgagga gaaggagaag gaggaggact aggaggagga ggacggcgac gaccagaaggggcccaagag agggggcgag
1.9(5)	ccgcgacgcg gaagtgaggt gcgtgcgggc tgcagcgcag accccggccc ggcccctccgagagcgtcct gggcgctccc tcacgccttg ccttcaagcc ttctgcct
1.46(1)	tt ccaccctcgt gagcggagaa ctgggagtgg ccattcgacg acaggttagcgggtttgcct cccactcccc cagcctcgcg tcgccggctc acagcggcct cctctggg
1.46(2)	ga cagtcccccc cgggtgccgc ctccgccctt cctgtgcgct ccttttccttcttctttcct attaaatatt atttgggaat tgtttaaatt tttttttt
1.46(3)	aaaaagagag aggcggggag gagtcggagt tgtggagaag cagagggact caggtaagta cctgtggatc taaacgggcg tctttggaaa tcctggagaa caccgggt

Table 3.1

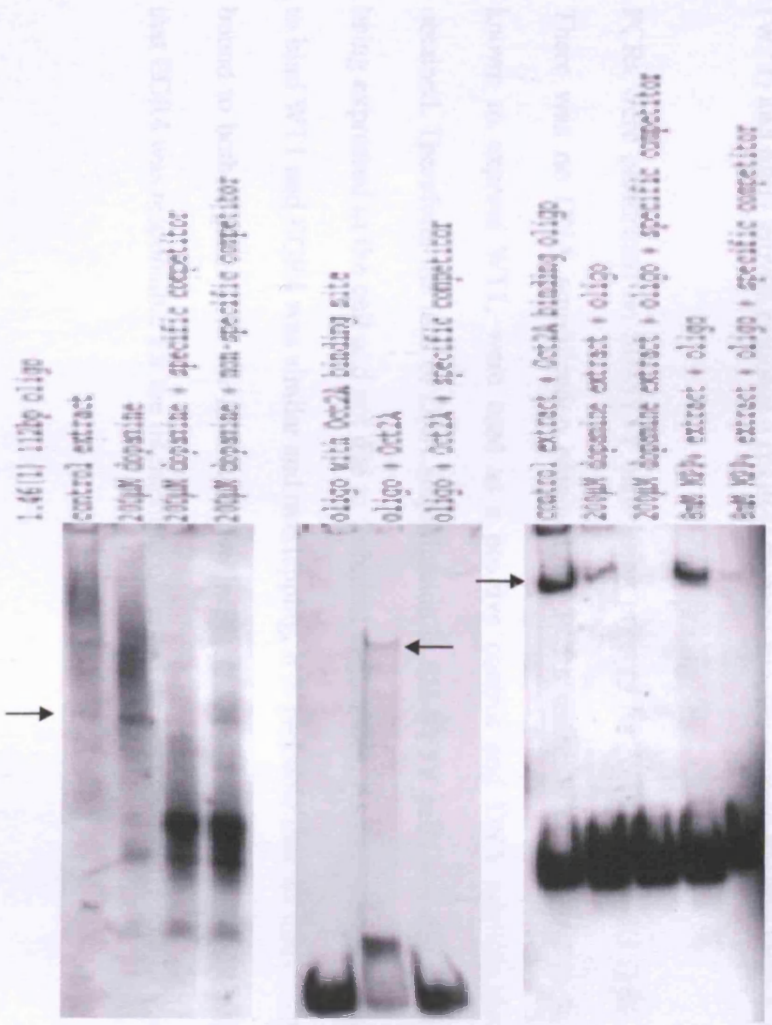


Figure 3.10 Electrophoretic mobility shift assay (EMSA) performed using DIG labelling.

A) 1.46(1), 112bp DIG-labelled dsoligo from the 1st 112bp of the 1.46 construct was incubated with control (untreated) nuclear extract, 200μM dopamine nuclear extract and specific unlabelled competitor oligos as well as non-specific unlabelled oligos. A retarded band was obtained which showed possible transcription factor binding.

B) DIG-labelled dsoligo containing binding sites for Oct2A was run alone or after incubation with Oct2A, and with specific competitor unlabelled oligo. A single retarded band showed specific binding of Oct2A to the oligo.

C) Oct2A binding labelled oligo incubated with control nuclear extract or 200μM dopamine or 8mM MPP+ nuclear extracts with or without specific unlabelled competitor oligos. A stronger band is obtained for lane with control extract as compared to that with dopamine or MPP+ extracts. Band is competed away in presence of specific competitors.

In response to the observed band present in Fig. 3.10a, further EMSAs were performed using smaller oligonucleotides from the region, 1.46(1), where the proteins were found to bind (Fig. 3.11). This would narrow down the binding region to better identify the binding sequence. No protein:DNA interaction was detected using these oligonucleotides (result not shown).

1.46(1) sequence was submitted to MatInspector for prediction of potential transcription factor sites (Table 3.1). 13 potential binding proteins and their respective binding sites were obtained (Table 3.2). Short 15bp oligonucleotides containing the 13 different binding sites were used for EMSAs. Oligonucleotides with binding sites for Wilms tumour suppressor 1 (WT1) and Early growth response 4 (EGR4) showed specific protein binding (Fig. 3.12).

PCRs were performed on SH-SY5Y cells using primers for WT1 and EGR4 (Fig. 3.13). There was no DNA amplification obtained for PCRs using WT1 primers. HEK cells, known to express WT1, were used as a positive control and DNA amplification was obtained. Therefore, the lack of DNA amplification in SH-SY5Y cells was due to WT1 not being expressed in the cell and not due to technical difficulties. As the sequence identified to bind WT1 and EGR4 was similar and overlapping, it is probable that the same protein(s) bound to both sequences in the EMSA shown in Fig. 3.12. As such, it is highly probable that EGR4 was responsible for the interactions observed on both the oligonucleotides used.

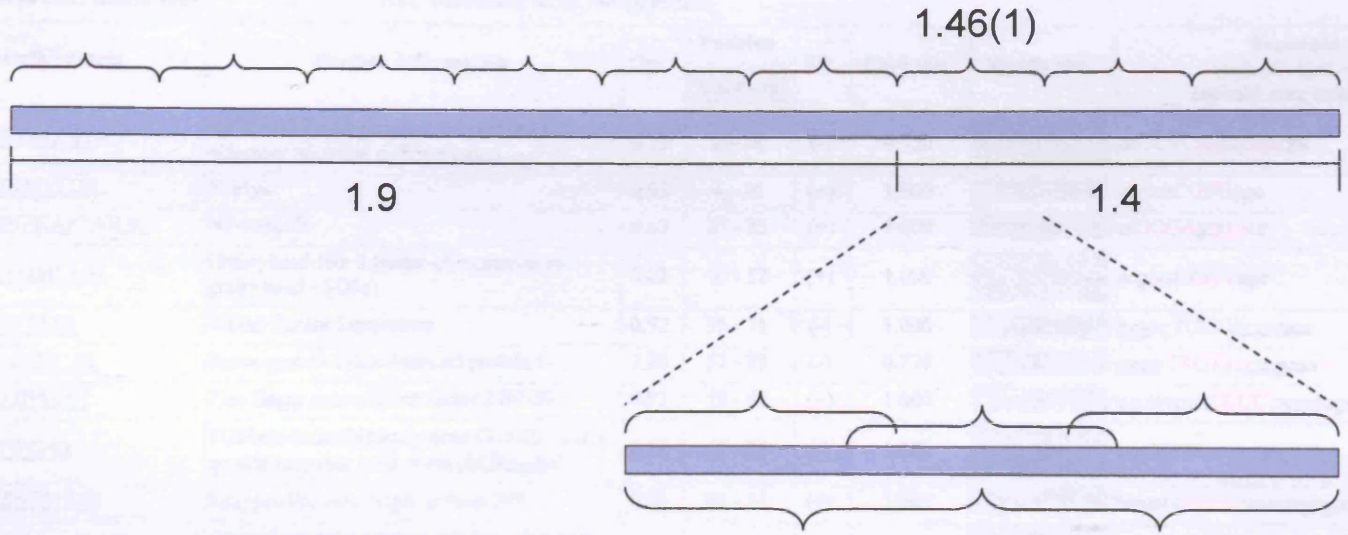


Figure 3.11 Schematic diagram of shorter EMSA oligonucleotides location on α-synuclein promoter
Shorter oligos subsequently used in EMSAs both as probe or competitors, to narrow down the location of binding by the protein/ protein complex as observed in previous EMSAs using 1.46(1) oligo.

Table 3.2

MatInspector Release professional 7.4.5, Dec 2006 **Wed Mar 21 17:38:55 2007**

Sequence file: 1_1.seq (110 bp)
Family matches: Yes
MatInspector library: Matrix Family Library Version 6.2 (October 2006)
Selected groups (core/matrix sim) ALL vertebrates.lib (0.75/Optimized)

Family/matrix	Further Information	Opt.	Position from - to	Str.	Core sim.	Matrix sim.	Sequence (red: ci-value > 60 capitals: core sequence)
V$OAZF/ROAZ.01	Rat C2H2 Zn finger protein involved in olfactory neuronal differentiation	0.73	2 - 18	(-)	0.750	0.778	ccGCTCacgagggtgga
V$EBOX/NMYC.01	N-Myc	0.92	4 - 16	(+)	1.000	0.922	caccctCGTGagc
V$NFKB/NFKAPPAB.02	NF-kappaB	0.82	23 - 35	(+)	1.000	0.822	ctGGGAgtggcca
V$GRHL/GRHL3.01	Grainyhead-like 3 (sister-of-mammalian grainyhead - SOM)	0.82	40 - 52	(+)	1.000	0.855	acgacaGGTTagc
V$EGRF/WT1.01	Wilms Tumor Suppressor	0.92	55 - 71	(-)	1.000	0.922	gggagTGGGaggcaaac
V$EGRF/NGFIC.01	Nerve growth factor-induced protein C	0.80	57 - 73	(-)	0.770	0.824	ggggGAGTgggaggcaa
V$ZBPF/ZBP89.01	Zinc finger transcription factor ZBP-89	0.93	59 - 81	(+)	1.000	0.944	gcctcccactCCCCcagcctcgc
V$SP1F/TIEG.01	TGFbeta-inducible early gene (TIEG) / Early growth response gene alpha (EGRalpha)	0.83	61 - 75	(-)	1.000	0.858	ctgGGGGagtgggag
V$ZBPF/ZNF219.01	Kruppel-like zinc finger protein 219	0.91	62 - 84	(+)	1.000	0.929	tcccactCCCCcagcctcgcgtc
V$ZBPF/ZNF202.01	Transcriptional repressor, binds to elements found predominantly in genes that participate in lipid metabolism	0.73	65 - 87	(+)	1.000	0.739	cactccCCCAgcctcgcgtcgcc
V$EGRF/EGR3.01	Early growth response gene 3 product	0.77	76 - 92	(+)	1.000	0.788	cctcGCGTcgccggctc
V$WHNF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation	0.95	76 - 86	(-)	1.000	0.964	gcgACGCgagg
V$PAX5/PAX5.03	PAX5 paired domain protein	0.80	78 - 106	(+)	0.789	0.827	tcgcgTCGCcggctcacagcggcctcctc

Reference for MatInspector:
Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T (2005)
MatInspector and beyond: promoter analysis based on transcription factor binding sites
Bioinformatics 21, 2933-42

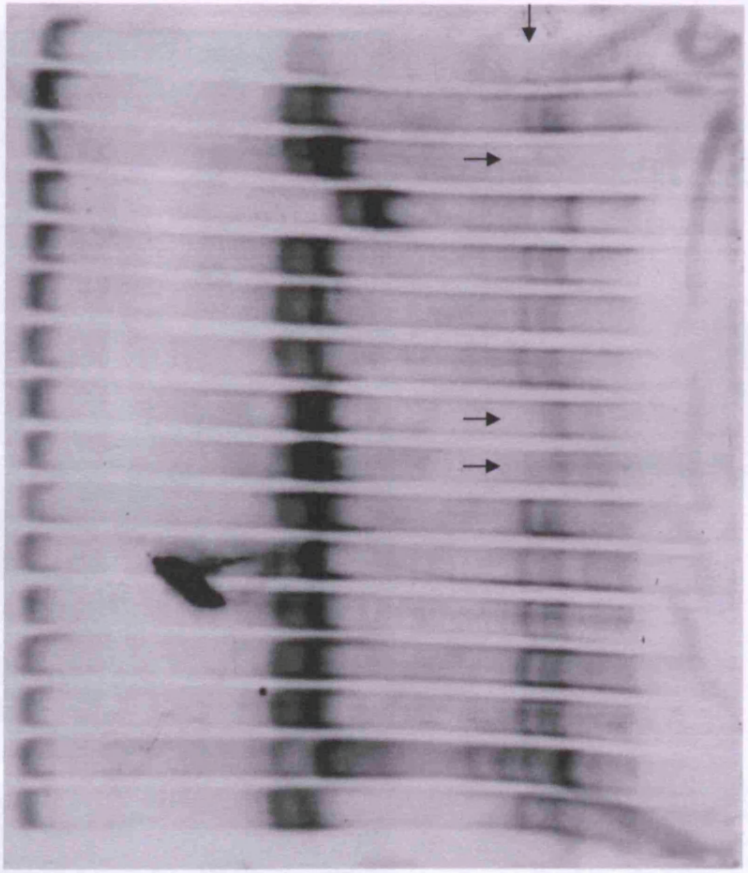


Figure 3.12 EMSA on oligonucleotides derived from MatInspector analysis
1.46(1), 112bp DIG-labelled dsoligo from the 1st 112bp of the 1.46 construct, was incubated with control (untreated) nuclear extract, 200μM dopamine nuclear extract and specific unlabelled competitor oligos as well as each of the 13 unlabelled oligos containing transcription factor binding sites as predicted by matInspector. C5 and C6 showed competition for the protein(s), similar to that obtained for the specific competitor.

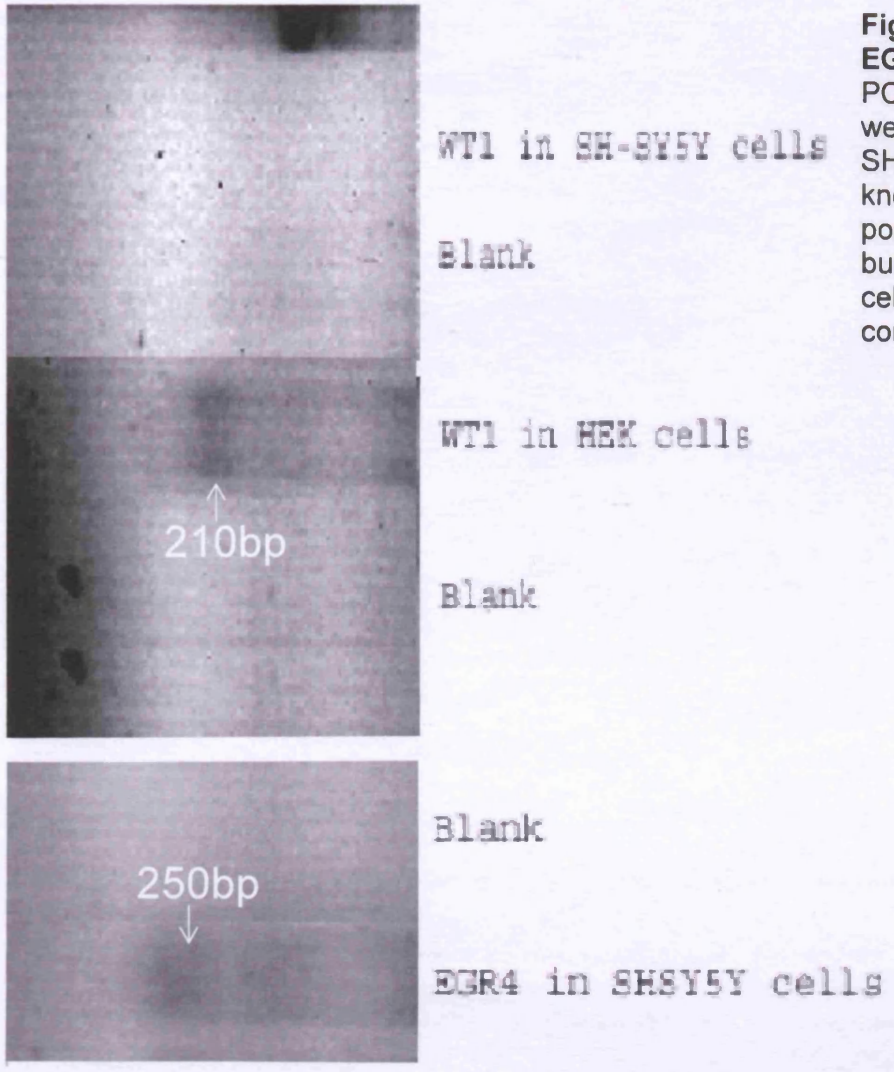


Figure 3.13 PCR detection of WT1 and EGR4
PCR using primers for WT1 and EGR4 were performed on cDNA made from SH-SY5Y RNA extracts. HEK cells, known to express WT1, were used as a positive control for WT1 PCRs. EGR4 but not WT1 was detected in SH-SY5Y cells. WT1 was detected in the positive control.

3.2.3.b Affinity isolation & silver staining

To identify the protein(s) observed binding to 1.46(1) sequence in Fig. 10a, affinity isolation of the binding factor(s) by magnetic beads with streptavadin-biotin bound 1.46(1) oligomers was used together with silver-staining. A silver-stained band present in dopamine stressed cells lane but not in the control lane was observed (Fig. 3.14). This band was cut out, processed and sent for protein sequencing by mass spectrometry. Mass spectrometry did not reveal the identity of the proteins as the concentration was too low even when protein purified from 4 different bands cut from 4 separate gels were pooled.

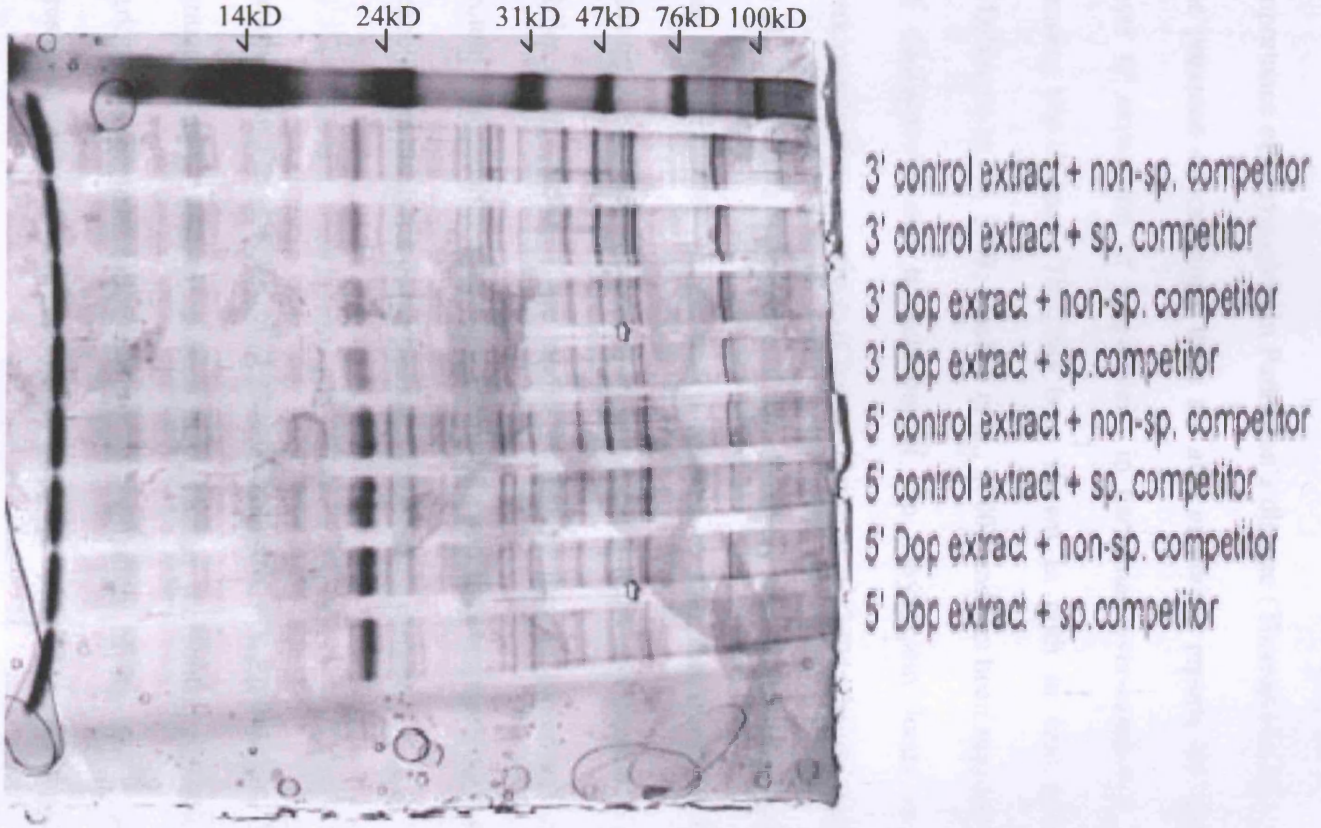


Figure 3.14 Affinity isolation and silver staining of DNA interacting factors
A silver-stained non-denaturing acrylamide gel electrophoresed with bead-DNA-protein mix is shown. Magnetic Streptavidin beads were used to pull down biotinylated DNA of the sequence corresponding to 1.46(1), which were incubated with nuclear extract of SH-SY5Y cells treated with 200μM dopamine. The hollow arrows indicate bands which indicate proteins binding to the DNA during stressed conditions. 3' and 5' indicate the use of 3' and 5' biotinylated-streptavidin bead bound 1.46(1) oligonucleotide.

3.3 Discussion

There exists a substantial amount of evidence in current literature that highlights the importance of α-synuclein in Parkinson's disease (Thomas and Beal, 2007). Other than the presence of mutations, there are also numerous reports that show that an altered level of expression of α-synuclein, in particular over-expression, was sufficient in causing the disease. This has been shown in both *in vivo* and *in vitro* systems (Hashimoto et al., 1998; Masliah et al., 2000), and has been supported by the discovery of duplications and triplications of the α-synuclein locus in affected familial Parkinson's disease patients (Chartier-Harlin et al., 2004; Ibanez et al., 2004a; Singleton et al., 2003). The importance of this discovery was boosted by studies which showed that the triplication of the region resulted in four functional copies of α-synuclein, causing a two-fold increase in α-synuclein mRNA and protein (Farrer et al., 2004; Miller et al., 2004). These studies confirmed that an increased level of α-synuclein protein was capable of causing the disease and thus emphasising the importance of α-synuclein levels in the development of Parkinson's disease.

However, in the absence of α-synuclein genetic abnormalities in sporadic cases, α-synuclein may, instead, play a role in the molecular pathogenesis of sporadic Parkinson's disease following the dysregulation of its expression. Environmental stresses as well as factors involved in aging are believed to be the main causations in the development of sporadic Parkinson's disease (Elbaz et al., 2007). The consensus among many researchers is that Parkinson's disease is a result of an interplay between susceptibility genes and environmental stress (Elbaz et al., 2007; Thomas and Beal,

2007). This hypothesis together with the evidence showing that oxidative stress increases the intracellular level of α-synuclein and promotes its aggregation (Przedborski et al., 2001; Vila et al., 2000), have directed us to look into the regulation of normal α-synuclein in the presence of oxidative stress.

From our studies using RT PCR and dual luciferase assays, we have confirmed that oxidative stresses such as dopamine and MPP+, caused an up-regulation of α-synuclein, and have further shown that this up-regulation was due to transcriptional activation of the promoter.

Electrophoretic Mobility Shift Assays (EMSAs) subsequently performed revealed a potential transcription factor(s) interaction with α-synuclein promoter during dopamine stressed conditions. Utilising an online transcription factor binding site search programme, MatInspector, (Cartharius et al., 2005; Cross et al., 1994) several potential transcription factor sites were found of which one particular site, a GC rich consensus site, was then confirmed by additional EMSAs to be the site of interaction during dopamine stress. As this site was shown by MatInspector to be shared by either WT1 or EGR4 (NGFIC), PCRs were performed on SH-SY5Y cell extracts to ascertain the presence of both the proteins in our cell model. This demonstrated that only EGR4 was present and not WT1, thus eliminating the possibility of WT1 interacting with α-synuclein promoter in our experiments.

Even though the MatInspector programme indicated EGR4 as a good candidate for the interaction with the tested oligomer, the identity of the transcription factor that is responsible for this interaction, however, could not yet be confirmed as further literature

search showed that all the EGR transcription family members were able to bind to the same consensus sequence (Beckmann and Wilce, 1997). In addition, affinity isolation of the binding protein followed by mass spectrometry could not identify the protein in question. Moreover, a GC rich sequence was also known to be able to be bound by a variety of transcription factors.

Consequently, to narrow down the list of transcription factors that would have to be examined for the interaction, a further search using two online transcription factor binding site search programmes, MatInspector (Table. 3.3) and TFSEARCH (Akiyama, ; Heinemeyer et al., 1998) (Table. 3.4), was performed and the results compared. Three matching transcription factors were identified by both the search programmes, when the search stringency was lowered in both from the default 8.0 to 7.5 so as to reveal more matches. These three transcription factors include the EGR family of transcription factors, in particular EGR4, myeloid zinc finger 1 factors and Ikaros zinc finger family.

EGR4 and its family members, specifically, were identified in MatInspector as a good match with an above optimised similarity score (highlighted in green under matrix similarity; Table. 3.3). Both myeloid zinc finger 1 and Ikaros zinc finger family of transcription factors are known to be involved in haematopoiesis and lymphocyte differentiation. Thus, they are unlikely to be the transcription factors responsible for interacting with α-synuclein promoter in neurons during stress. The EGR family of transcription factors, on the other hand, are immediate early genes that have been found to be able to be induced by diverse extracellular stimuli within the nervous system (Beckmann and Wilce, 1997), and therefore most likely to be our candidate

MatInspector Release professional 7.7.3, February 2008 **Tue Apr 22 17:26:22 2008**

Sequence file: 1_2.seq (17 bp)
Family matches: Yes
MatInspector library: Matrix Family Library Version 7.0 (October 2007)
Selected groups (core/matrix sim) ALL vertebrates.lib (0.75/0.75)

Check transcription factor <-> matrix family assignment			Opt.	Position	Str.	Core sim.	Matrix sim.	Sequence (red: ci-value > 60 capitals: core sequence)
Family	Further Family Information	Matrix		from - to				
V$EBOX	E-box binding factors	V$ATF6.01	0.93	1 - 13	(-)	1.000	0.880	ctcCCACtccccc
V$EGRF	EGR/nerve growth factor induced protein C & related factors	V$NGFIC.01	0.80	1 - 17	(+)	0.770	0.824	ggggGAGTgggaggcaa
V$EKLF	Basic and erythroid krueppel like factors	V$BKLF.01	0.95	1 - 17	(+)	0.750	0.810	ggGGGAgtgggaggcaa
V$MAZF	Myc associated zinc fingers	V$MAZR.01	0.88	1 - 13	(+)	0.754	0.772	gggggaGTGGgag
V$TFII	GTF2I repeat domain-containing factors	V$GTF3R4.01	0.97	2 - 12	(+)	0.750	0.767	gggGAGTggga
V$MAZF	Myc associated zinc fingers	V$MAZ.01	0.90	3 - 15	(+)	0.800	0.759	gggaGTGGgaggc
V$IKRS	Ikaros zinc finger family	V$IK2.01	0.98	5 - 17	(+)	1.000	0.936	gagtGGGAggcaa
V$MZF1	Myeloid zinc finger 1 factors	V$MZF1.02	0.99	6 - 16	(+)	0.761	0.805	agTGGGaggca
V$BTBF	BTB/POZ (broad complex, TramTrack, Bric à brac/pox viruses and zinc fingers) transcription factor	V$KAISO.01	0.92	7 - 17	(-)	0.750	0.751	ttgcCTCCcac

Table 3.3

Reference for MatInspector:
Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T (2005)
MatInspector and beyond: promoter analysis based on transcription factor binding sites
Bioinformatics 21, 2933-42

Table 3.4

TFSEARCH Search Result

```
** TFSEARCH  ver.1.3 **          (c)1995 Yutaka Akiyama (Kyoto Univ.)

 This simple routine searches highly correlated sequence fragments
 versus TFMATRIX transcription factor binding site profile database
 by E.Wingender, R.Knueppel, P.Dietze, H.Karas (GBF-Braunschweig).

  <Warning> Scoring scheme is so straightforward in this version.
            score = 100.0 * ('weighted sum' - min) / (max - min)
            The score does not properly reflect statistical significance!

 Database:  TRANSFAC MATRIX TABLE, Rel.3.3 06-01-1998
 Query:     1.46(1)c6 (17 bases)
 Taxonomy:  Vertebrate
 Threshold: 75.0 point


 TFMATRIX entries with High-scoring:

   1 GGGGGAGTGG GAGGCAA                          entry         score
     ------------->                              M00084 MZF1    80.6
          --------->                             M00141 Lyf-1   79.2
         ------------>                           M00087 Ik-2    78.5
       ------------>                             M00244 NGFI-C  75.7
      ---------->                                M00008 Sp1     75.3


 Total 5 high-scoring sites found.
```

Max score: 80.6 point, Min score: 75.3 point

factor. Hence, we focused our efforts on deciphering if any of the EGR transcription factors were responsible for the observed interaction (Chapter 4).

In conclusion, this is a novel study showing that wild-type α-synuclein expression could be influenced by selective oxidative stresses and that this regulation is a transcriptional event. The binding of transcription factors to the α-synuclein promoter indicates the possible mechanism responsible for this regulation and possible identities of these transcription factors have been deduced. More work to conclusively identify the transcription factors involved is discussed in the next chapter.

Chapter 4: Regulation of α-synuclein expression in oxidative stress by Early Growth Response family of transcription factors

4.1 Introduction

4.1.1 Early Growth Response family of transcription factors

Early Growth Response (EGR) family of transcription factors belong to a subclass of immediate early genes (Beckmann and Wilce, 1997). These genes are inducible by a diverse variety of stimuli and usually lead to rapid and often transient transcriptional induction. The four members of the family, EGR1-4, share a homologous zinc finger DNA binding domain that recognise the same consensus DNA sequence, GCG(G/T)GGGCG (Beckmann and Wilce, 1997). Variation in affinities of each member to slightly different sequences has also been found (Skerka et al., 1997). EGR proteins themselves may be regulated by transcriptional, translational, or post-translational events as well as protein-protein interactions (Beckmann and Wilce, 1997). These regulations and/or modifications of EGR would therefore subsequently affect their transcription regulation activity.

Expression of the EGR proteins occurs throughout the nervous system at varying levels, but substantial levels of all four members are present in the striatum, where dopaminergic neurons are found (Beckmann and Wilce, 1997). To date, studies on the effects of dopamine on expression have only covered EGR1 (Beckmann and Wilce, 1997; Snyder-Keller et al., 2002). These studies revealed the relationship between dopamine and EGR1, in that the activation of dopamine receptors increases the basal expression of EGR1 (Snyder-Keller et al., 2002).

4.1.2 Regulation of α-synuclein by EGR transcription factors

Following the identification of putative transcription factors that may bind the GC rich region, 5'(GGGGGAGTGGGAGGCAA)3', of the α-synuclein promoter following dopamine stress, as discussed in the previous chapter, the EGR family of transcription factors were selected for further investigation. This group of trans-acting factors were selected for the following reasons; their high scores in consensus prediction by two different transcription factor binding site search programmes, being inducible by extracellular stimuli and their known associations with the nervous system.

4.1.3 Hypothesis & Aims

Experimental observations from Chapter 3 and the results from transcription site prediction software indicate that one member of the EGR family of transcription factors may bind to the α-synuclein promoter in the presence of dopamine stress and thus regulate the expression of α-synuclein.

Aims of project:

4. To determine if any of the EGR transcription factors are regulated by dopamine stress

5. To investigate which of the EGR transcription factors are capable of modulating the α-synuclein promoter function.

4.2 Results

4.2.1 Quantitation of Early Growth Response genes in dopamine stress

Changes in EGR mRNA have been shown as an accurate representation of EGR-mediated transcriptional activity (O'Donovan et al., 1999; Skerka et al., 1997). On this basis, the expression of each of the EGR family members was examined during dopamine stress to test whether any of them respond to the stress. Real-time Polymerase Chain Reaction (RT PCR) was used to quantify each EGR transcript levels in the presence and absence of dopamine stress. Results were normalised onto β2microglobulin and βactin housekeeping control genes. The identity and specificity of the product of each RT PCR was confirmed after the experiments (Fig 4.1)

All the EGR genes 1-4 showed up-regulation with dopamine stress (Fig. 4.2; similar results for both normalisations were obtained, one representative graph is shown). EGR2 mRNA expression increased to the least extent of 2.5 fold and EGR3 mRNA increased the most, 9 fold, in the presence of dopamine stress (Fig. 4.2b, c). EGR1 and EGR4 both increased 6 fold with dopamine treatment (Fig. 4.2a, d).

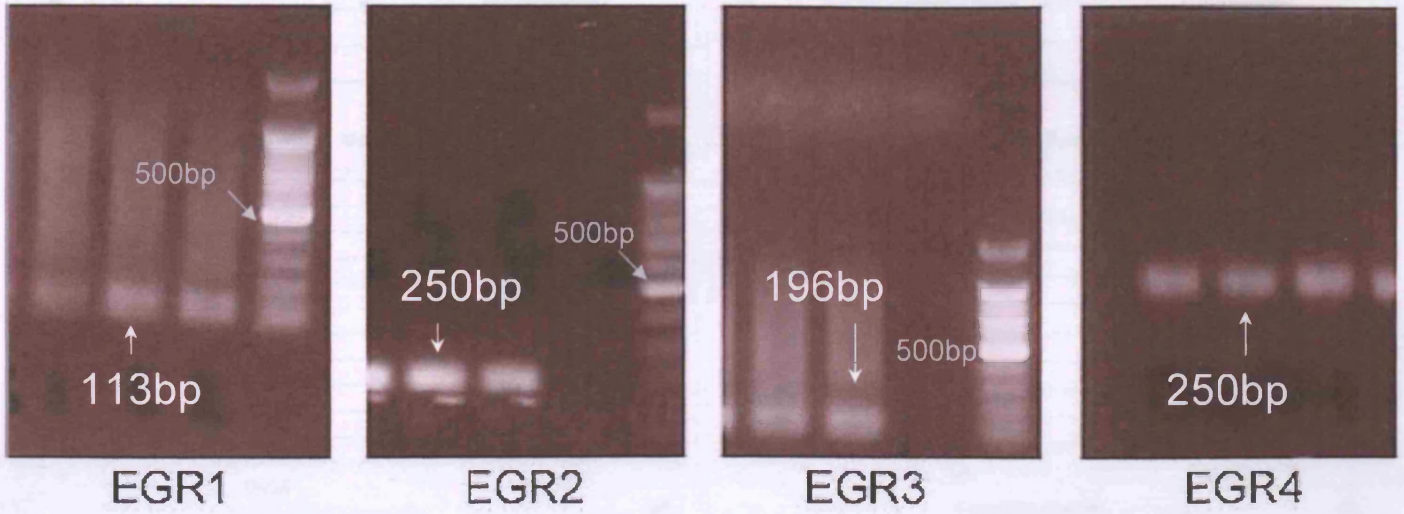


Figure 4.1 RT PCR EGR1-4 product agarose gel check
Agarose gel check on RT PCR products to confirm product identity and PCR specificity. Specific product of the correct size was obtained for each EGR primer set used. Amplified product identity was confirmed by sequencing.

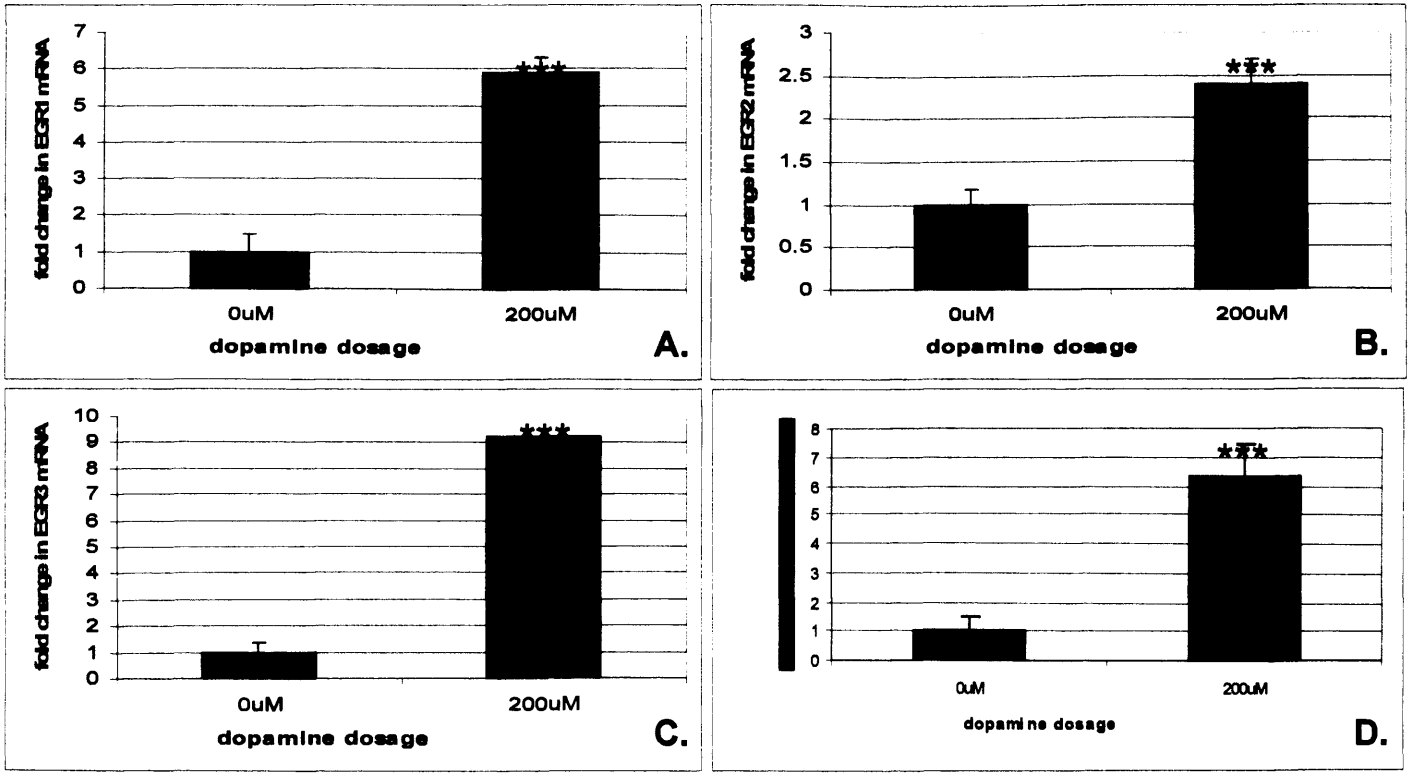


Figure 4.2 Effect of dopamine on EGR1-4 mRNA expression

Cells were incubated for 24hrs in 0μM and 200μM dopamine in full media culture media before mRNA retrieval. The error bars indicate the standard error of the mean of 3 experiments. Differences were analysed by paired T-test. (***p<0.001)

A) EGR1 mRNA levels increased with dopamine stress.

B) EGR2 mRNA levels also increased with dopamine stress, but to much smaller extent compared to EGR1, 3 and 4.

C) EGR3 mRNA levels showed the greatest increase with dopamine stress

D) EGR4 mRNA levels increased to a similar extent as EGR1 with dopamine stress

4.2.2 Regulation of α-synuclein expression by Early Growth Response gene 4 (EGR4)

Of all the EGR members, EGR4 (NGFI-C) was selected by MatInspector as the potential interacting transcription factor to our target sequence. As such, studies into the regulation of α-synuclein expression by the EGR transcription factors were prioritized for EGR4.

4.2.2.a Effect of EGR4 on α-synuclein mRNA expression

To investigate if EGR4 might regulate α-synuclein expression during dopamine stress, RT PCR was used to examine the effect of knocked-down EGR4 on endogenous α-synuclein mRNA expression in cells. This was done by transiently transfecting SH-SY5Y cells with small-interfering EGR4 RNA. Levels of EGR4 mRNA in the cells were then quantified by RT PCR to ensure knock-down before α-synuclein mRNA expression was quantified. Normalisations to 2 house-keeping genes, β2-microglobulin and βactin were performed to ensure results were consistent.

EGR4 increased 6 fold from basal expression levels when SH-SY5Y cells were subjected to 200μM dopamine stress (Fig. 4.3a). Knock-down of EGR4 using short-interference RNA caused a 50% decrease of EGR4 expression in normal conditions but a 70% decrease when dopamine stressed. The knock-down of EGR4 in each condition resulted in a significant increase of α-synuclein expression by 30% and 79% in the presence and absence of dopamine respectively (Fig. 4.3b).

The effect of over-expression of EGR4 on endogenous α-synuclein mRNA expression was also examined. SH-SY5Y cells were transiently transfected with EGR4 over- expressing construct and levels of EGR4 and α-synuclein mRNA in the cells were quantified by RT PCR. Normalisations to 2 house-keeping genes, β2-microglobulin and NADH cytochrome b5-reductase were performed. Over-expression of EGR4 in the cells did not cause any significant difference in α-synuclein expression (Fig. 4.4).

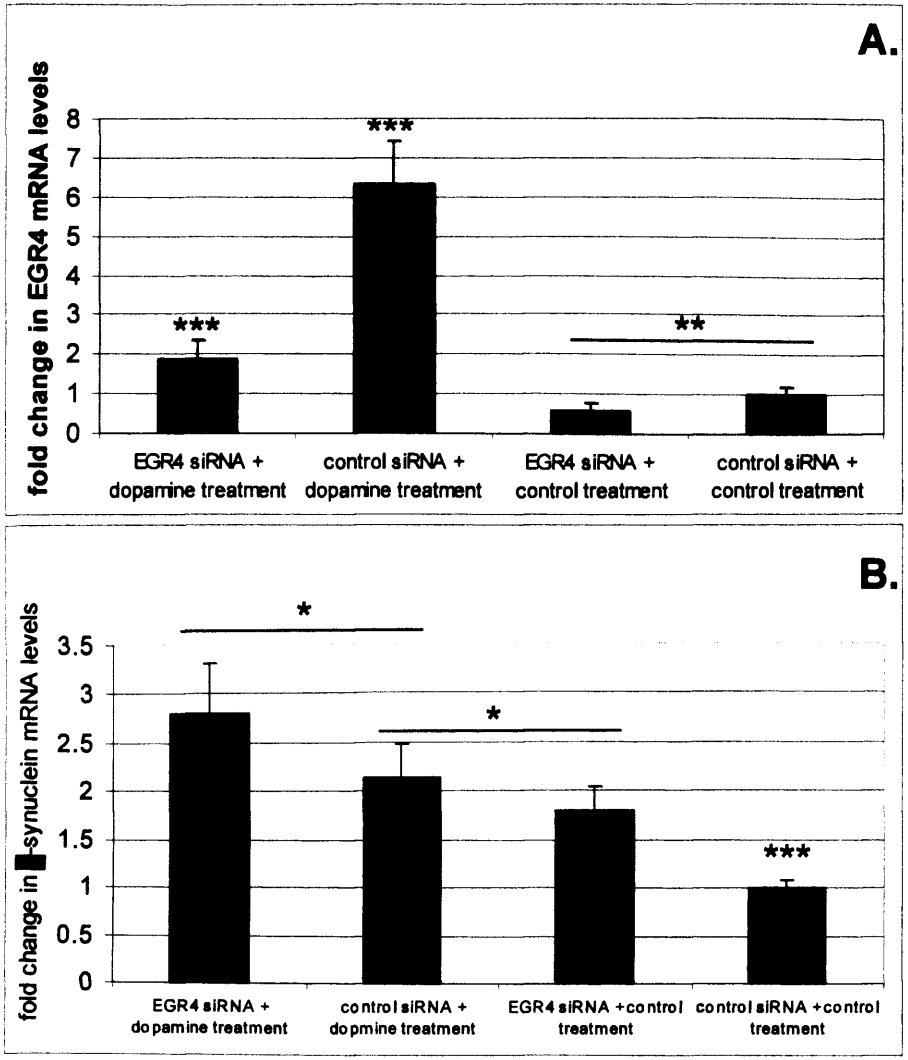


Figure 4.3 Effect of EGR4 knock-down on α-synuclein mRNA expression
EGR4 siRNA and non-specific siRNA transfected cells were incubated for 24hrs in 0μM and 200μM dopamine in full media culture media before mRNA retrieval. The error bars indicate the standard error of the mean of 3 experiments. Differences were analysed by paired T-test.

A) EGR4 mRNA levels were measured. All the differently transfected and treated cells had significantly different levels of EGR4 mRNA expressed (**$p<0.01$, ***$p<0.001$ when compared to all other samples).

B) α-synuclein mRNA levels were measured. α-synuclein levels were significantly lower in untreated cells transfected with non-specific siRNA compared to all other samples (***$p<0.001$). The other samples were also significantly different, but to a lesser extent (*$p<0.05$).

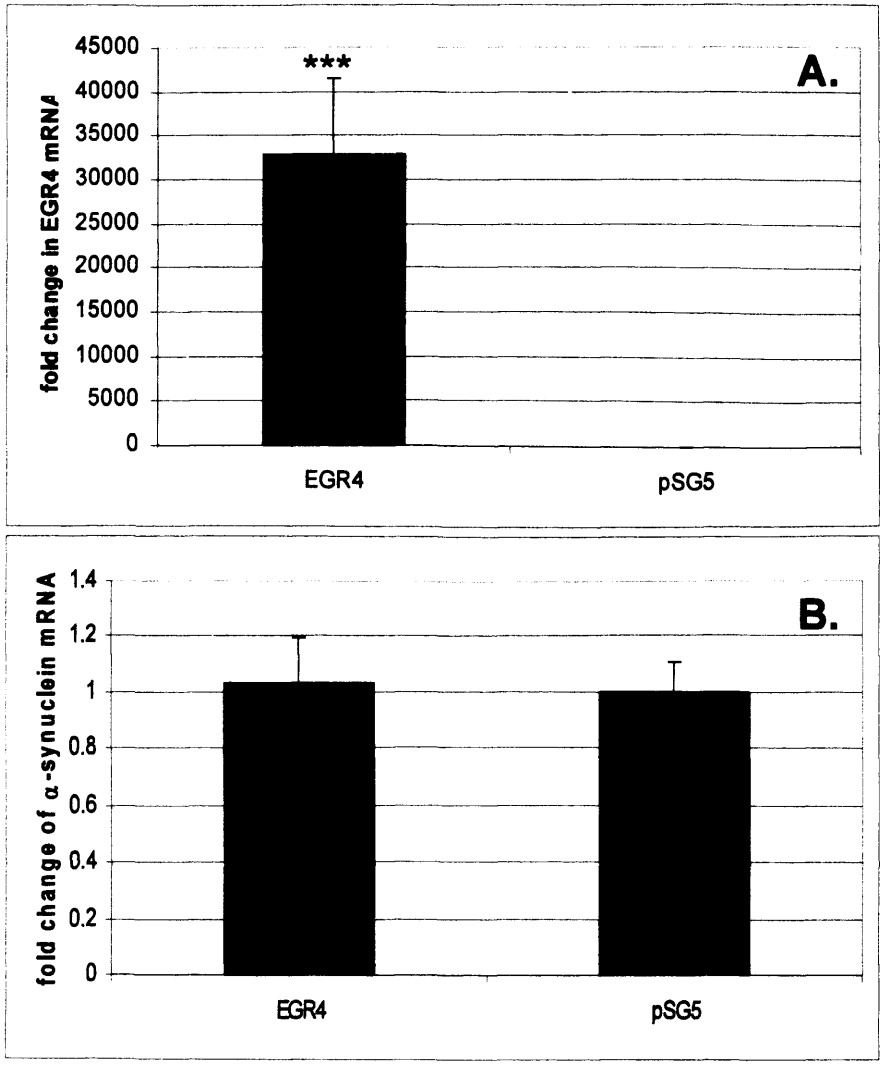


Figure 4.4 Effect of EGR4 over-expression on α-synuclein mRNA expression

EGR4 overexpressing and empty pSG5 constructs were transfected into SH-SY5Y cells were incubated for 24hrs in full media culture media before mRNA retrieval. The results of the RT PCR was normalised onto results obtained using housekeeping gene primers – β2-microglobulin and NADH cytochrome b5-reductase. Results were similar for both normalisations. The error bars indicate the standard error of the mean of 3 experiments. Differences were analysed by paired T-test.

A) EGR4 mRNA levels were measured. EGR4 overexpressing construct transfected cells had significantly higher EGR4 levels compared to those transfected with pSG5 (***$p<0.001$).

B) α-synuclein mRNA levels were measured. α-synuclein levels were not different between those transfected with EGR4 over-expressing construct and pSG5.

4.2.2.b Effect of EGR4 on α-synuclein promoter activity

Dual luciferase assays was performed on extracts from cells co-transfected with α-synuclein promoter luciferase constructs, SV40 renilla construct and EGR4 siRNA to investigate the effect of EGR4 knock-down on α-synuclein promoter activity. The 1.9 α-synuclein promoter luciferase construct was used as it contained both the predicted EGR4 binding site as well as the α-synuclein core promoter region to confer efficient transcription.

In contrast to results obtained from RT PCR experiments described previously, luciferase constructs containing ~1.9kb of α-synuclein promoter responded negatively to EGR4 knock-down (Fig. 4.5). The 1.9kb promoter activity decreased by 50% when EGR4 was knocked down in both dopamine treated and control conditions. There was no significant change in the activity of the 1.46kb promoter in all the samples.

The effect of EGR4 over-expression on α-synuclein promoter activity was also examined. Extracts from cells co-transfected with α-synuclein promoter luciferase constructs, SV40 renilla construct and EGR4 over-expressing construct were used. The 1.9 α-synuclein promoter luciferase activity increased 2.5 fold with EGR4 over-expression (Fig. 4.6). There was also no change detected on the 1.46 promoter activity.

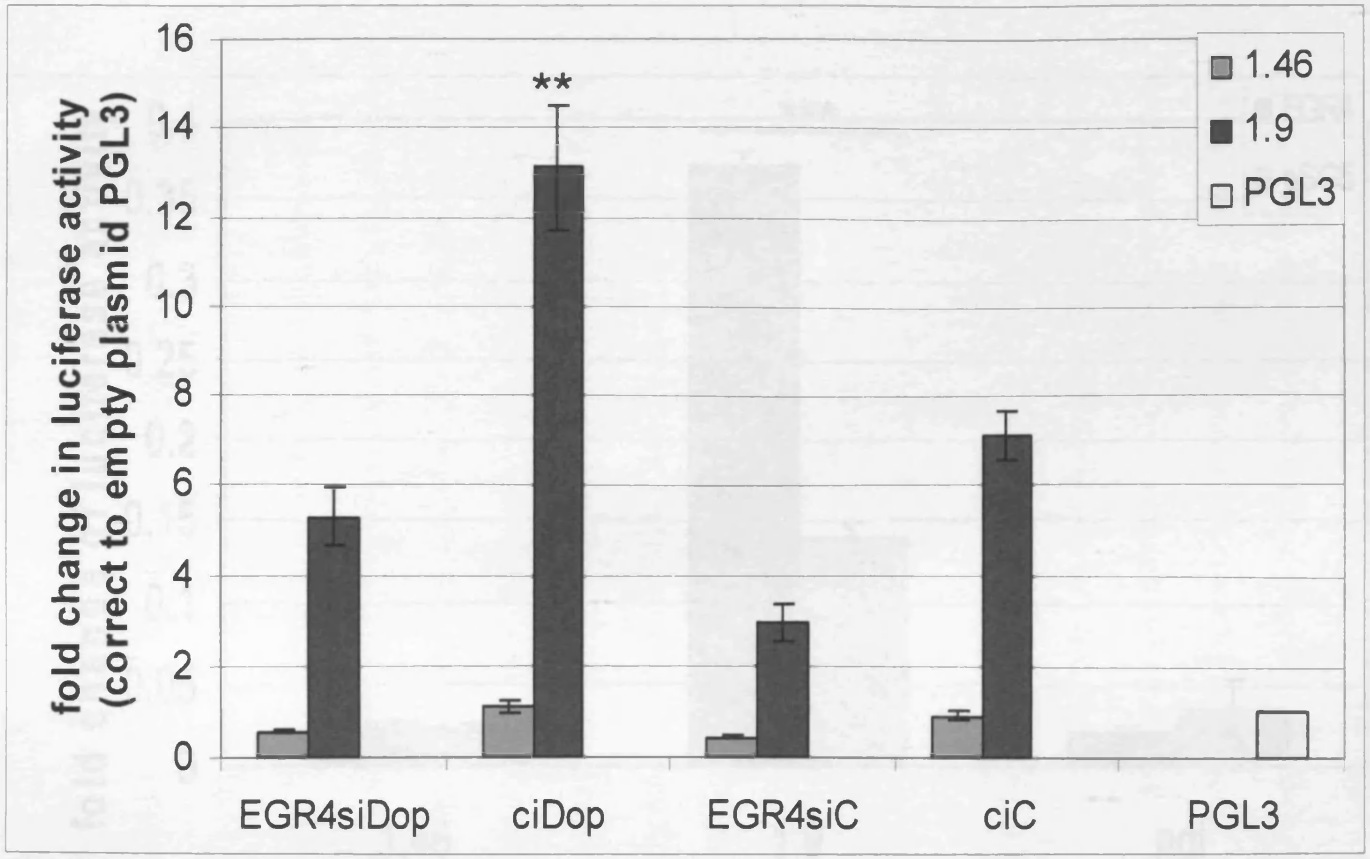


Figure 4.5 Effects of EGR4 knock-down and dopamine on α-synuclein promoter activity
SH-SY5Y cells were transfected with 1.46, 1.9 α-synuclein promoter construct or PGL3 empty construct and EGR4 siRNA or non-specific RNA and incubated for 24hrs with 0μM or 200μM dopamine in full culture media. Cells transfected with non-specific RNA and treated with dopamine had significantly higher luciferase activity compared to all the other samples (**p<0.01). Data was analysed by paired T-test. EGR4siDop indicate cells co-transfected with EGR4siRNA and treated with dopamine; ciDop: cells co-transfected with control siRNA and treated with dopamine; EGR4siC: non-treated cells co-transfected with EGR4siRNA; ciC: non-treated cells co-transfected with control siRNA. PGL3 indicates level of normalised luciferase activity when cells are co-transfected with empty plasmid PGL3.

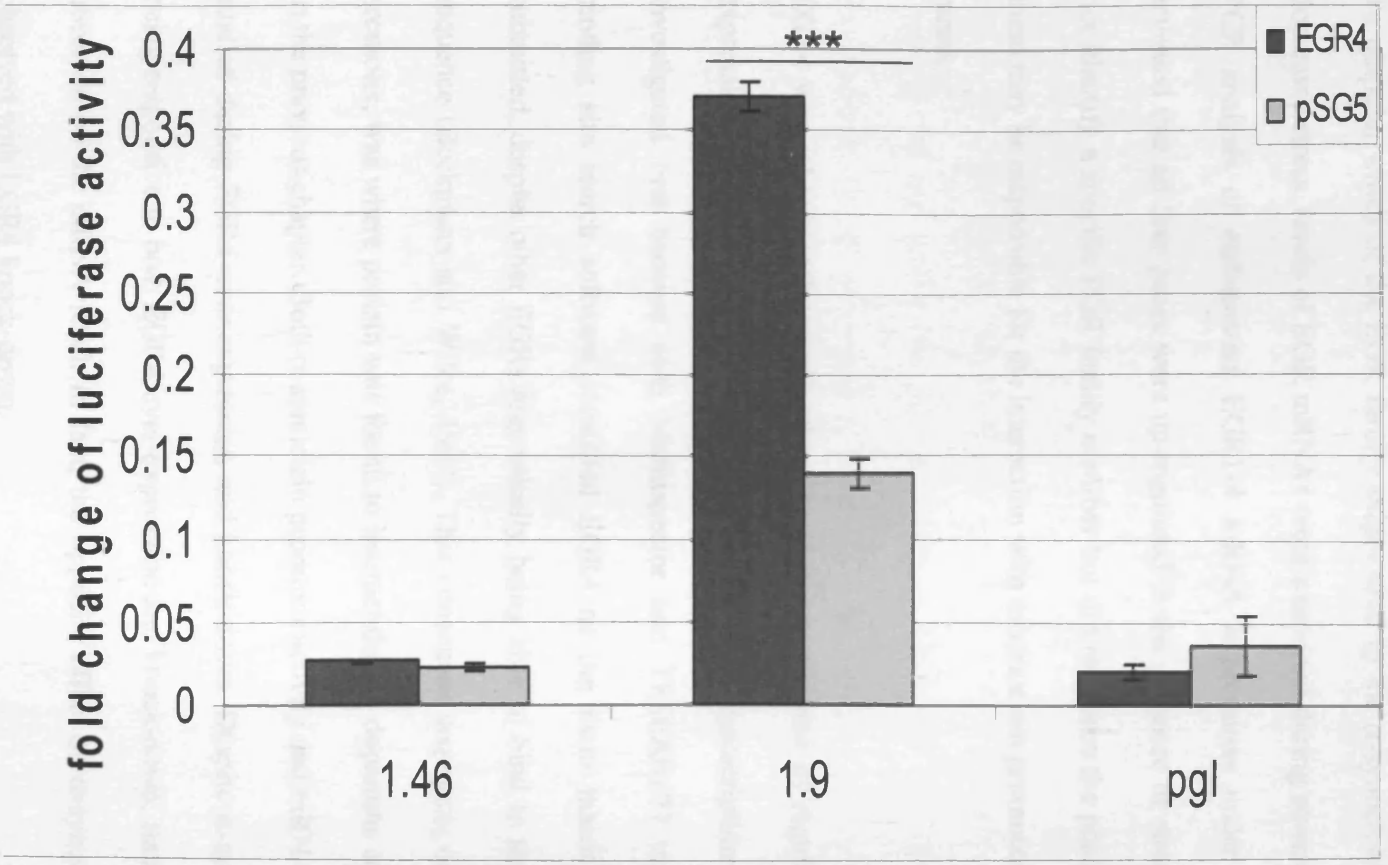


Figure 4.6 Effects of EGR4 over-expression on α-synuclein promoter activity
Cells were transfected with 1.46, 1.9 α-synuclein promoter construct or PGL3 empty construct and EGR4 overexpressing construct or empty pSG5 construct. Cells co-transfected with 1.9 α-synuclein promoter construct and EGR4 overexpressing constructs had significantly higher luciferase activity compared to their counterpart with empty pSG5 constructs (***$p<0.001$). Data was analysed by paired T-test.

4.3 Discussion

To pinpoint which of the EGR family might bind to the α-synuclein promoter during dopamine stress, levels of EGR mRNAs were examined during stressed conditions. RT PCR analysis of endogenous EGR1-4 mRNA expressions under dopamine stress revealed that all four genes were up-regulated in the presence of stress. This result did not identify a specific EGR family member but did reinforce the possibility that one of them may be responsible for the interaction with α-synuclein promoter during dopamine stress.

EGR4 was subsequently examined to see if it was capable of regulating α-synuclein expression during dopamine stress. EGR4-driven transcription regulation was investigated first because both MatInspector and TFSEARCH transcription factor binding site search software identified EGR4 as the main match to the sequence submitted, despite other EGRs theoretically being able to bind to the same consensus sequence (Beckmann and Wilce, 1997). This consensus sequence, on the α-synuclein promoter, was where protein was found to interact during dopamine stress, as discussed in the previous chapter. Both α-synuclein promoter activity and mRNA expression were studied during EGR4 over-expression and knock-down. Despite α-synuclein promoter being responsive to both EGR4 over-expression and knock-down, such effects were not observed on the mRNA level. In fact, the opposite effect on α-synuclein mRNA was observed with EGR4 knock-down.

The two experimental approaches, RTPCR and promoter reporter analysis, produced conflicting results. Due to the limitation of transfection efficiency, only a portion of cells in the total sample examined by RTPCR would have been transfected with EGR4 siRNA or EGR4 over-expressing constructs. Since the transfected cells could not be specifically selected, the extent of the modulation on α-synuclein expression may be understated. In this regards, luciferase reporter assays allow for the selection of transfected cells, since the cells producing α-synuclein promoter-regulated luciferase were also co-transfected with EGR4 siRNA or EGR4 over-expressing construct. Thus the results presented in this chapter indicate towards EGR4 being involved in the regulation of α-synuclein both with and without dopamine. In addition, the promoter reporter analysis results relates to the EMSA results obtained earlier in Chapter 3 section 3.2.3.a, in that, a protein-DNA interaction band was observed with dopamine treatment but not in control.

Similar studies on the effects of EGR1-3 on α-synuclein expression and promoter activity using short-interference RNA knockdown and also over-expression of each of the EGR1-3 genes would be the next step in this study. Subsequently, supershift assays could also be carried out using antibodies specific for the EGR gene. This would confirm the identity of the interacting EGR protein and also if the regulational effects produced on α-synuclein by the transcription factor is direct.

Chapter 5: Regulation of α-synuclein expression by Nurr1 (NR4A2)

5.1 Introduction

5.1.1 Early Growth Response Nurr1 (NR4A2)

Nurr1 *(NR4A2)* is a transcription factor which belongs to the orphan nuclear receptor superfamily and is also categorized under immediate-early genes, whose expression and activity are regulated in a cell-specific manner by a variety of extracellular mitogenic, apoptotic and differentiation stimuli (Jankovic et al., 2005).

Le et al. (Le et al., 2003) were the first to report two mutations (–291Tdel and –245T/G) in the 5'-untranslated region (UTR) of the Nurr1 gene in families with autosomal dominant Parkinson's disease. Since then, only four additional mutations (-253C/T, -223C/T, Ser125Cys and -309C/T) have been discovered, indicating that Nurr1 mutations are rare (Abou-Sleiman et al., 2008; Grimes et al., 2006; Hering et al., 2004; Tan et al., 2004b; Wellenbrock et al., 2003). To date, functional data have only been obtained for 3 of the 6 mutations (–291Tdel and –245T/G and -309C/T) (Abou-Sleiman et al., 2008; Le et al., 2003). The 3 mutations studied showed decreased Nurr1 expression levels *in vitro* and in patient lymphocytes or brain. In addition, α-synuclein-rich lewy bodies were also found in the brain of a patient with the -309C/T mutation (Abou-Sleiman et al., 2008). So far however, no functional link between Nurr1 and α-synuclein has been reported.

Only a few Nurr1 regulated genes have been identified so far (Hermanson et al., 2006; Luo et al., 2007; Sacchetti et al., 2001; Sakurada et al., 1999; Volpicelli et al., 2007), mostly due

to the difficulty in the study of the regulatory functions of Nurr1 as knock-out mice fail to develop midbrain dopaminergic neurons altogether. In addition to being a transcription factor, Nurr1 is also classified as an immediate early gene due to its rapid induction in response to stimuli such as growth factors, ischemia, seizures and inflammation (Crispino et al., 1998). These factors have led us to consider the possibility of Nurr1 involvement in the regulation of α-synuclein. α-synuclein is another gene strongly associated with Parkinson's disease, and can be regulated in response to stress, especially oxidative stresses, and subtle changes in cellular levels could lead to genesis of Parkinson's disease (Abou-Sleiman et al., 2006b). This hypothesis was further supported by a study by Baptista et.al., which showed that the expression of Nurr1 in cells decreased in response to transfection with either WT or mutant α-synuclein, *in vitro* (Baptista et al., 2003).

In the previous two chapters, the effect of oxidative stress on α-synuclein expression has been characterised and one of the EGR family of proteins was identified as a possible trans-acting factor. However, in a further study of additional transcription factors with known association with either Parkinson's disease (Nurr1) or neuronal tissue (Brn3) was prepared. This selection was used to examine their possible role in the regulation of α-synuclein expression, in relationship to the healthy and diseased states. In this chapter the regulation of α-synuclein by Nurr1 was examined.

5.1.2 Hypothesis & Aims

Le et al. 2003 demonstrated that the pathogenicity of the two Nurr1 5' untranslated region (UTR) mutations they found mainly lay in expression changes (Le et al., 2003). As such, this study was initiated to examine if the newly found 5' UTR -309C/T Nurr1 mutation, together with other Parkinson's disease pathogenic signatures, also lead to similar alterations in expression level. In addition, since Nurr1 functions as a transcription factor, and changes in its expression as well as sequence mutations can lead to Parkinson's disease, this study was extended to include investigations into whether Nurr1 might regulate α-synuclein.

Aims of project:

6. To investigate if Nurr1 expression was altered by the -309C/T mutation

7. To then examine if the difference in expression of Nurr1 would in turn affect the expression of its target genes

8. To investigate if Nurr1 could transcriptionally regulate α-synuclein

5.2 Results

5.2.1 *In vitro* analysis of -309C/T Nurr1 mutation

5.2.1.a Quantitation of -309C/T Nurr1 mRNA

SH-SY5Y cells were transfected with wild type (WT) and mutant Nurr1 cDNA and real-time PCR methods were used to compare the mRNA expression *in vitro* of -309C/T Nurr1 cDNA with mRNA levels from pCMX vector, WT Nurr1 and the -245T/G Nurr1 mutant (previously reported by Le et al. 2003 (Le et al., 2003)). Co-transfection of a GFP reporter with Nurr1 constructs, in parallel wells, revealed no difference in transfection efficiency between constructs (Fig. 5.1). The identity and specificity of the product of each RT PCR was confirmed after the experiments (Fig 5.2)

The mRNA expression level of the -245T/G Nurr1 mutant was significantly reduced by approximately 80-90% compared to WT, in agreement with a previous study (Le et al., 2003). We observed an approximate 50% reduction in mRNA expression of the -309C/T Nurr1 mutant that was significantly different from both the WT and the -245T/G Nurr1 mutant respectively (Fig. 5.3).

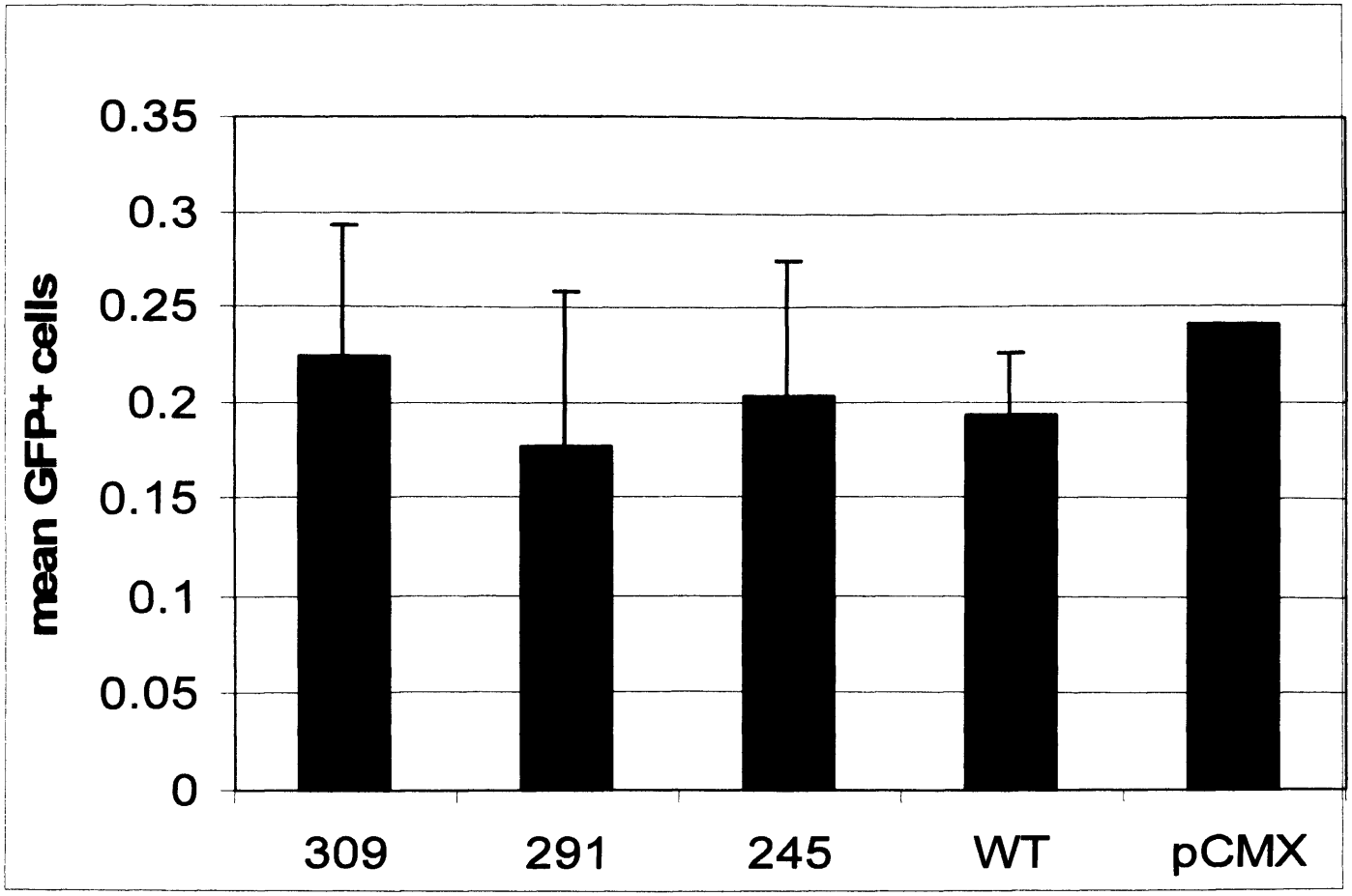


Figure 5.1 Transfection efficiency of Nurr1 constructs
Mean GFP positive cells shown corrected to pCMX. The cells were co-transfected with Nurr1 (WT/mut) construct, POMC promoter luciferase fusion construct, sv40 renilla construct and GFP construct. 309 and 245 are -309C/T and -245T/G mutants of N*urr1*, and pCMX indicates the empty vector. There were no significant differences in transfection efficiencies.

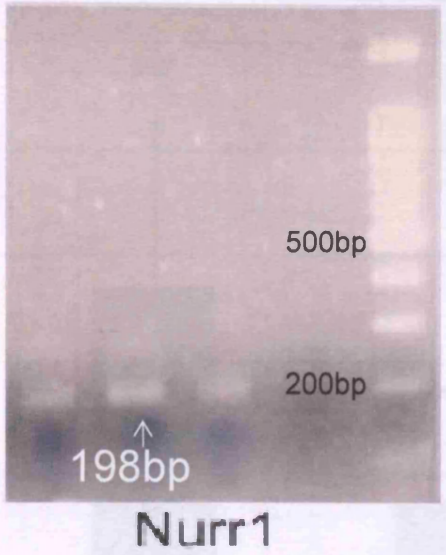


Figure 5.2 RT PCR Nurr1 product agarose gel check
Agarose gel check on RT PCR Nurr1 product to confirm product identity and PCR specificity. Specific product of the correct size was obtained. Amplified product was confirmed by sequencing to be Nurr1.

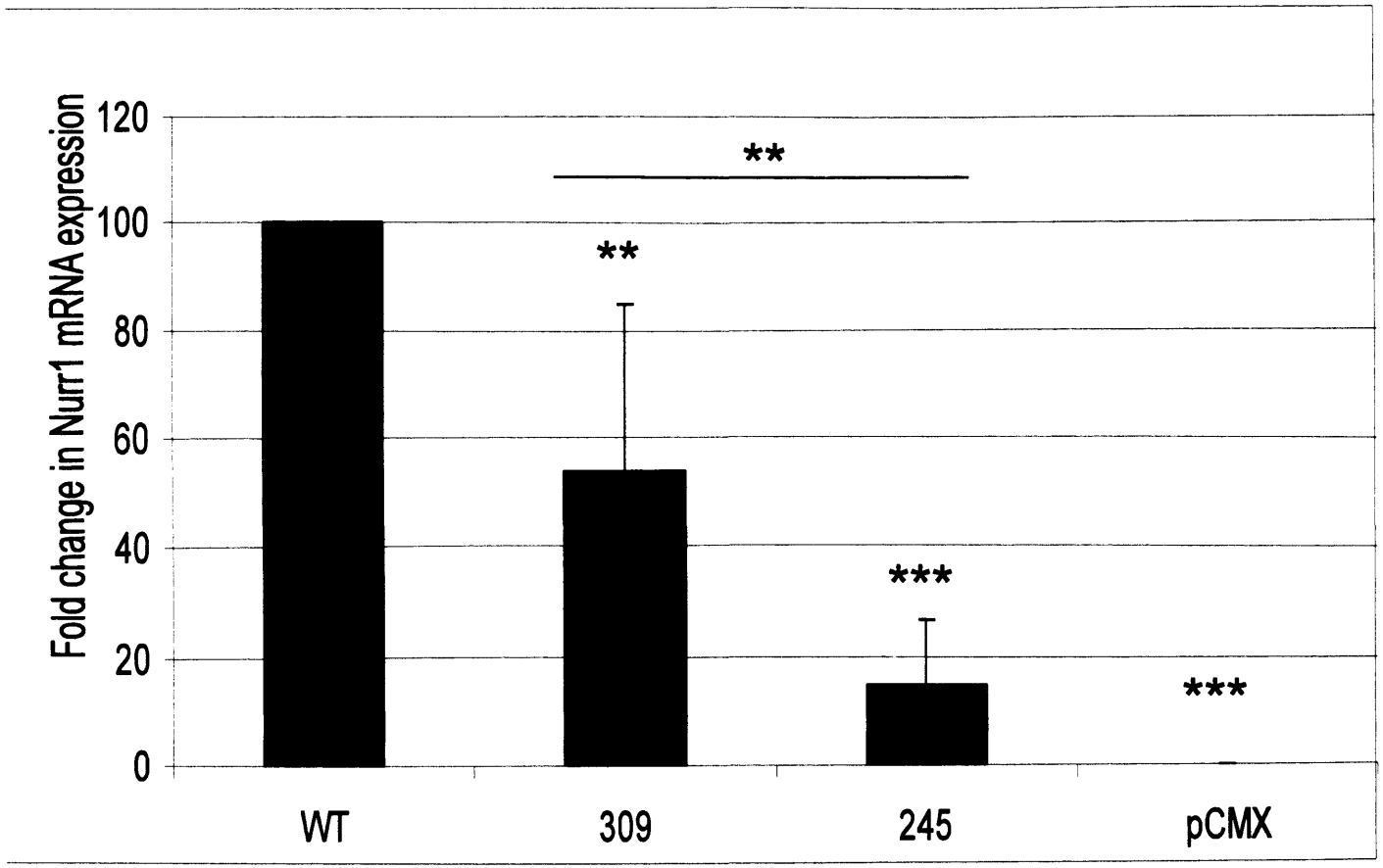


Figure 5.3 mRNA expression of WT, -309C/T and -245T/G Nurr1 constructs
Mean mRNA expression shown as a percentage of wild type (WT) Nurr1 construct (100%) co-transfected in SH-SY5Y cells, with POMC promoter luciferase fusion construct and sv40 renilla construct. The error bars indicate the standard error of the mean of 3 experiments in triplicates each. The significance of differences was analysed by ANOVA and Bonferroni post hoc test. WT versus -309C/T or -245T/G (**$p<0.01$, ***$p<0.001$) and -309C/T vs -245T/G (**$p<0.01$)

5.2.1.b -309C/T Nurr1 functional activity

The ~50% decrease in -309C/T Nurr1 mRNA expression was then investigated to determine if it was sufficient to alter its trans-activating properties on downstream target genes. We co-transfected Nurr1 constructs with a construct containing the proopiomelanocortin gene (POMC) promoter that contains tandem NuRE (Nur77 response element) sites fused to luciferase. A SV40 renilla plasmid was also co-transfected to control for variation in transfection efficiency. The luciferase activities induced by the -309C/T and -245T/G Nurr1 mutants were both significantly lower than WT Nurr1 by ~60%, but not significantly different from each other (ANOVA, overall $p < 0.0001$, Fig. 5.4). These *in vitro* data confirmed that the -309C/T mutant was sufficient to affect the trans-activating role of Nurr1 by reducing its expression.

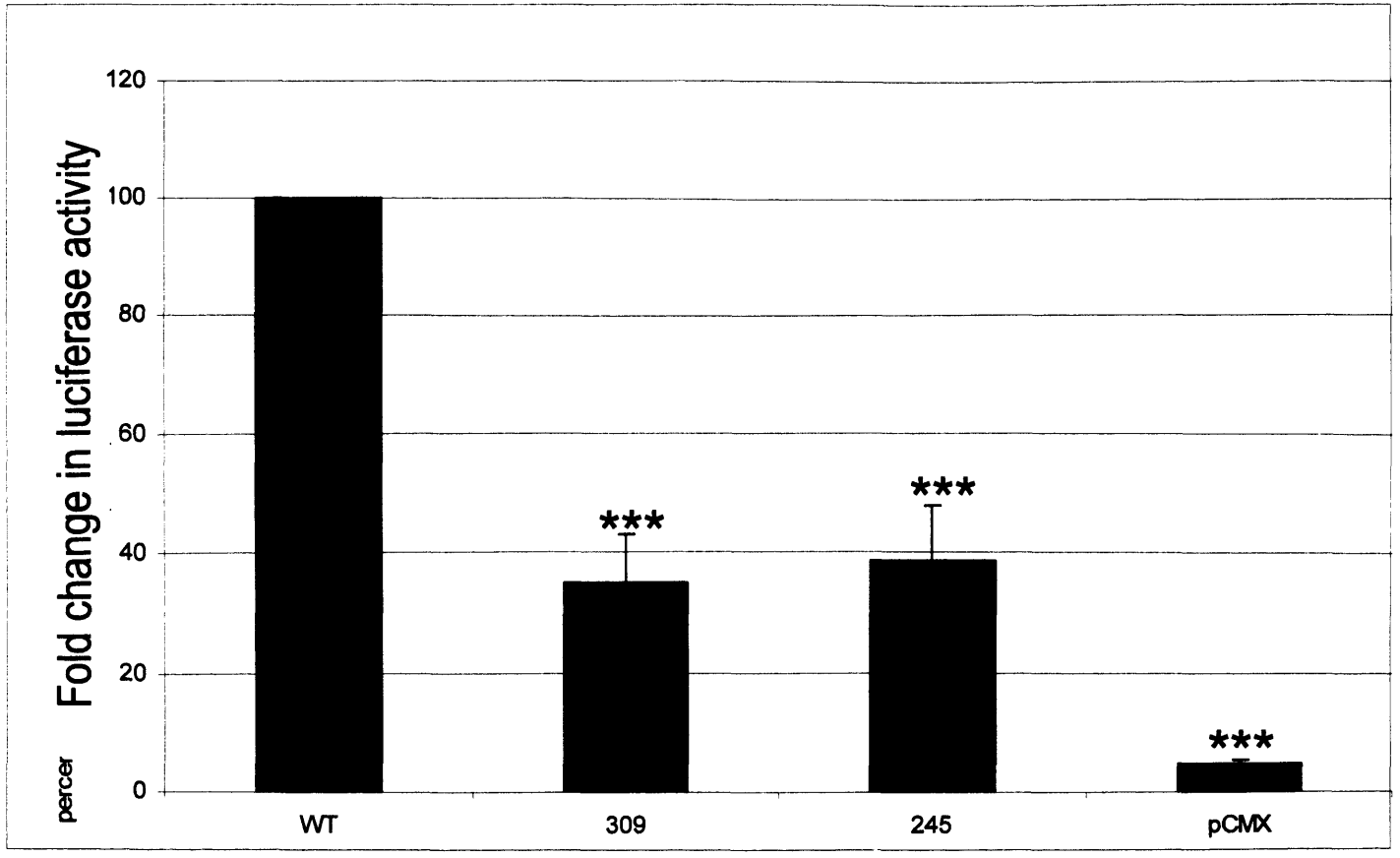


Figure 5.4 Transactivation abilities of WT, -309C/T and -245T/G Nurr1 constructs
Mean luciferase activities shown as a percentage of wild type (WT) Nurr1 construct (100%) transfected in SH-SY5Y cells, with POMC promoter luciferase fusion construct and SV40 renilla construct. The error bars indicate the standard error of the mean of 3 experiments in triplicates each. The significance of differences was analysed by ANOVA and Bonferroni post hoc test. -309C/T vs WT and -245T/G vs WT were both significantly different (***$p<0.001$)

5.2.2 Nurr1 transcriptional regulation of α-synuclein

5.2.2.a Nurr1 regulation on expression of PD associated genes

To mimic the loss of expression caused by 5' UTR Nurr1 mutation as found in some patients with Parkinson's disease, endogenous Nurr1 was knocked-down in SH-SY5Y cells using siRNA targeting *Nurr1*. Analysis by real-time PCR of mRNA levels present in transfected cells, demonstrated siRNA knockdown of endogenous *Nurr1* mRNA by approximately 50% in the cells. This significantly up-regulated endogenous *α-synuclein*, *parkin* and *PINK1* mRNA expressions by 3 fold, 3 fold and 2 fold respectively (Fig. 5.5).

The effect of Nurr1 over-expression was also tested by the transient transfection of Nurr1 expression construct. The over-expression of Nurr1 did not result in any significant change in the expression of endogenous *α-synuclein* and *parkin* transcript levels. A slight decrease in the expression of *PINK1* transcript was observed (Fig. 5.6).

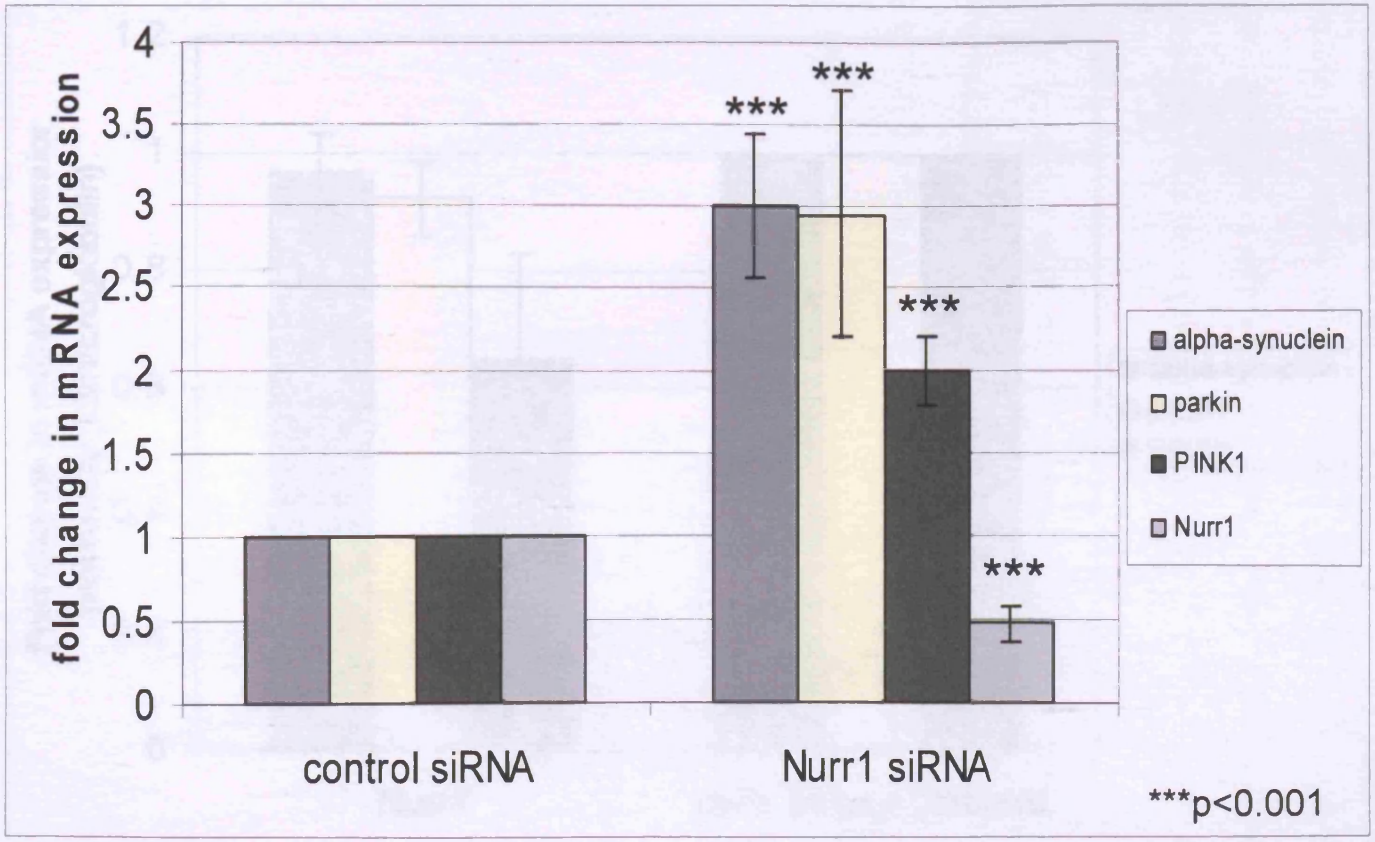


Figure 5.5 Effect of Nurr1 knock-down on Parkinson's disease associated genes
mRNA from SHSY-5Y cells transfected with siRNA targeting Nurr1 mRNA or non-specific mRNA were measured for α-synuclein, parkin, PINK1 mRNA and Nurr1 mRNA using RT-PCR. Nurr1 mRNA levels were knocked-down by ~ 60% with Nurr1 targeting siRNA compared to control. α-synuclein and parkin mRNA levels, in turn, increased 3 fold and PINK1 mRNA levels doubled when Nurr1 was knocked-down.

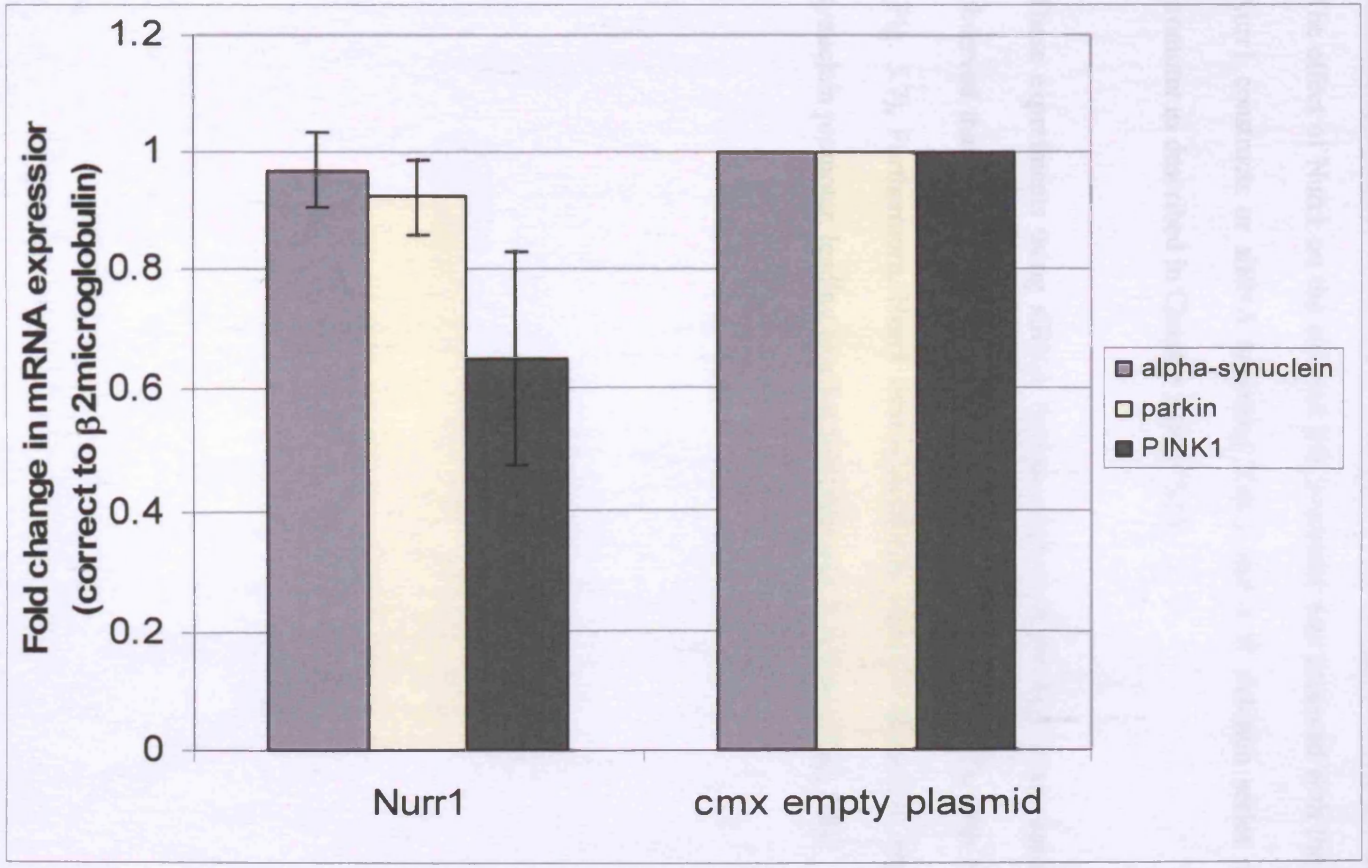


Figure 5.6 Effect of Nurr1 over-expression on Parkinson's disease associated genes
mRNA from SHSY-5Y cells transfected with Nurr1 expressing construct or empty vector were measured for α-synuclein mRNA and Nurr1 mRNA using RT-PCR. The Nurr1 expression construct overexpressed Nurr1 by 15000 fold but this resulted only to modest decreases in α-synuclein, parkin and PINK1 expressions.

5.2.2.b Nurr1 regulation of α-synuclein promoter activity

The effect of Nurr1 on the α-synuclein promoter was assessed with the co-transfection of Nurr1 constructs or siRNA targeting Nurr1 and a 5' deletion series of the α-synuclein promoter as described in Chapter 3 (Fig. 3.5).

These experiments using siRNA further reinforced the data from real-time PCRs. It was observed that α-synuclein promoter activity increased by ~30% with Nurr1 knock-down (Fig. 5.7), Furthermore, Nurr1 over-expression, caused substantial repression to the α-synuclein promoter, leading to a 3-4 fold decrease in activity (Fig. 5.8).

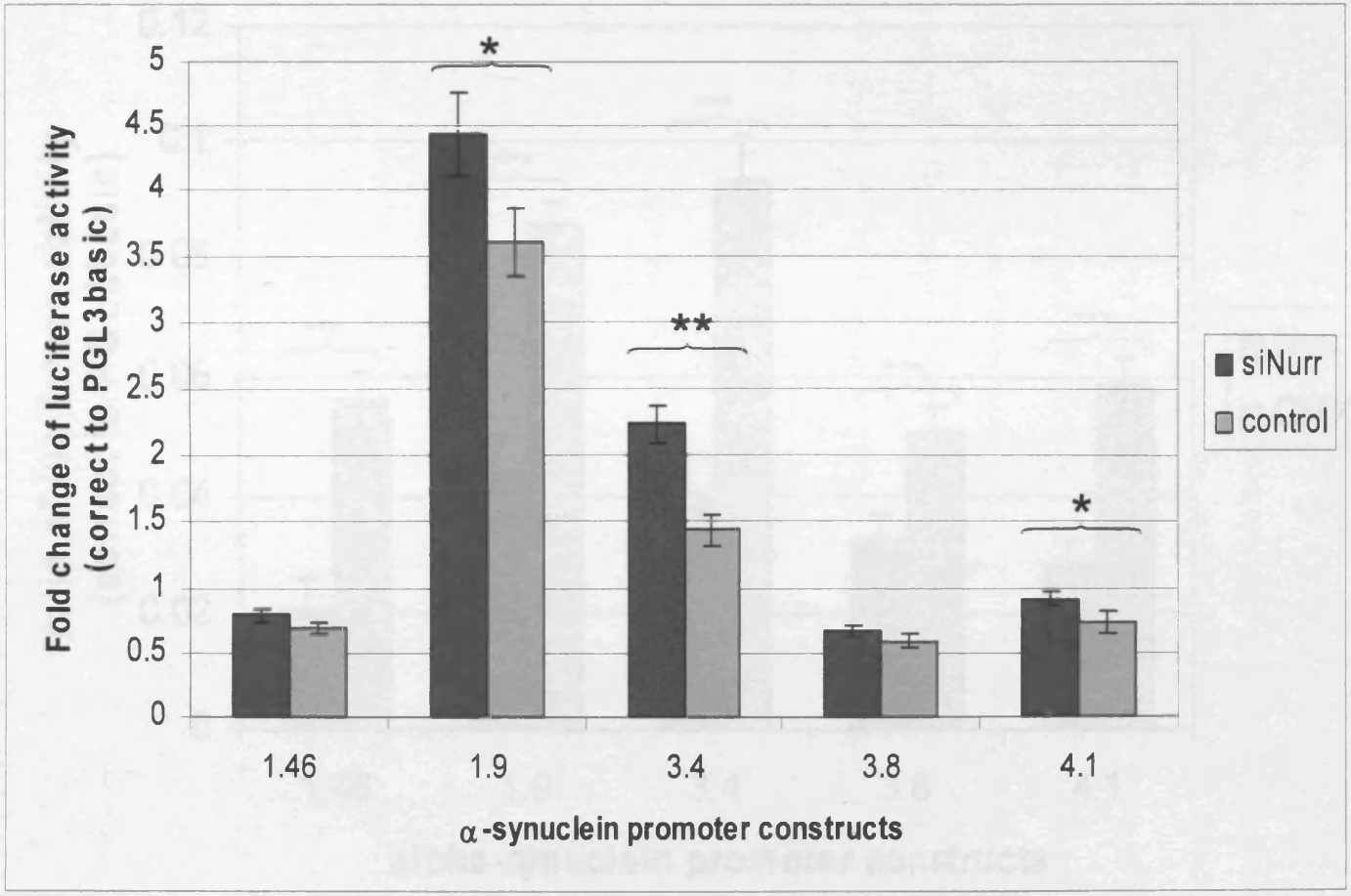


Figure 5.7 Effects of Nurr1 knock-down on α-synuclein promoter activity
SH-SY5Y cells were co-transfected with siRNA targeting Nurr1 or non-specific mRNA and with different α-synuclein promoter constructs and incubated in full culture media for 24hrs. A significant increase in promoter activity was obtained for constructs 1.9, 3.4 and 4.1 (*$p<0.05$, **$p<0.01$).

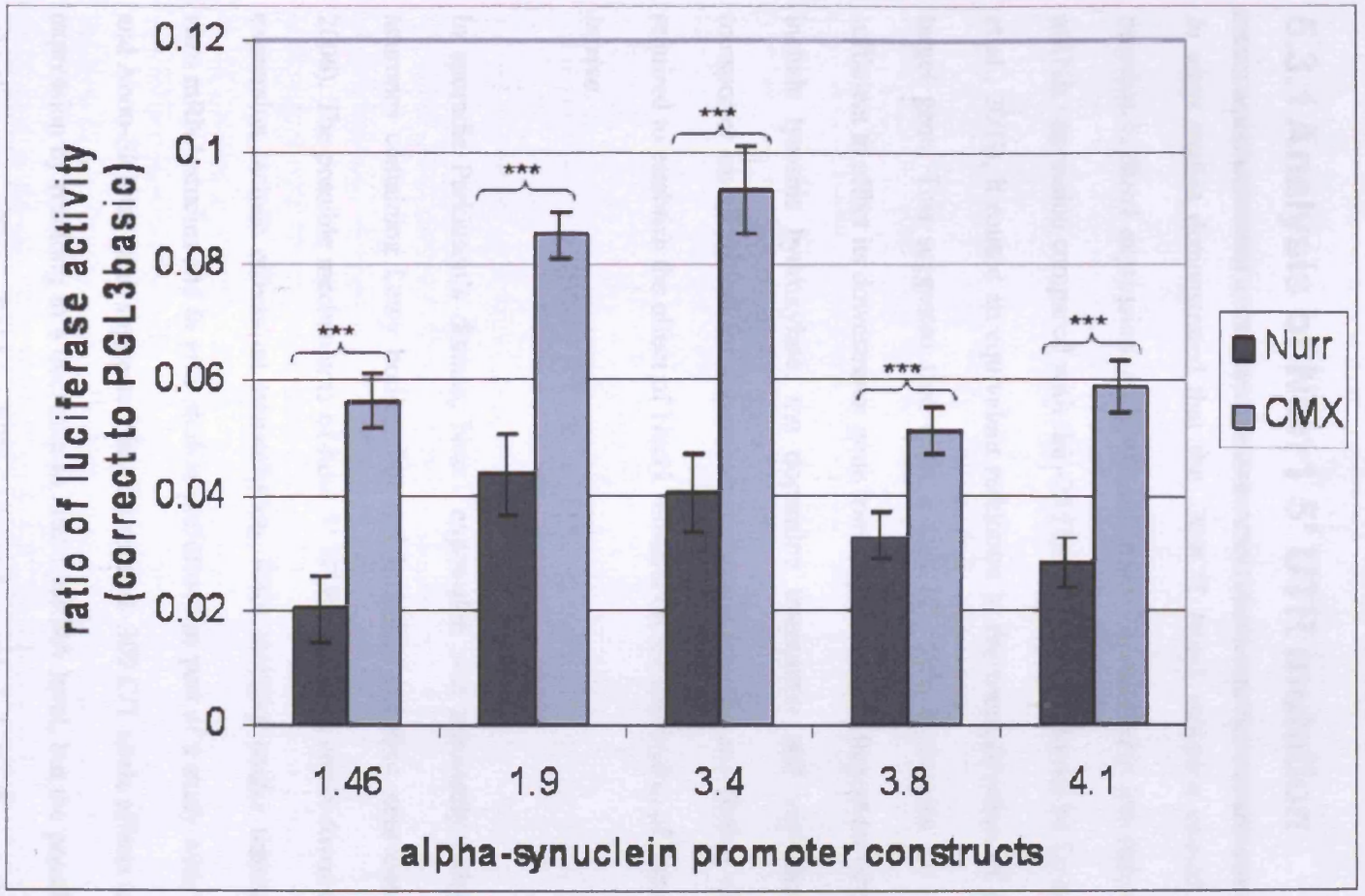


Figure 5.8 Effects of Nurr1 over-expression on α-synuclein promoter activity
Cells were co-transfected with Nurr1 WT or empty vector and with different α-synuclein promoter constructs. Significant decreases in promoter activity were obtained for all the Nurr1 transfected cells compared to control cells (***p<0.001).

5.3 Discussion

5.3.1 Analysis of Nurr1 5' UTR mutation

In vitro studies demonstrated that the -309C/T Nurr1 mutation caused a significant decrease in Nurr1 expression. Although this mutation resulted in less reduction in Nurr1 mRNA expression compared with the -245T/G mutant discovered by Le *et.al.* 2003 (Le et al., 2003), it caused an equivalent reduction in the transactivation of a downstream target gene. This suggested that even a loss of ~50% expression by –309C/T was sufficient to affect its downstream gene transcriptional role. Important targets for Nurr1 include tyrosine hydroxylase, the dopamine transporter and vesicular monoamine transporter are all critical for dopaminergic neuron survival and further work would be required to establish the effect of Nurr1 mutants on the expression of these target genes *in vivo*.

In sporadic Parkinson's disease, Nurr1 expression was reportedly reduced in nigral neurones containing Lewy bodies, but not inclusion negative neurones (Chu et al., 2006). The possible mechanisms of how 5' UTR mutations could downregulate Nurr1 expression include effects on transcription, RNA stability and/or translation. Our *in vitro* mRNA studies and *in vivo* studies performed as part of a study with Healy, Muqit and Abou-Sleiman et.al. suggested that the mutant -309 C/T allele affects transcriptional expression by resulting in a decrease in Nurr1 mRNA level, but the possibility that the mutation may affect mRNA stability cannot be excluded. Further studies would be required to elucidate the functional role of the 5' UTR region of Nurr1 (Abou-Sleiman et al., 2008).

5.3.2 Nurr1 regulation of α-synuclein expression

To simulate Nurr1 mutations found in Parkinson's disease patients, which result in reduction of Nurr1 levels, Nurr1 was down-regulated using Nurr1 targeted siRNA. This resulted in a significant increase in α-synuclein expression. Furthermore, activity of α-synuclein promoter constructs increased with decreased Nurr1. These results extend substantial research done on both genes so far on the pro- and anti-Parkinson's disease properties of Nurr1 and α-synuclein respectively. Heterozygous Nurr1 knock-down mice showed increased sensitivity to stresses such as MPTP (Le et al., 1999) whilst α-synuclein was found to be increased in the presence of such stresses (Abou-Sleiman et al., 2006b). Moreover, decreased functional Nurr1 results in the genesis of Parkinson's disease (Le et al., 2003) while increased α-synuclein, due to factors such as multiplication of the gene locus, also result in Parkinson's disease (Eriksen et al., 2005).

To investigate if the reverse were true, Nurr1 was overexpressed in SH-SY5Y cells and the endogenous expression of α-synuclein as well as its promoter activity was examined. Endogenous α-synuclein showed a modest decrease in expression with Nurr1 overexpression, whereas its promoter constructs were strongly repressed by Nurr1 increase. Several factors could explain this discrepancy, e.g. constitutive levels of Nurr1 could function to invoke a maximal suppression of α-synuclein gene expression, thus, any further increase in Nurr1 expression does not cause a further repression whereas its removal lifts the blockade and allows α-synuclein expression. It could also be due to the efficiency of transient transfection, with only 40% of the cells over-expressing Nurr1 (result not shown). As the effect of increased Nurr1 is small compared to constitutive levels of Nurr1 already present, this renders the change in α-synuclein expression

insignificant. Nonetheless, the α-synuclein promoter luciferase assays showed that Nurr1 was able to repress the promoter.

Concurrently, electrophoretic mobility shift assays were also used to assess any possible binding of Nurr1 to the α-synuclein promoter from the core promoter region to the translation start site which is Nurr1-responsive. No interactions were detected, suggesting that as in dopamine transporter gene, Nurr1 acts indirectly and not via a specific binding site.

Here, we have shown for the first time, linkage between the transcriptional activity of Nurr1 and the regulation of α-synuclein. Further studies are needed to investigate whether an increase in α-synuclein is the cause of Parkinson's disease in patients with mutations causing decreased Nurr1 activity or whether other effects of Nurr1 are also involved.

Chapter 6: Regulation of α-synuclein expression by Brn3 transcription factors

6.1 Introduction

Following on from the α-synuclein promoter regulation study (Chapters 3 and 4), an investigation of transcription factor regulation of α-synuclein was performed. A selection of candidate transcription factors were studied including Nurr1 (Chapter 5) and, in this chapter, Brn3. Additionally, the relationship between Nurr1 and Brn3 as regards α-synuclein was investigated.

6.1.1 Brn3 family of transcription factors

Brn3 subfamily of factors, consisting of Brn3a, Brn3b and Brn3c, belong to the POU (Pit-Oct-Unc) family of transcription factors. These three factors are transcribed from separate genes and they all contain a conserved POU domain, but otherwise show little homology (Latchman, 1999).

Brn3a is important for the differentiation and survival of sensory as well as motor neurons. Knock-out Brn3a mice suffer from severe neuronal developmental defects and are usually non-viable (McEvilly et al., 1996). Brn3a exists as two forms, the short and long form, which are generated by alternative splicing at the 5' end of *Brn3a* RNA (Theil et al., 1993). The presence of a longer N-terminal in Brn3a long enables it to activate not only genes such as neurofilamnets and SNAP-25, activated by the POU domain, but also others, such as α internexin and the protective Bcl-2 by its additional N-terminal activation domain. Brn3a long has, in consequence, been found to have a protective effect on neurons against

apoptosis (Smith et al., 1997a; Smith et al., 1997b). Although Brn3a has not been previously associated with any disease states, its neural protective properties make it an interesting factor to study for potential regulation of anti-Parkinson mechanisms, since the development of Parkinson's disease entails the enhanced apoptosis of specific neurons, leading to loss of neurological function (Latchman, 1998).

Brn3b and Brn3c are involved mainly in the differentiation and maintenance of retinal and vestibular neurons of the inner ear respectively. Although neither Brn3b nor c appear to cause any other neurological defects other than in retinal and vestibular neurons respectively and seems unlikely to be involved in survival of neurons important in Parkinson's disease, Brn3b being known to be antagonistic (Latchman, 1999) with Brn3a, makes it worth examining alongside Brn3a.

6.1.2 Hypothesis & Aims

In view of the importance of the Brn3a transcription factor in the survival of motor neurons and its role in protecting neurons against apoptosis, we were interested to characterise the properties of Brn3a in relation to the progression and development of Parkinson's disease. The first step was to investigate the regulation effects of Brn3a on α-synuclein. This was followed by studying the transcriptional effect of Brn3a on Nurr1, another Parkinson's disease associated gene. Brn3b was also included in the experiments to assess any antagonistic effects to Brn3a.

Aims of project:

9. To investigate if Brn3a and b could regulate α-synuclein expression

10. To investigate if Brn3a and b could regulate Nurr1 expression

11. If regulation was established for the first two aims, to further probe into the link between Brn3, Nurr1 and α-synuclein

6.2 Results

6.2.1 Brn3 regulation of α-synuclein expression

6.2.1.a Presence of Brn3 in brain region important in Parkinson's disease

To first establish if Brn3a and Brn3b may indeed be important in Parkinson's disease, PCR detection of Brn3a and Brn3b was carried out in 8 different cDNA samples obtained from control human substantia nigra region of brains (Fig. 6.1). Brn3a but not Brn3b was detected in this region of the human brain.

6.2.1.b Effect of Brn3 on endogenous α-synuclein expression

Although only Brn3a and not Brn3b was detected in the substatia nigra of human brains, Brn3b was still included in subsequent experiments as other regions in the brain are also affected in Parkinson's disease and the antagonistic effects of Brn3b on Brn3a may reveal interesting regulation control of α-synuclein.

To investigate if Brn3 might influence endogenous α-synuclein expression, α-synuclein mRNA was quantified, using RT PCR, from SH-SY5Y cells transfected with constructs expressing different forms of Brn3, Brn3a short, Brn3a long, Brn3b short and Brn3b long (Fig. 6.2). Normalisations were performed to housekeeping genes, β2-microglobulin and β-actin, with similar results.

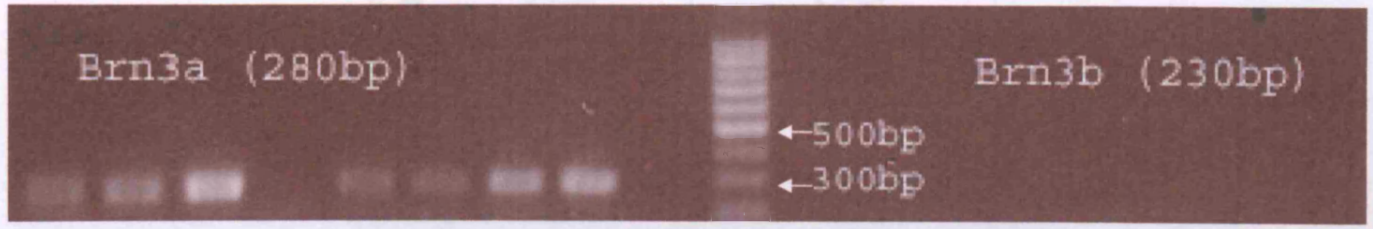


Fig. 6.1 PCR detection of Brn3a and b in substantia nigra brain region
Gel check on PCR amplification of Brn3a and Brn3b in 8 cDNA samples from human substantia nigra regions. Brn3a but not Brn3b was amplified from the samples.

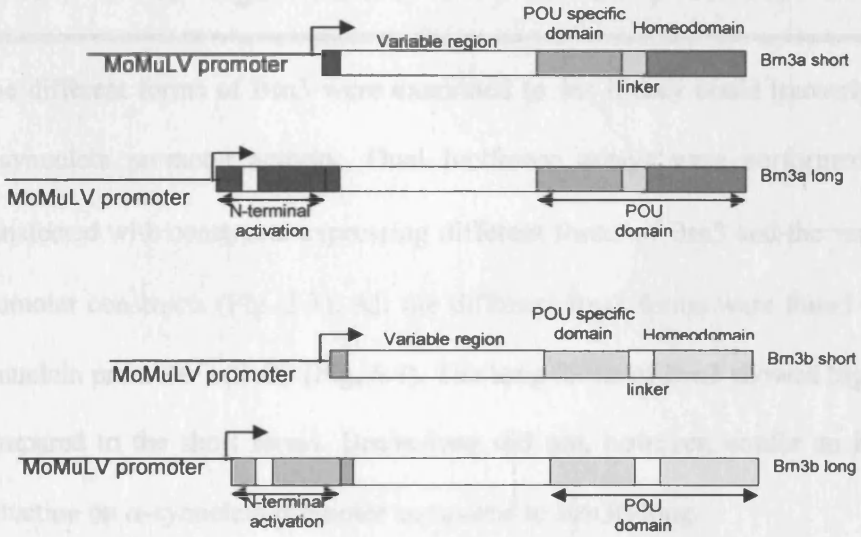


Figure 6.2 Schematic diagram of Brn3a and b isoforms in LTR plasmid
Brn3a and b long constructs contain Brn3a and b long cDNA sequences and Brn3a and b short contain Brn3a and b short gene sequences inserted into the LTR plasmid

Brn3a-short, a-long and b-long were found to increase α-synuclein expression significantly (Fig. 6.3). Brn3a-short and Brn3b-long caused similar increases in α-synuclein expression by 1.4-1.5 fold. Brn3a-long was shown to have the greatest effect on α-synuclein expression, causing a 2 fold increase. No change in α-synuclein expression was observed with Brn3b-short.

6.2.1.c Brn3 regulation of α-synuclein promoter activity

The different forms of Brn3 were examined to see if they could transcriptionally regulate α-synuclein promoter activity. Dual luciferase assays were performed using cells co-transfected with constructs expressing different forms of Brn3 and the various α-synuclein promoter constructs (Fig. 3.3). All the different Brn3 forms were found to up-regulate α-synuclein promoter activity (Fig. 6.4). The long forms of Brn3 showed higher up-regulation compared to the short forms. Brn3a-long did not, however, confer an increased level of induction on α-synuclein promoter compared to Brn3b-long.

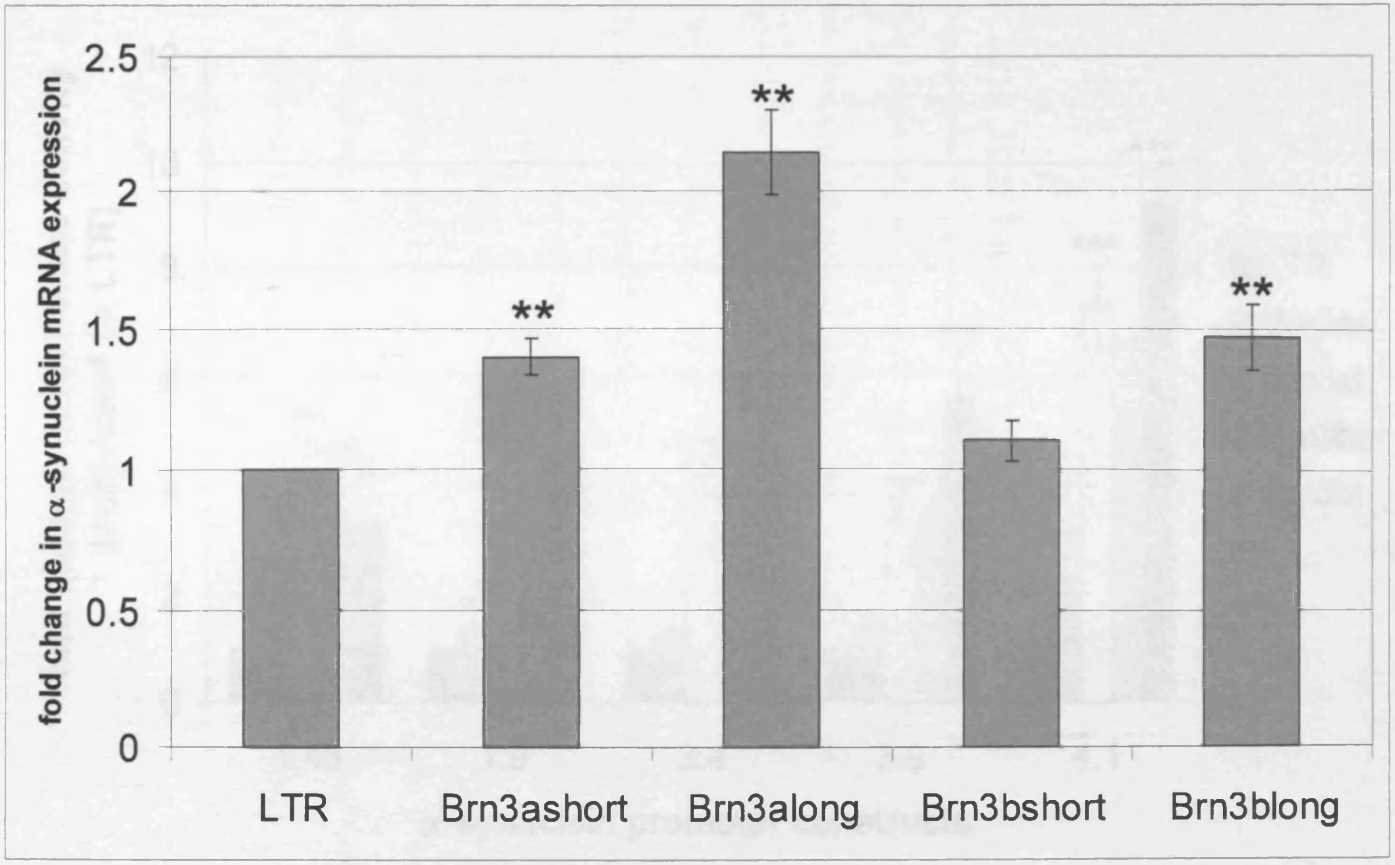


Figure 6.3 Effects of Brn3a and b isoforms over-expression on α-synuclein mRNA expression
Cells were transfected with control LTR construct, brn3a-short, a-long, b-short or b-long constructs. α-synuclein mRNA expression in cells transfected with brn3a-short, a-long and b-long were significantly higher than those transfected with LTR or brn3b-short (** $p<0.01$). Cells transfected with Brn3a-long had α-synuclein mRNA levels significantly higher than all the other samples. Data was analysed by paired T-test.

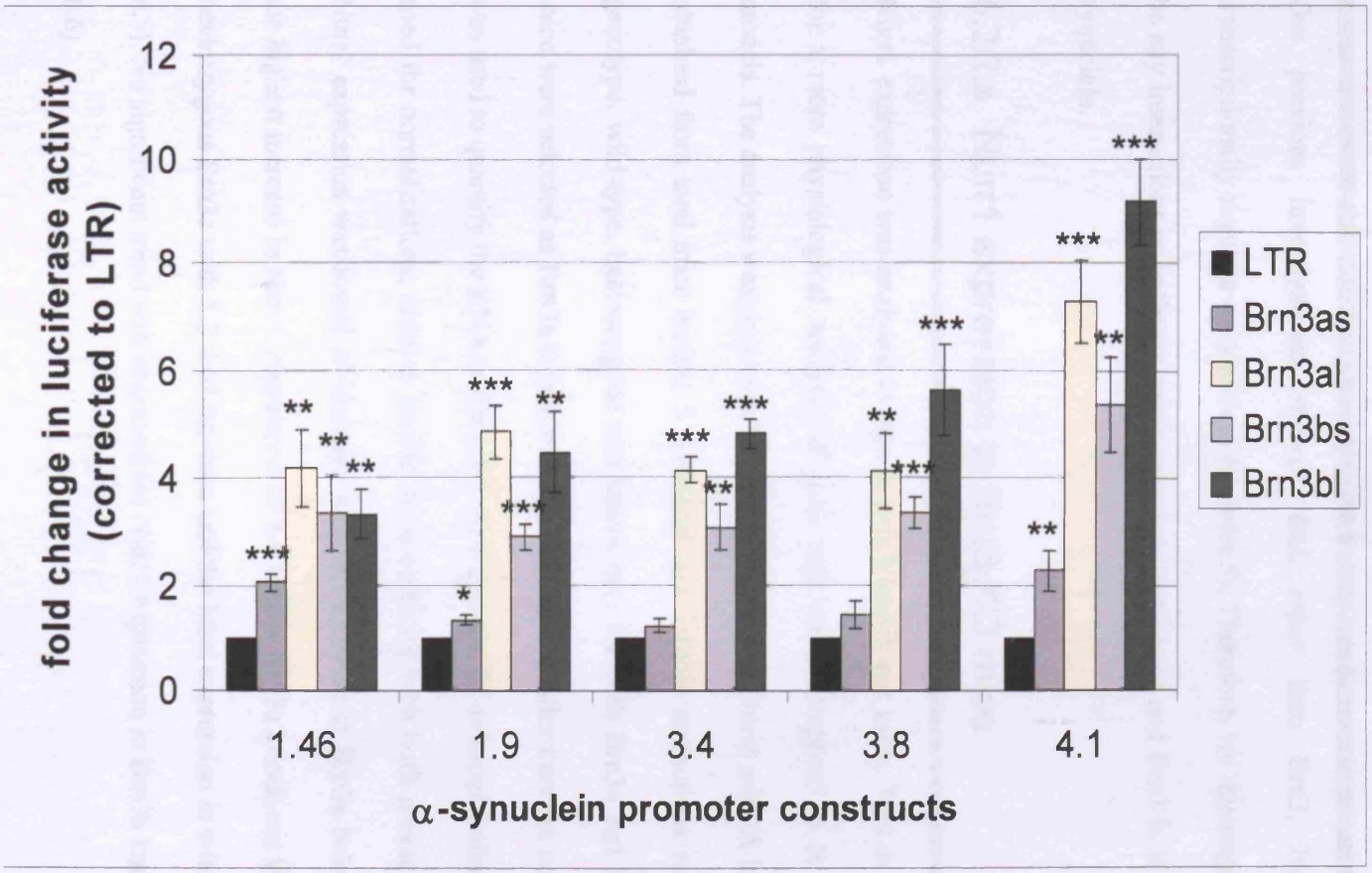


Figure 6.4 Effects of Brn3a and b isoforms over-expression on α-synuclein promoter activity
Cells were co-transfected with control LTR construct, brn3a-short, a-long, b-short or b-long constructs and with different α-synuclein promoter constructs. Significant increases in promoter activities were obtained when cells were co-transfected with a brn3 construct compared to LTR construct for almost all the α-synuclein constructs (*p<0.05; **p< 0.01; ***p<0.001).

6.2.2 Brn3 regulation of Nurr1 expression

Our previous investigations showed that, other than Brn3, Nurr1 could also transcriptionally regulate α-synuclein (chapter 5). Therefore, we investigated if there might be any interactions or pathway cross over between Nurr1 and Brn3 in the regulation of α-synuclein.

6.2.2.a Nurr1 expression in Brn3 KO mice

Nurr1 expression was analysed in Brn3a and b knock-out mice. This *in vivo* study allows for a more physiological analysis of gene regulation compared to *in vitro* cell culture models. The analysis was performed through quantifying Nurr1 mRNA in RNA extractions obtained from total mice brains. 5 postnatal day 1 brain extractions were done for each genotype, wild-type, heterozygous and knock-out, for both Brn3a and b. Postnatal day 1 mice were selected as Brn3a knock-out mice die shortly after they are conceived. RT PCR was used to quantify the RNA and housekeeping genes, β2-microglobulin and β-actin, were used for normalisations, similar results were obtained with both means of normalisation. Nurr1 expression was found to increase with the decrease in Brn3a being expressed, with the highest increase in Nurr1 expression of 1.6 fold in Brn3a knock-out brains, followed by heterozygous Brn3a with 1.2 fold increase and the least expression in wild-type brains (Fig. 6.5). No significant trend was observed for Nurr1 expression in Brn3b knock-out mice (Fig. 6.6).

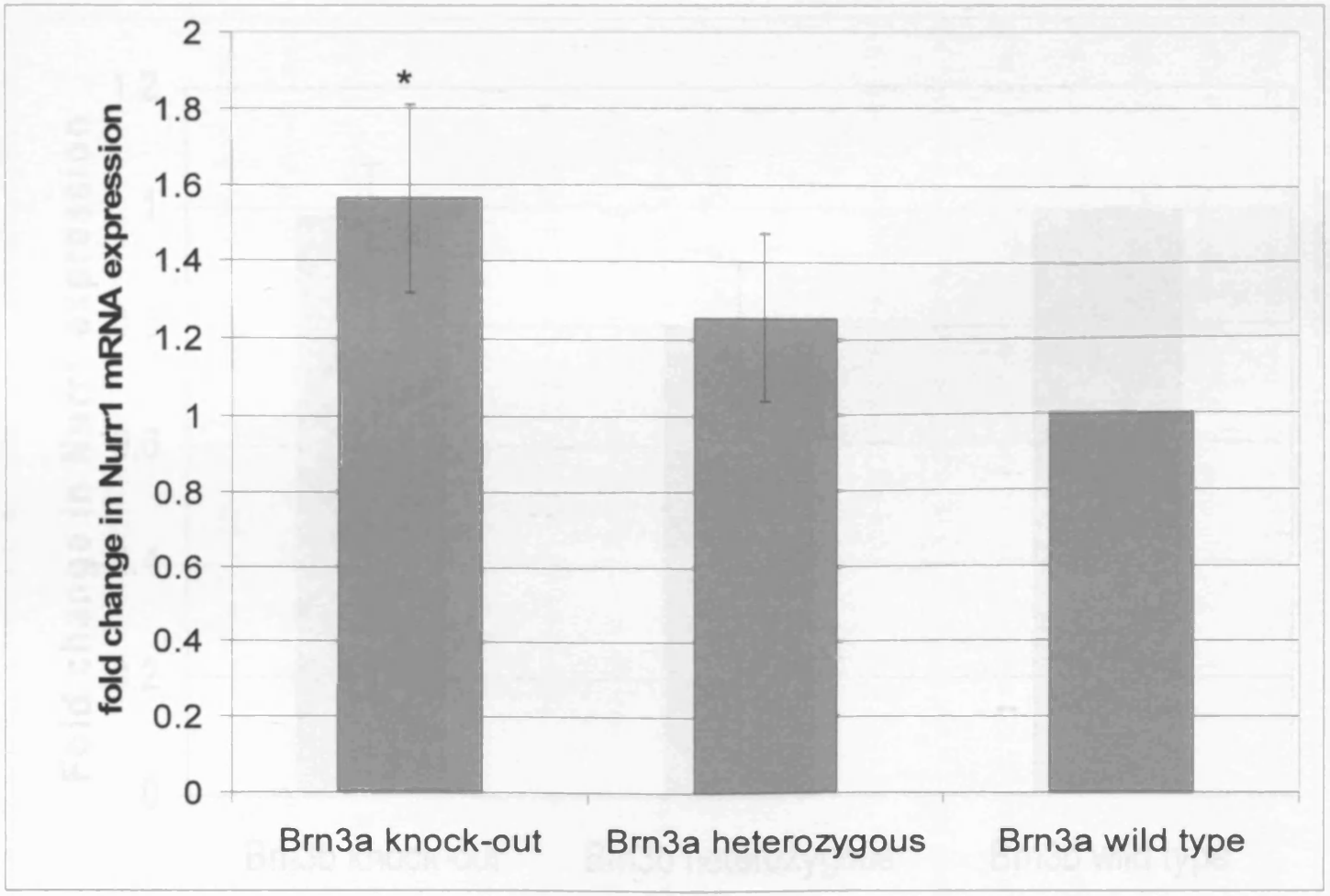


Figure 6.5 Nurr1 mRNA expression in Brn3a KO mice
Nurr1 mRNA was quantified in RNA extractions from 9 Brn3a mice, 3 for each genotype. Nurr1 expression increased with decrease in Brn3a dosage, and was significantly higher in Brn3a knock-out mice compared to the other 2 genotypes (*$p<0.05$).

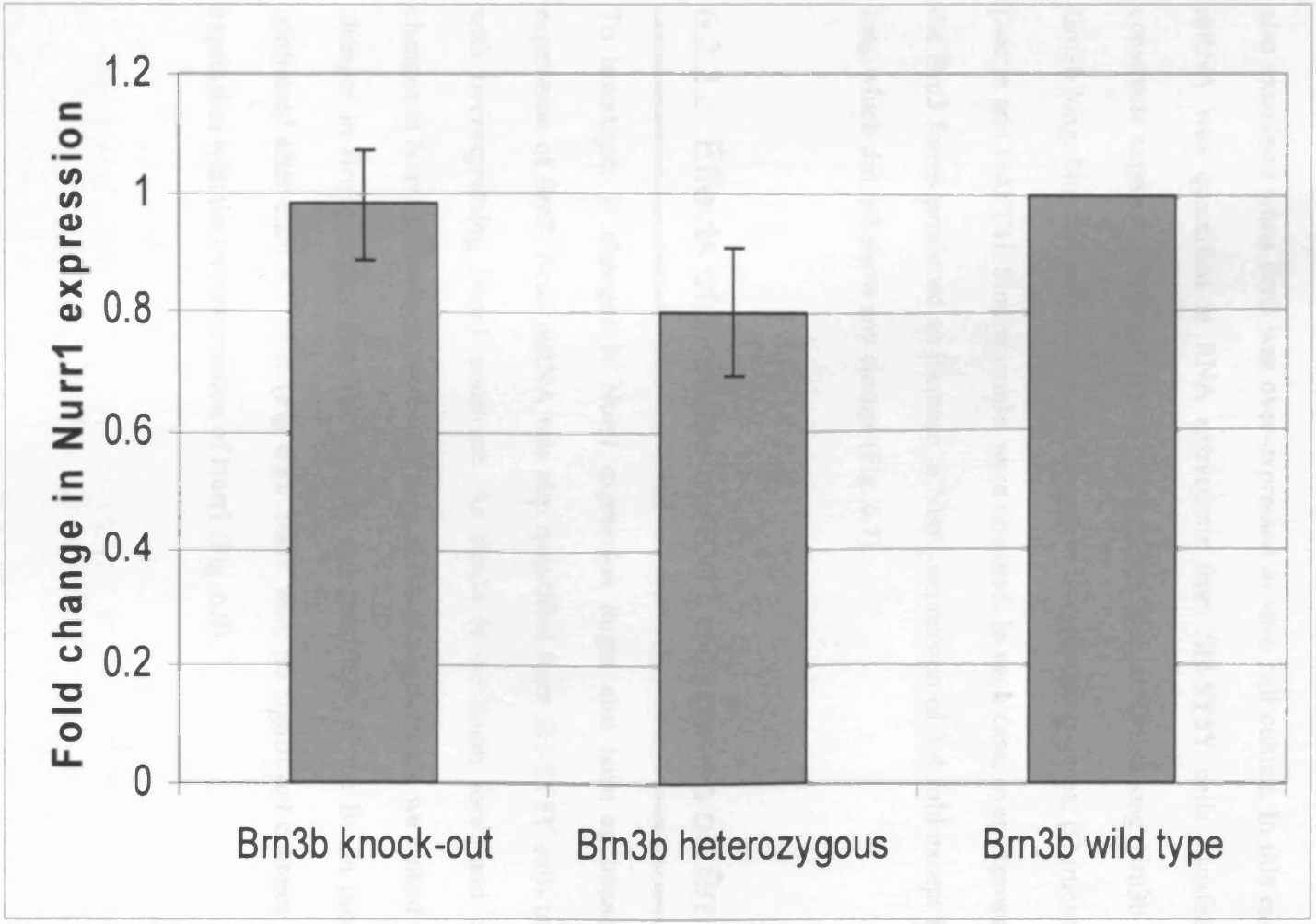


Figure 6.6 Nurr1 mRNA expression in Brn3b KO mice
Nurr1 mRNA was quantified in RNA extractions from 15 Brn3b mice, 5 for each genotype. No significant trend in Nurr1 expression was observed.

6.2.2.b Effects of Brn3 overexpression on Nurr1

To follow on the results obtained from the Brn3 knock-out mice, Nurr1 expression was also examined when Brn3 was over-expressed *in vitro* cell culture. In this case, Nurr1 mRNA was quantified in RNA extractions from SH-SY5Y cells transfected with constructs expressing different forms of Brn3, Brn3a short, Brn3a long, Brn3b short and Brn3b long. Normalisations were performed to housekeeping genes, β2-microglobulin, β-actin and GAPDH. Similar results were obtained. In each case, over-expression of all the Brn3 forms produced an increase in Nurr1 expression of 3-4 fold except for Brn3a long which did not show any change (Fig. 6.7).

6.2.2.c Effects of increase in Nurr1 expression on Brn3

To investigate if changes in Nurr1 expression might also have an impact on the expression of Brn3. Brn3 mRNA was also quantified from SH-SY5Y cells transfected with overexpressing Nurr1 construct. As Brn3a is our main focus and consistent changes in Nurr1 expression were only seen in Brn3a experiments, we looked mainly at changes in Brn3a expression. The identity and specificity of the Brn3a product was confirmed after each RT PCR (Fig 6.8). There were no significant changes in Brn3a expression with the overexpression of Nurr1 (Fig. 6.9).

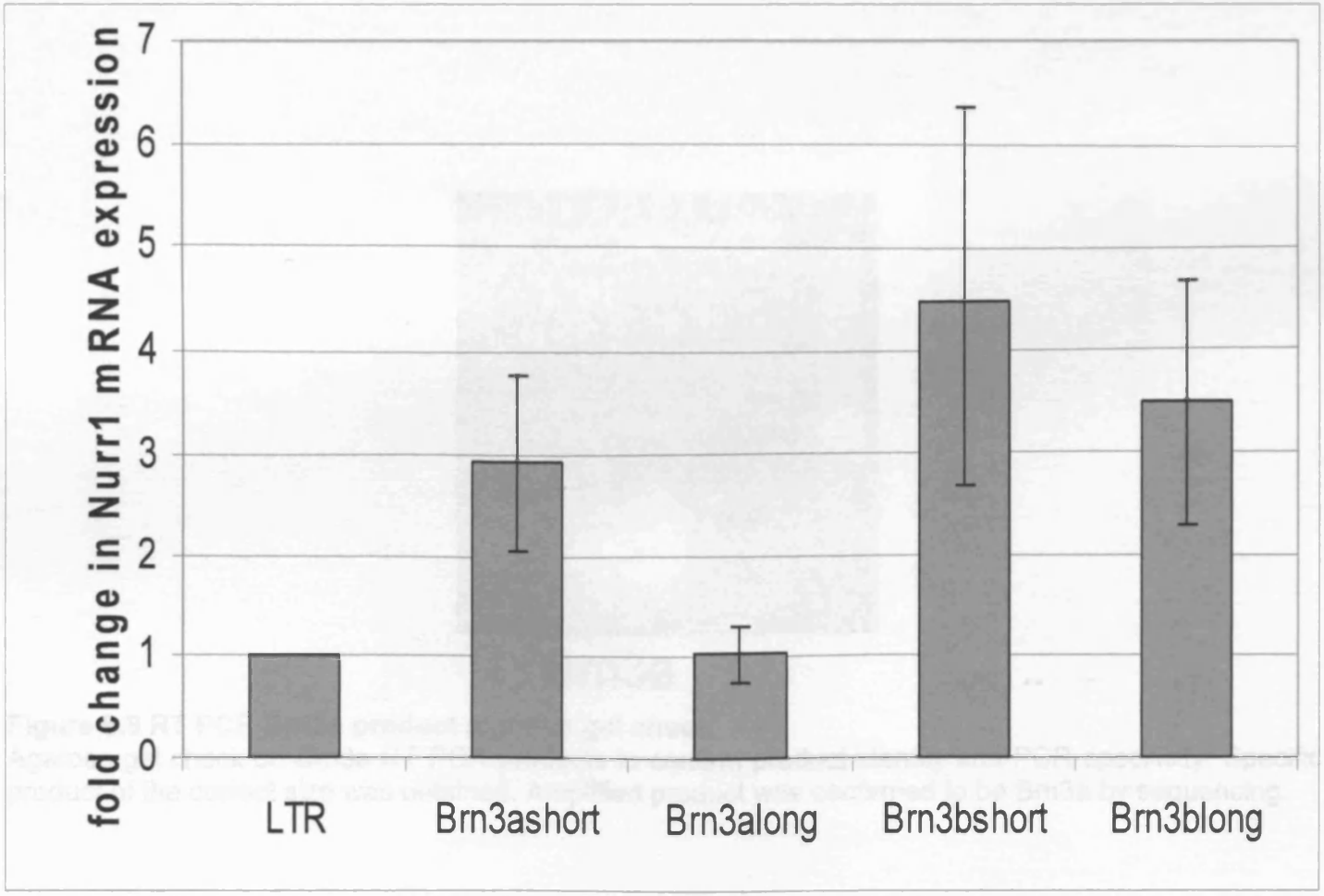


Figure 6.7 Effects of Brn3a and b isoforms over-expression on Nurr1 mRNA expression
Nurr1 mRNA levels were quantified in RNA extractions from SH-SY5Y cells transfected with different Brn3 forms and empty control plasmid. Increases in Nurr1 expression were obtained for cells transfected with all the different forms for Brn3 except for Brn3a long.

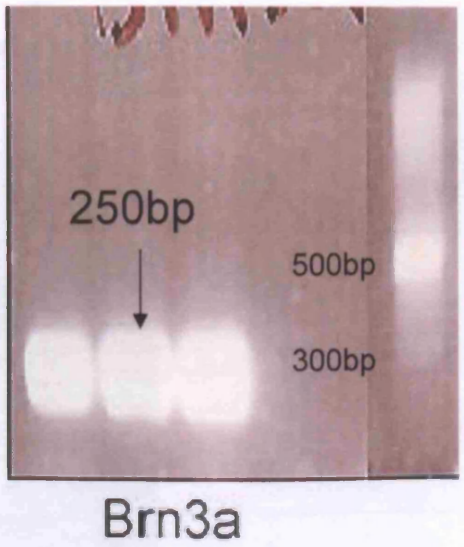


Figure 6.8 RT PCR Brn3a product agarose gel check
Agarose gel check on Brn3a RT PCR products to confirm product identity and PCR specificity. Specific product of the correct size was obtained. Amplified product was confirmed to be Brn3a by sequencing.

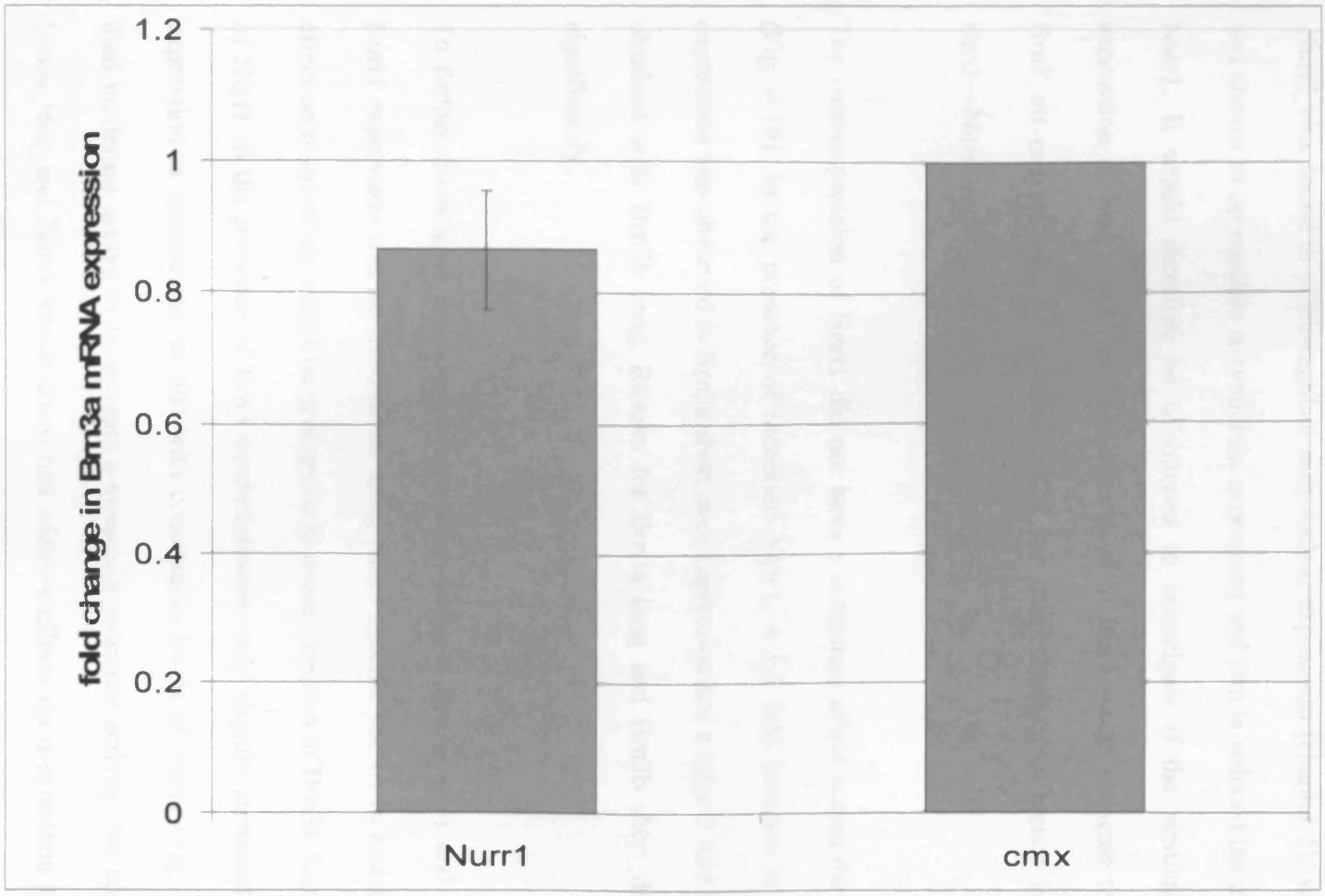


Figure 6.9 Effect of Nurr1 over-expression on Brn3a mRNA expression
Brn3a mRNA expression was quantified in mRNA extractions from SH-SY5Y cells transfected with Nurr1 overexpressing construct or control construct transfected SH-SY5Y cells. There was no significant difference in the Brn3a expression between the samples.

6.2.3 Regulation of α-synuclein by Brn3 & Nurr1

Nurr1 was found to down-regulate α-synuclein expression (chapter 5), whereas Brn3 was shown to up-regulate α-synuclein expression and Brn3a reduced the expression of Nurr1. It would therefore be of interest to investigate if the simultaneous over-expression of Nurr1 with the over-expression of Brn3 would abrogate the effects of Brn3 on α-synuclein, i.e. to find out if the transcriptional influence is sequential: Brn3→Nurr1→α-synuclein.

The over-expression of Nurr1 did not have a consistent effect across the Brn3 forms (Fig. 6.10). In the presence of increased Nurr1, a 1.5 fold increase in α-synuclein expression was observed in Brn3a short over-expression and a large 3 fold decrease was obtained with Brn3b long. Results for Brn3a long and Brn3b short did not differ significantly.

To further investigate the possible interaction between Brn3, Nurr1 and α-synuclein, Nurr1 expression was knocked-down using Nurr1 specific siRNA to assess whether its effect on α-synuclein might be synergistic to overexpression of Brn3a. Knocking-down of Nurr1 in the presence of Brn3 over-expression only slightly increased a-synuclein expression in comparison to that with constitutive levels of Nurr1 (Fig. 6.11). Using dual luciferase assays to investigate a-synuclein promoter activity, we confirmed that Brn3a long and Nurr1 knock-down had additive effects on α-synuclein promoter up-regulation (Fig. 6.12). It was observed that α-synuclein 1.9 construct promoter activity increased 1.3 fold with Nurr1 knock-down, 2 fold with Brn3a-long over-expression and

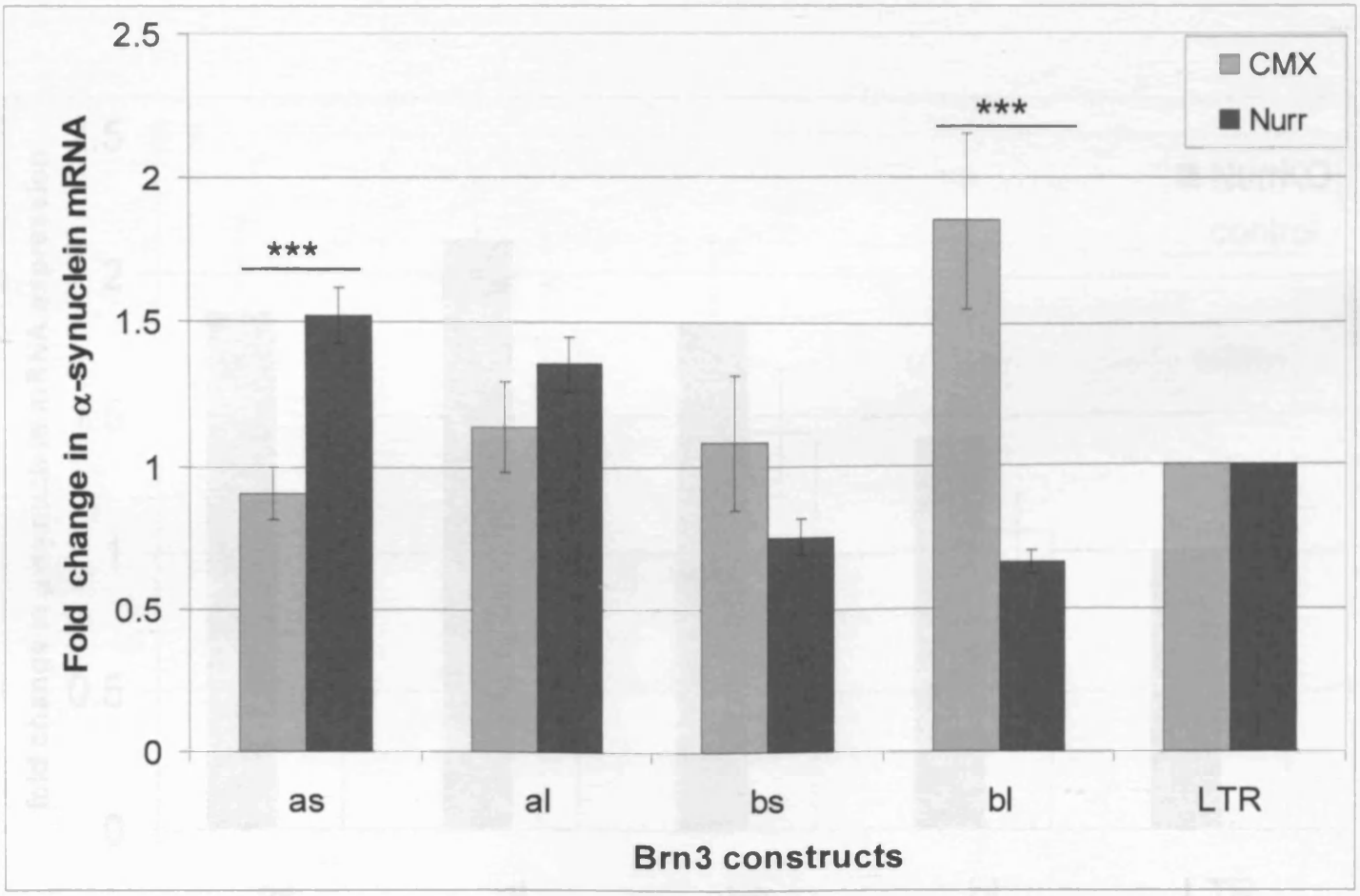


Figure 6.10 Effects of Brn3 and Nurr1 over-expression on α-synuclein mRNA expression
α-synuclein mRNA was quantified in RNA extractions from SH-SY5Y cells co-transfected with the different Brn3 constructs and Nurr1 constructs. The presence of Nurr1 increased α-synuclein expression in Brn3a overexpressing cells, and decreased α-synuclein expression in Brn3b overexpressing cells (***$p<0.001$).

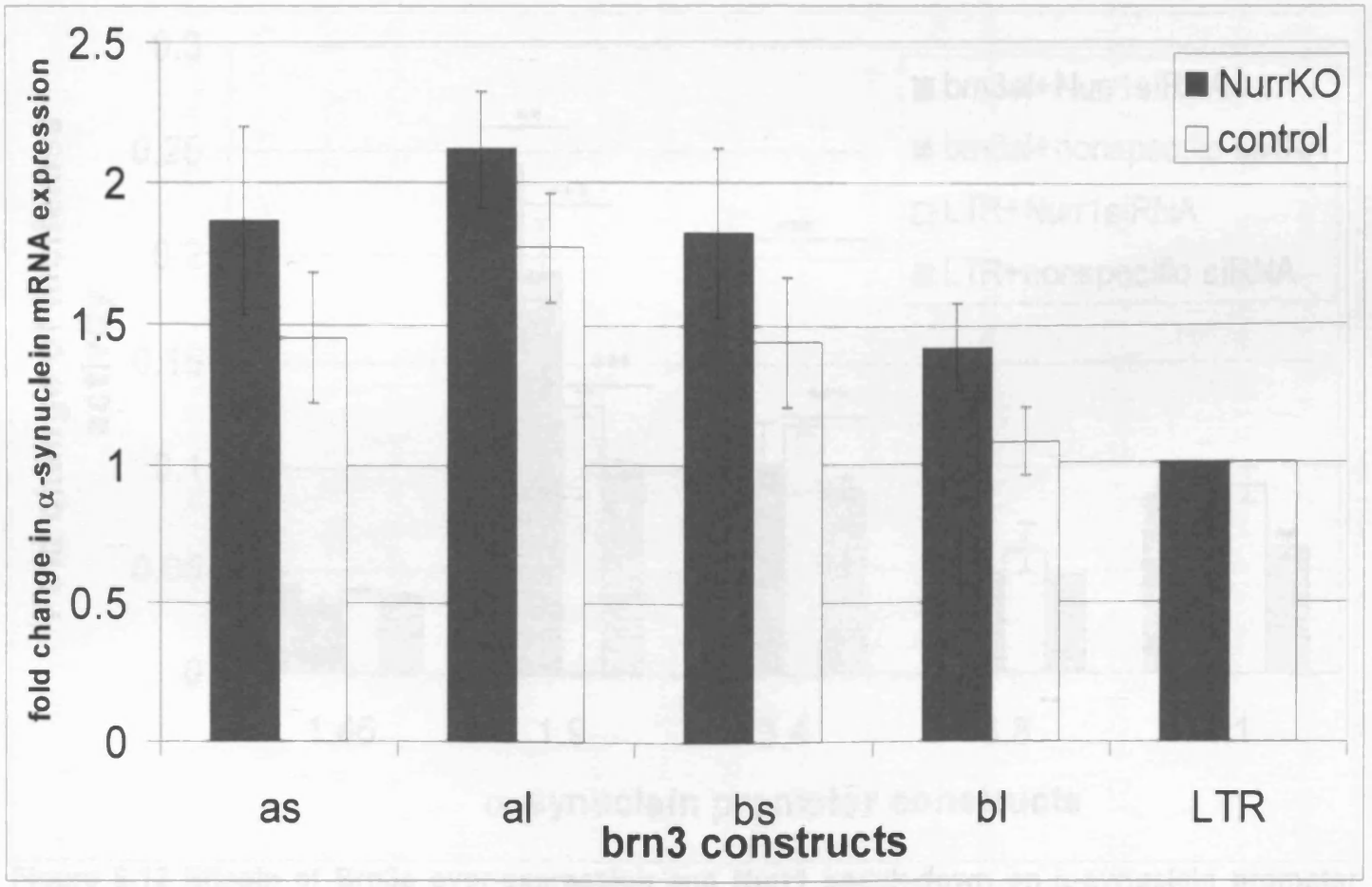


Figure 6.11 Effects of Brn3 over-expression and Nurr1 knock-down on α-synuclein mRNA expression

α-synuclein mRNA was quantified in RNA extractions from SH-SY5Y cells co-transfected with the different Brn3 constructs and Nurr1 siRNA or nonspecific siRNA. The knockdown of Nurr1 slightly increased α-synuclein expression in all Brn3 overexpressing cells.

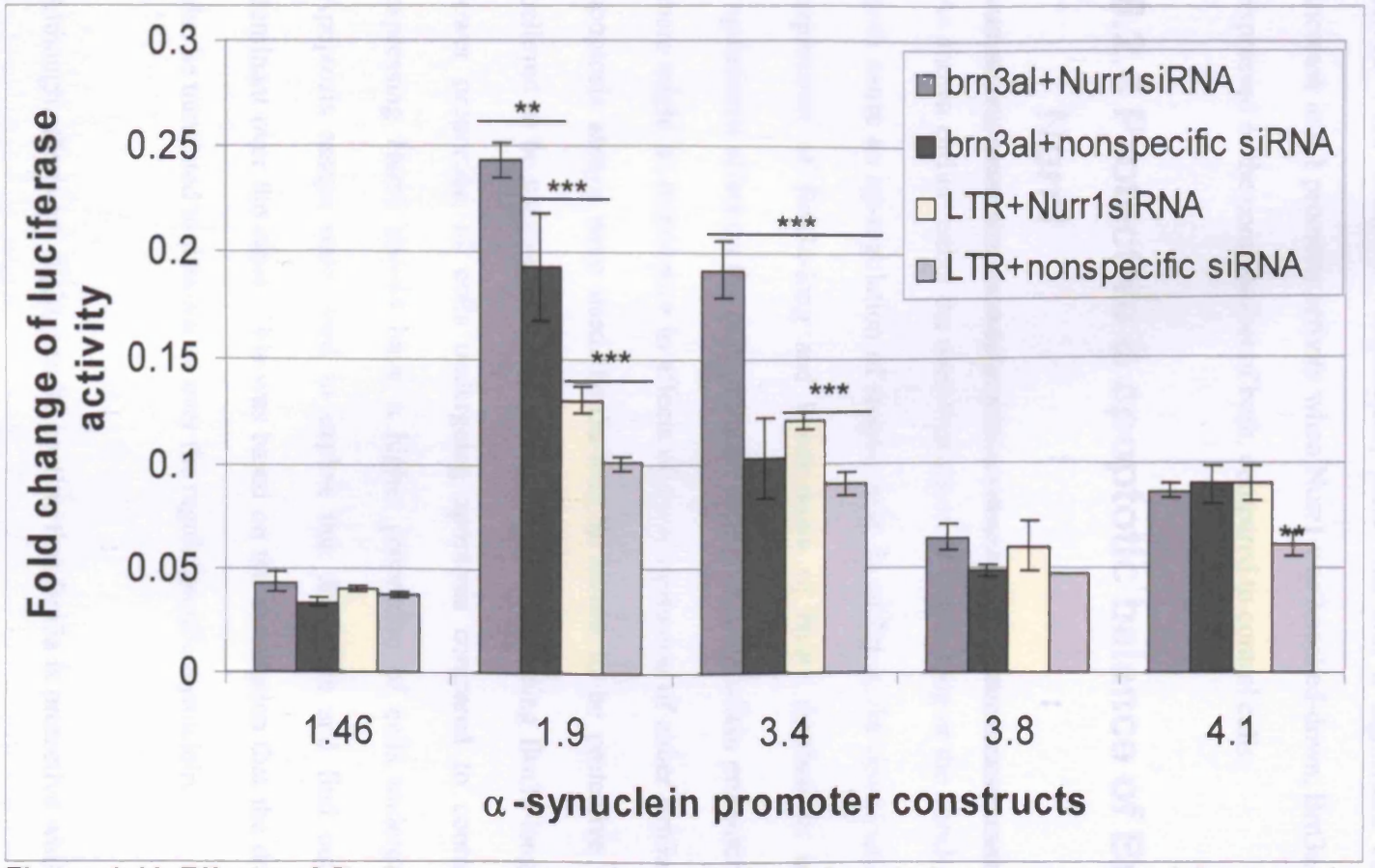


Figure 6.12 Effects of Brn3a over-expression and Nurr1 knock-down on α-synuclein promoter activity

SH-SY5Y cells were co-transfected with a-synuclein promoter luciferase plasmids and Brn3a long and Nurr1 specific siRNA or their respective control plasmids or siRNA. 1.9 a-synuclein promoter activity showed additive increase with the overexpression of Brn3a and Nurr1. The additive effect diminished with longer a-synuclein promoter (**p<0.01; ***p<0.005).

2.5 fold with both. However, with the increase in α-synuclein promoter length, the up-regulatory effects of Brn3a-long and Nurr1 knock-down diminished and no additive effects were observed. Nonetheless, there was still a significant but similar 30% increase in 4.1 promoter activity when Nurr1 was knocked-down, Brn3a-long was over-expressed or the combination of both, compared to control cells.

6.2.4 Protective & apoptotic balance of Brn3 & Nurr1

As shown earlier, either the over-expression of Brn3a-long or the knock-down of Nurr1 both cause an up-regulation of α-synuclein. In addition, the combination of the over-expression of Brn3a-long and knock-down of Nurr1 resulted in an additive up-regulational effect on the core promoter region of α-synuclein promoter. To examine if there might be dominance in effects of over-expression of either Brn3a-long or Nurr1, apoptosis assays were used. Brn3a-long is known to be protective while Nurr1 is believed to be pro-apoptotic, as such, cells over-expressing Brn3a-long should have a lower proportion of cells undergoing apoptosis compared to control while over-expressing Nurr1 should have a higher proportion of cells undergoing apoptosis. Apoptosis assays were used to exploit this difference and find out if either was dominant over the other. This was based on the assumption that the dominance could also be translated to dominance over the regulation of α-synuclein.

Although effects are slight, results confirm that Brn3a is protective while Nurr1 is pro-apoptotic (Fig. 6.13). When SH-SY5Y cells were co-transfected with both Brn3a and Nurr1, the apoptosis level was found to be similar to control cells, showing that neither Brn3a nor Nurr1 were functionally dominant over the other.

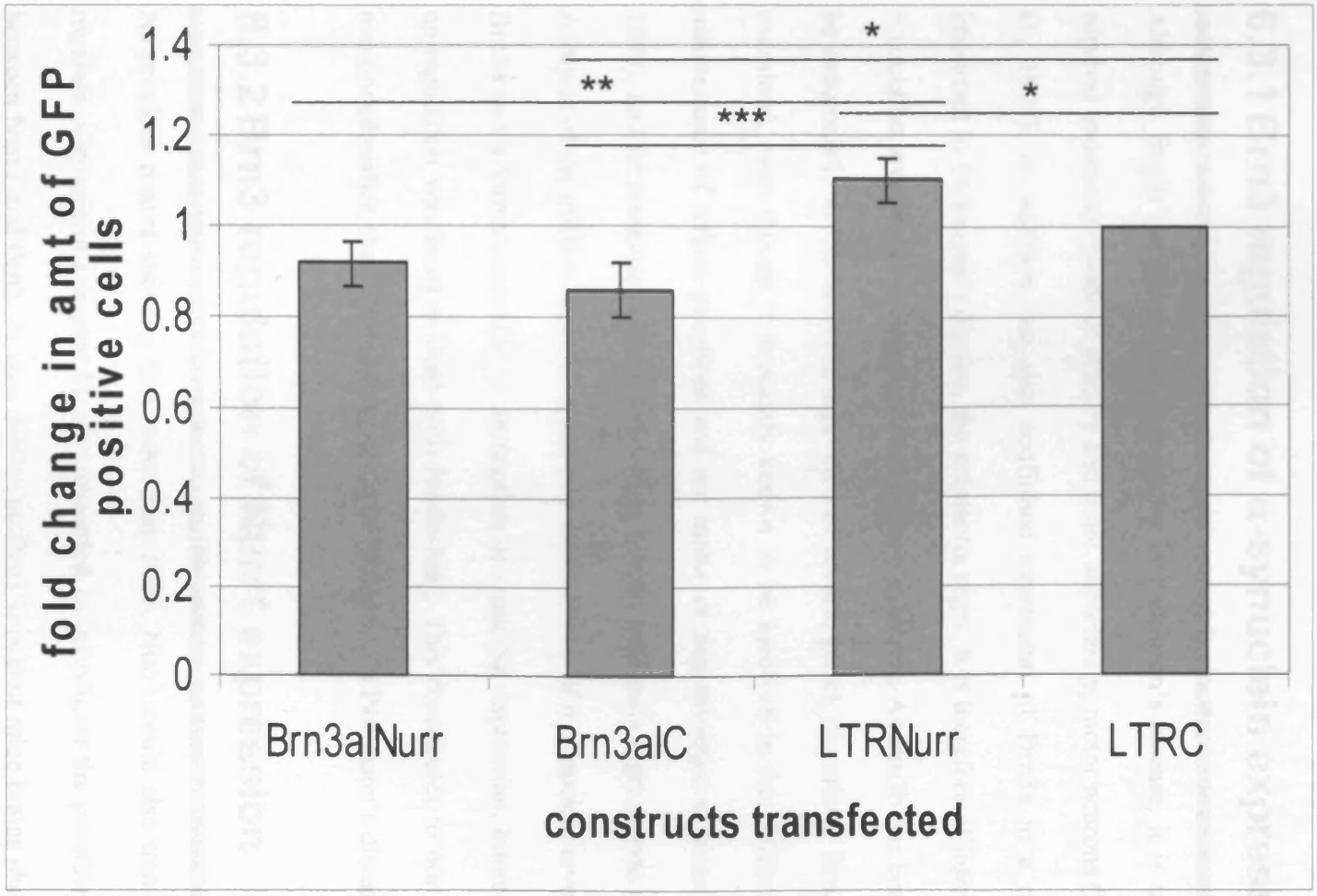


Figure 6.13 Apoptotic effect of Brn3a and Nurr1
SH-SY5Y cells transfected with Brn3a long were less apoptotic compared to control cells while those transfected with Nurr1 were more apoptotic. Co-transfection of both Brn3a long and Nurr1 still reduced the number of early apoptotic cells slightly compared to control cells

6.3 Discussion

6.3.1 Brn3 regulation of α-synuclein expression

Although Brn3a has never been implicated in Parkinson's disease, it is important in survival and maintenance of sensory and more importantly, motor neurons (McEvilly et al., 1996). In addition, we also confirmed expression of Brn3a in a region most important in Parkinson's disease, the substantia nigra. It is therefore of interest whether it could be involved in the genesis of Parkinson's disease. As Brn3b has been shown to be antagonistic to Brn3a in the regulation of several genes, the role of Brn3b was also examined, even though it is mainly known to be involved in the differentiation and maintenance of retinal ganglions and not motor or dopaminergic neurons (Latchman, 1999), and its presence was not detected in human substantia nigra region of the brain. Analysis of the mRNA expression and promoter activity of α-synuclein revealed that all Brn3a and b forms were able to up-regulate α-synuclein expression. Interestingly, this up-regulation was most evident with Brn3a-long. This contributes to our hypothesis, mentioned earlier, that Brn3a-long could be protective in Parkinson's disease.

6.3.2 Brn3 regulation of Nurr1 expression

As previous results showing that, other than Brn3, Nurr1 could also transcriptionally regulate α-synuclein (chapter 5), we proceeded to investigate the possible interaction between Nurr1 and Brn3. *In vivo* studies on Brn3 knockout mice brains showed an up-regulation of Nurr1 with the knockdown of Brn3a. *In vitro* Brn3a over-expression, however, did not lead to down-regulation of Nurr1. In fact, Brn3a short over-expression

led to Nurr1 up-regulation. Brn3a short, on the other hand, did not alter the expression of Nurr1. It is plausible that Brn3a long is responsible for the regulation of Nurr1 in Brn3a knockout mice. The over-expression of Brn3a long does not show an opposite effect to its knockdown could simply be due to constitutive levels of Brn3a long functioning to invoke a maximal suppression of Nurr1gene expression, thus, any further increase in Brn3a long expression does not cause a further repression whereas its removal lifts the blockade and allows Nurr1 expression. It could also be due to the efficiency of transient transfection. With only a portion of the cells over-expressing Brn3a long, the effect of increased Brn3a long is nominal compared to constitutive levels of Brn3a long already present, this renders the change in Nurr1 expression insignificant. This case scenario is similar to that observed earlier in the regulation of α-synuclein by Nurr1 (Chapter 5).

As Nurr1 has been shown to regulate Brn3a, the reverse regulation was also tested to determine if Nurr1 could also regulate the expression of Brn3a, in a feed back loop. Although the over-expression of Nurr1 did seem to slightly down-regulate Brn3a, the change was not significant. More experiments would have to be performed to conclusively identify the relationship between Brn3a and Nurr1.

6.3.3 Regulation of α-synuclein by Brn3 & Nurr1

With over-expression of Brn3a and knock-down of Nurr1 both up-regulating the expression of α-synuclein, and that the knock-down of Brn3a increasing the expression of Nurr1, we sought to find out if Brn3a and Nurr1 could act synergistically on the expression of α-synuclein. Predictably, the over-expression of Brn3a long together with the over-expression of Nurr1 did not result in any change in α-synuclein expression.

The knocking-down of Nurr1 in the presence of Brn3a long over-expression, surprisingly, however, only very slightly increased a-synuclein expression compared to when Nurr1 was not knocked-down. Studying α-synuclein promoter activity, confirmed that Brn3a long and Nurr1 knock-down had additive effects on α-synuclein promoter up-regulation. However, with the increase in α-synuclein promoter length, the up-regulatory effects of Brn3a long and Nurr1 knock-down diminished and additive effects were no longer observed. The decrease in activation of the promoter was undoubtedly due to the presence of repressor sites further upstream of the gene, thus dampening the up-regulation caused. Yet, it is not clear why the up-regulation of α-synuclein could not be observed on the endogenous mRNA level when either the over-expression of Brn3a long or the knock-down of Nurr1 alone could. Nonetheless, this is a novel display of interplay between Brn3a-long, Nurr1 and α-synuclein gene regulations.

The regulation of α-synuclein by Brn3a and Nurr1 might occur through two different models:

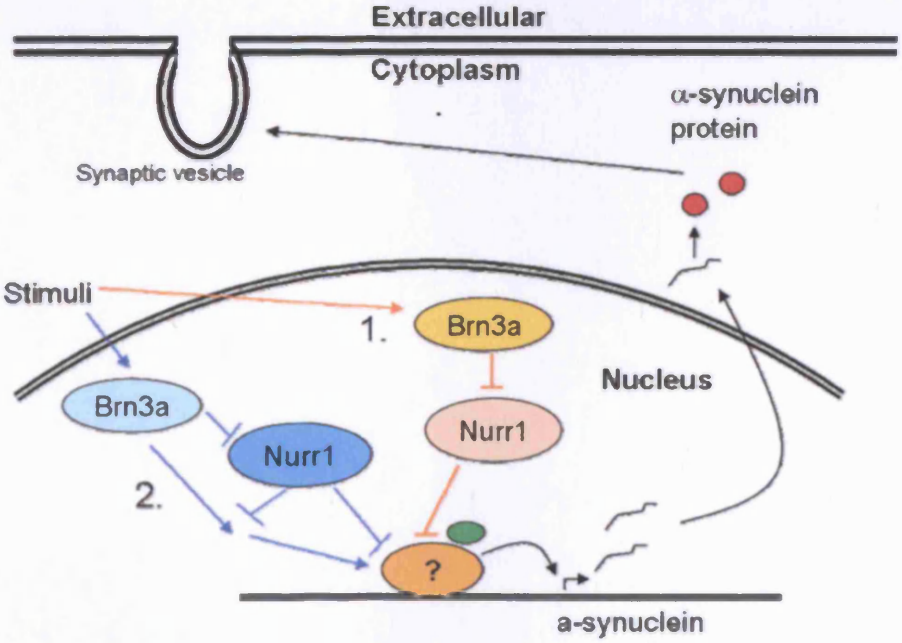


Figure 6.14 Possible regulation models of α-synuclein by Brn3 and Nurr1.
Two possible pathways to regulate a-synuclein by Brn3a and Nurr1 may occur. Pathway of model 1 is indicated by orange arrows while pathway of model 2 is indicated by blue arrows.

From our results in the apoptosis assays, dominance of either Brn3a or Nurr1 over the other was not found. Moreover, additive rather than synergistic effects were obtained from α-synuclein promoter assays when Brn3a and Nurr1 were used. As such, it is deduced that both Brn3a and Nurr1 might act seperately on the α-synuclein promoter, and the second model could better represent the regulation effects of Brn3a and Nurr1 on α-synuclein.

Chapter 7: Regulation of Parkin in oxidative stress

7.1 Introduction

In the previous result chapters, a detailed analysis into the regulation of Parkinson's disease associated gene, α-synuclein, in the presence of oxidative stress or in normal conditions was carried out. The oxidative stresses used evoked a robust transcriptional up-regulation of α-synuclein. As these stresses appear to play an important role in the development of Parkinson's disease, we also examined their effects on another Parkinson's disease associated gene, Parkin.

7.1.1 Oxidative stress, Parkin & Parkinson's disease

The majority of parkin mutations are found to either impair its binding to putative substrates or render its ligase activity defective, thus resulting in a decrease in its activity. This loss-of-function mechanism leads to neurodegeneration and PD (Imai and Takahashi, 2004). Recent studies of parkin knock out models have suggested that parkin loss-of-function may lead to mitochondrial dysfunction and oxidative stress (Greene et al., 2003; Palacino et al., 2004).

Oxidative stress has long been implicated in the pathogenesis of PD. Dopamine may be the major contributor since it can be generated endogenously by the substantia nigral neurons (Barzilai et al., 2001; Sherer et al., 2002). An increase in dopamine may lead to mitochondrial dysfunction and impaired proteolysis via its reactive metabolites,

dihydroxyphenylacetic acid and dopamine-quinones, or directly due to its oxidative nature (Khan et al., 2001; Kruger et al., 1998; Pearson et al., 2001; Stokes et al., 1999). The neurotoxin, MPP+, a reactive metabolite of MPTP (1-methyl-4-phenyl-1.2.3.6-tetrahydropyridine), is a mitochondrial complex I inhibitor and is selectively taken up into dopaminergic neurons via the dopamine transporter leading to severe oxidative damage and neuronal degeneration resulting in Parkinsonism in rodents, primates and humans (Javitch et al., 1985; Javitch and Snyder, 1984; Mizuno et al., 1987; Ramsay et al., 1986).

Parkin knock-out mice develop mitochondrial deficits (Palacino et al., 2004) and parkin knock-down in cell lines renders cells more vulnerable to oxidative stress (MacCormac et al., 2004). Parkin's ubiquitin ligase activity is also modified by nitric oxide mediated oxidative stress. It has have recently reported (LaVoie et al., 2005; Tan et al., 2004b; Yao et al., 2004) that the reactive metabolite of dopamine, dopamine quinone may decrease the solubility of endogenous parkin by covalently binding to cysteine residues of parkin. This results in the loss of parkin's activity. Furthermore, this study found increased insoluble parkin in the caudate nucleus of PD patients (LaVoie et al., 2005). It was also previously shown that endogenous parkin localizes to aggregates following exposure to dopamine in neuroblastoma cells (Muqit et al., 2004).

7.1.2 Hypothesis & Aims

Oxidative stress has been found to affect the expression of a variety of proteins implicated in PD, e.g. α-synuclein (Karamohamed et al., 2005). However, no studies to date have examined the effect of oxidative stress on the regulation of parkin at the level of transcription. To examine whether there is any up-regulation of parkin in the face of oxidative stress that might represent a compensatory mechanism following a reduction in parkin solubility, we studied the effects of two well characterized oxidants, dopamine and MPP+, on the regulation of parkin expression.

Aims of project:

12. To determine if the presence of extracellular dopamine, MPP+ or MG132 could alter the expression of parkin mRNA.

13. To investigate if the change in parkin mRNA expression is reflected in its protein levels, and also to confirm if parkin protein solubility is affected by the stresses used.

14. If the expression of parkin mRNA is altered, to confirm if this event is transcriptional.

7.2 Results

7.2.1 Quantitation of parkin mRNA

To determine if dopamine might have an effect on the transcription of parkin, real-time PCR was used to measure the amount of parkin mRNA in SHSY5Y cells incubated with 0μM, 62.5μM, 125μM and 250μM dopamine for 24hrs. Similar results were obtained in normalisations to three different control house-keeping genes, β2-microglobulin, β-actin and NADH cytochrome b5-reductase (one representative graph is shown). The identity and specificity of parkin product was confirmed after each RT PCR (Fig 7.1).

RT PCR results demonstrated that dopamine caused a dose-dependent increase in endogenous parkin mRNA (Fig. 7.2). A maximal increase of 120 fold was achieved with 250μM dopamine treatment.

To find out if this was specific to dopamine or a general response to oxidative stress another oxidative stressor, MPP+, was used to carry out a similar quantitative analysis. Cells were incubated with 0mM, 1mM, 5mM and 10mM MPP+ for 6hrs. The results from real-time PCR were similarly normalised and a dose-dependent increase of 3-4 fold in mRNA was also observed (Fig. 7.3).

Figure 7.1 RT PCR parkin product agarose gel check
Agarose gel check on parkin RT PCR products to confirm product identity and PCR specificity. Specific product of the correct size was obtained. Sequencing of amplified product confirmed the identity of product as parkin.

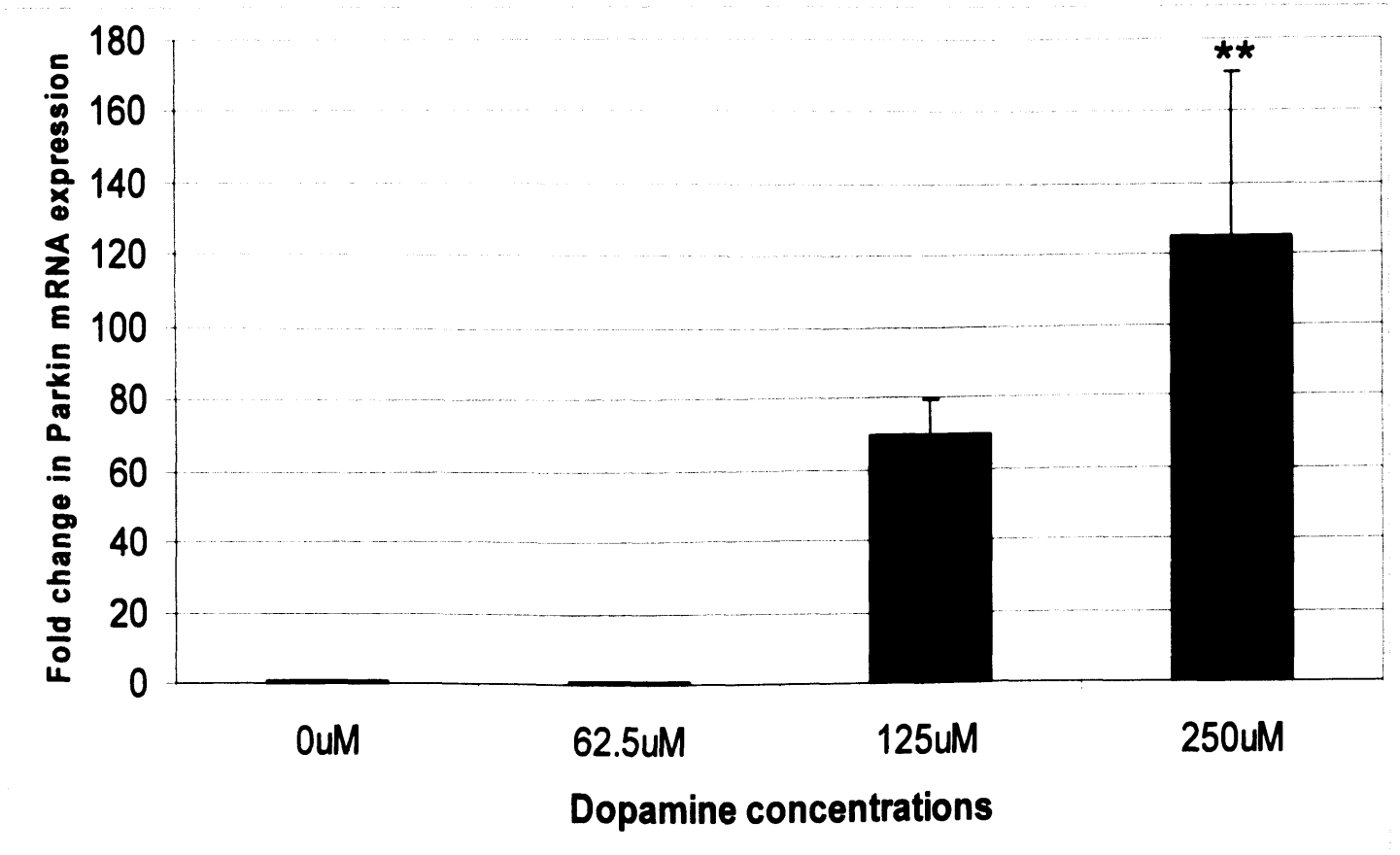


Figure 7.2 Effects of dopamine on parkin mRNA expression
Cells were incubated for 24hrs in 0μM, 62.5μM, 125μM and 250μM dopamine in full culture media, before mRNA was retrieved. The error bars indicate the standard error of the mean of 3 experiments performed in triplicate. Differences was analysed by ANOVA + Bonferroni post hoc test. The amount of parkin mRNA in cells stressed with 250mM dopamine was significantly higher than those in unstressed cells and cells stressed with only 62.5mM dopamine (** $p<0.01$).

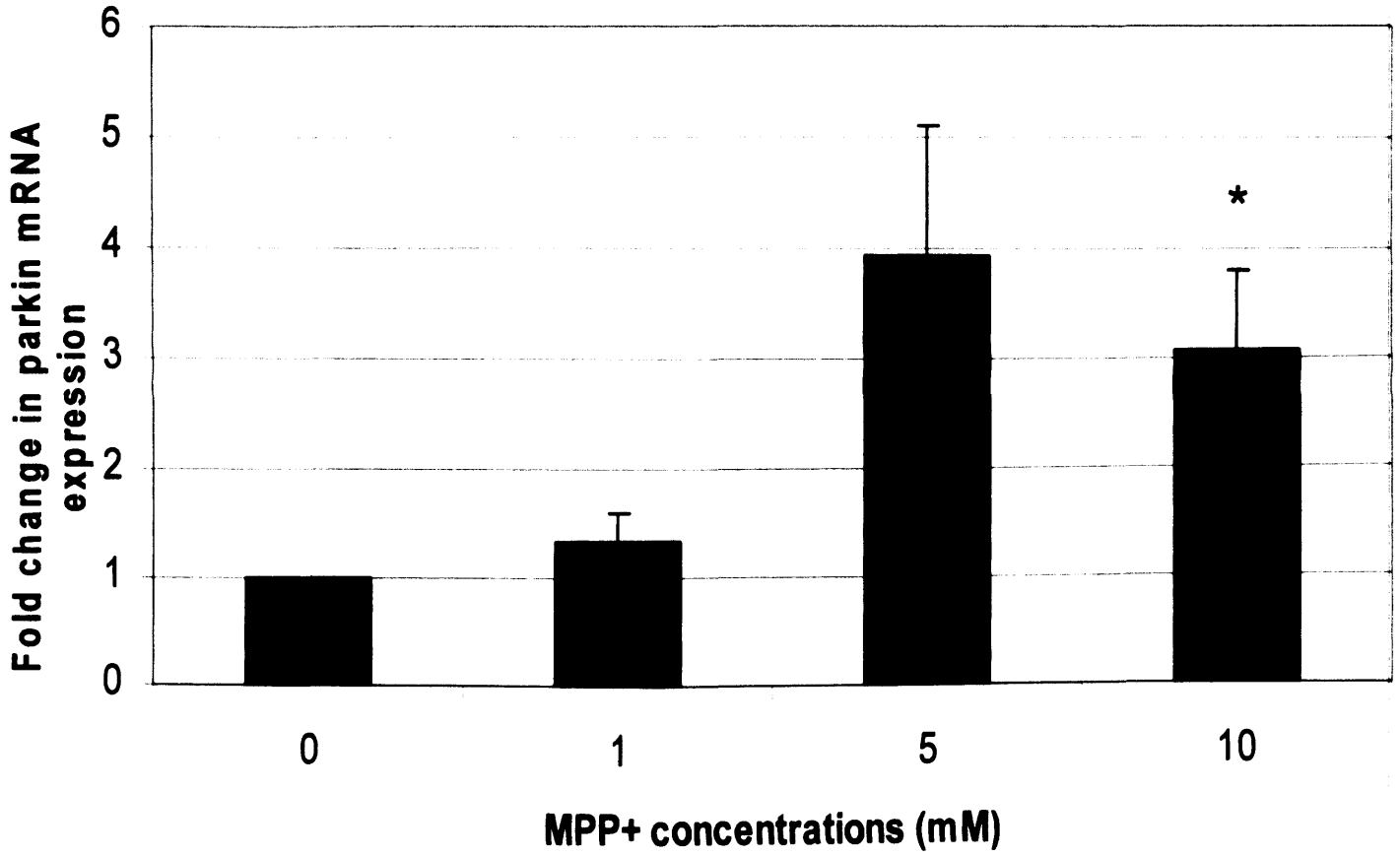


Figure 7.3 Effects of MPP+ on parkin mRNA expression
Cells were incubated for 6hrs in 0mM, 1mM, 5mM and 10mM MPP+ in full media culture media before mRNA retrieval. The error bars indicate the standard error of the mean of 5 experiments. Differences was analysed by ANOVA + Bonferroni post hoc test. There was a significant dose-dependent increase in parkin mRNA for stressed cells (*$p<0.05$).

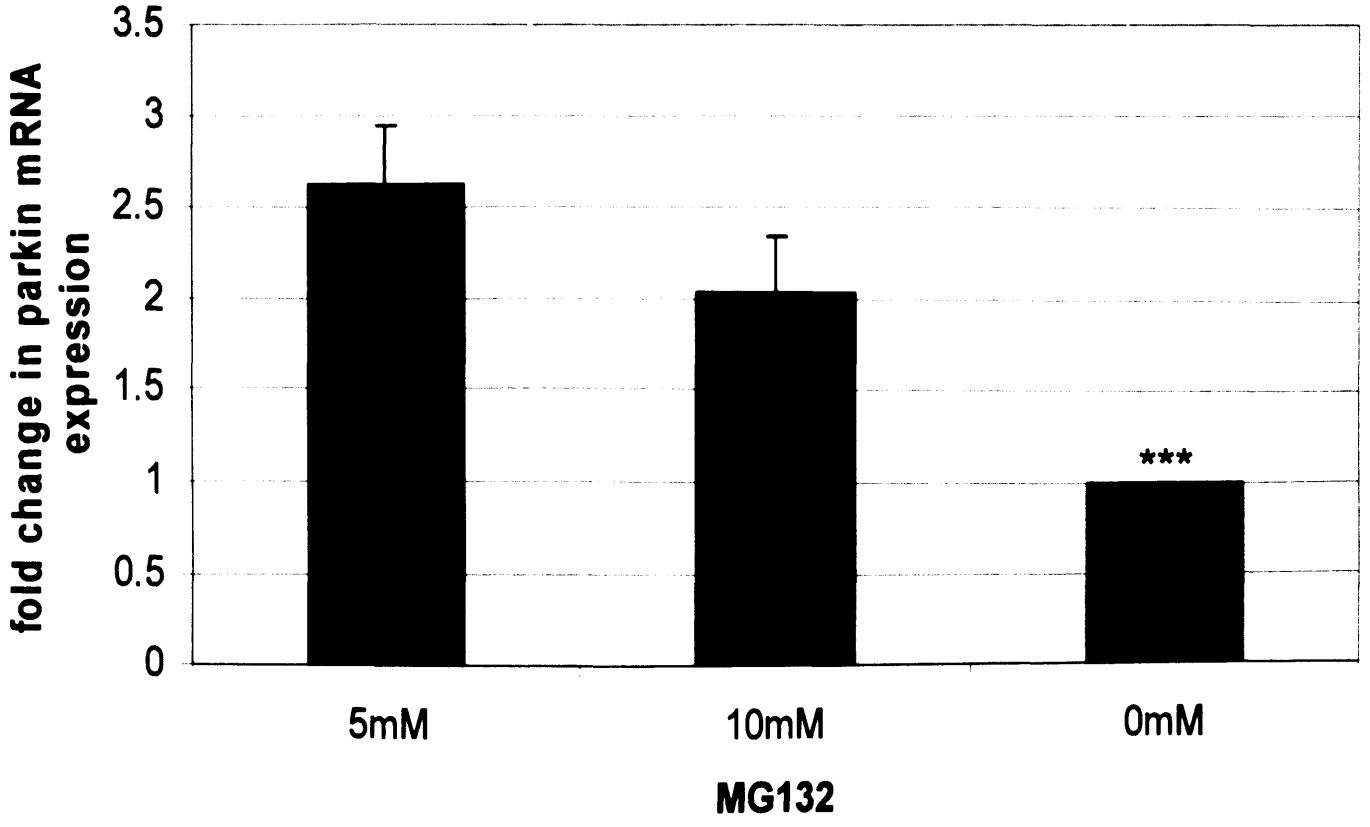


Figure 7.4 Effects of MG132 on parkin mRNA expression
Cells were incubated for 24hrs in 0mM, 5mM and 10mM MG132 in full media culture media before mRNA retrieval. There was a significant increase in parkin mRNA in stressed cells compared to untreated cells (***$p<0.001$). The error bars indicate the standard error of the mean of 3 experiments. Differences was analysed by ANOVA + Bonferroni post hoc test.

To further investigate the regulation of parkin expression by stresses, proteasomal inhibitor, MG132, was also used. This is of relevance as parkin is an E3 ligase involved in the ubiquitin proteasomal system. Using a proteasomal inhibitor would allow us to understand if the same principle applies to general cellular detrimental stresses, which may lead to proteasomal dysfunction, but does not target the proteasome specifically could lead to parkin up-regulation in a bid to overcome the dysfunction, and a stress that specifically targets and inhibits proteasomes. Cells were incubated with 0mM, 5mM and 10mM MG132 for 24hrs. The results from RT PCR were similarly normalized as with dopamine and MPP+ experiments and a significant increase of 2 and 2.5 fold in mRNA was observed in 5mM and 10mM MG132 treated cells compared to untreated cells (Fig. 7.4).

The RT PCR results show that both oxidative stresses and proteasomal inhibitors could increase parkin expression, however, this did not indicate if the increase in parkin mRNA was due to transcriptional or post-transcriptional regulation. It also did not reveal if the increase was reflected on the protein level.

7.2.2 Protein analysis

Western blot analysis was performed to determine if the regulation of parkin by oxidative stress observed at the mRNA level could also be seen at the protein level. In addition, in light of recent work showing altered parkin solubility by oxidative stress (LaVoie et al., 2005; Petit et al., 2005), parkin protein concentrations were analysed in both total (soluble and insoluble) and soluble cell extracts. 250μM dopamine and 10mM MPP+ was used to stress the cells. A significant increase in total parkin protein was obtained following dopamine stress (55% increase in signal). This was consistent with our mRNA results above. However, there was a significant decrease in soluble parkin protein (35% decreases in signal) suggesting that the increased parkin was sequestered into the insoluble fraction (Fig. 7.4). In MPP+ stressed cells, an increase in total parkin protein compared to control was also observed (100% increase in signal), but in contrast to dopamine, there was no change in the proportion of soluble parkin protein (Fig. 7.5).

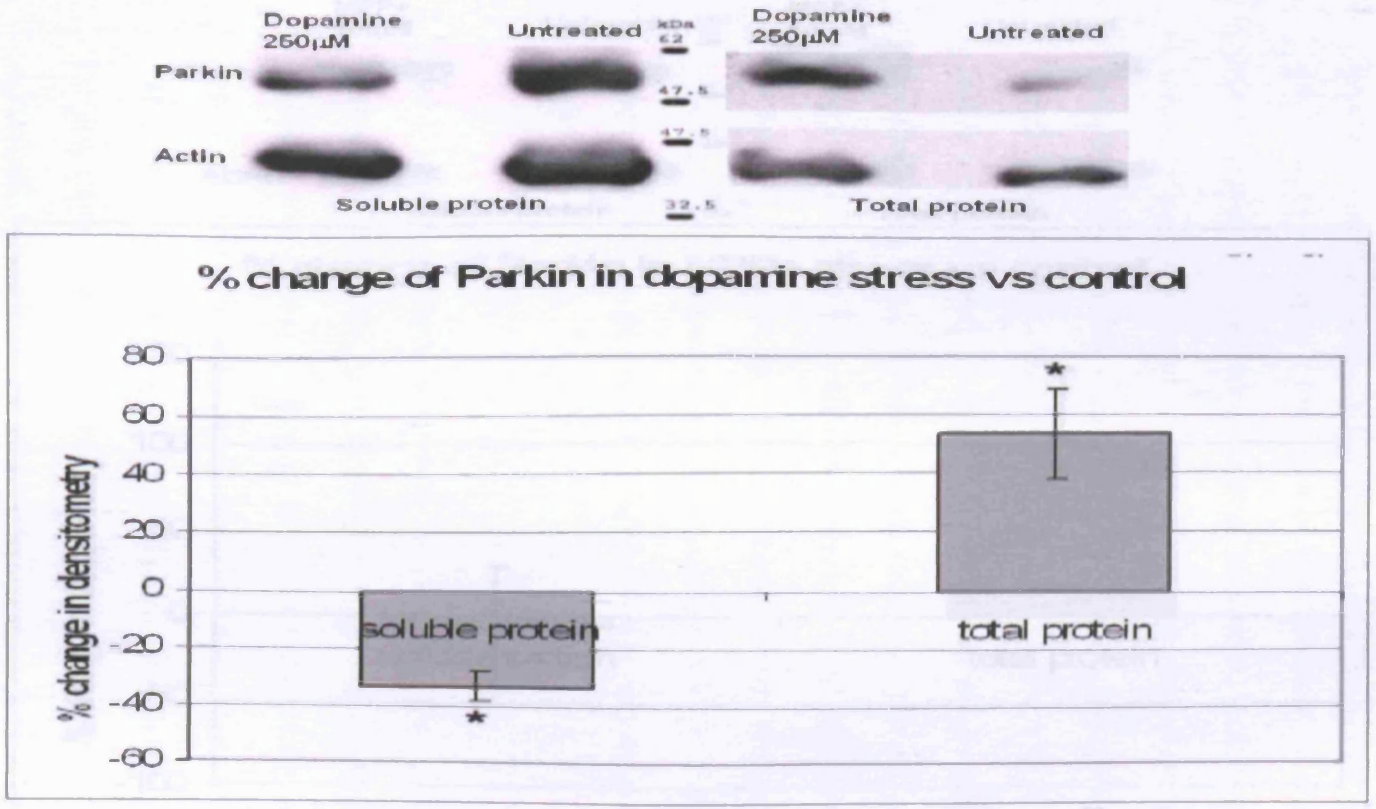


Figure 7.5 Effects of dopamine on parkin protein levels
Cells were incubated for 24hrs in 0mM and 250mM dopamine in full culture media before lysing in either RIPA buffer or Laemmli buffer to extract soluble and total protein respectively. The exposed bands were analysed using GS-800 BioRad densitometer and the results were normalised with β-actin protein and shown as a percentage change compared to untreated cells. Differences were analysed by paired T-test. Dopamine stressed soluble parkin showed a significant decrease while total parkin showed a significant increase compared to untreated samples (* $p<0.05$).

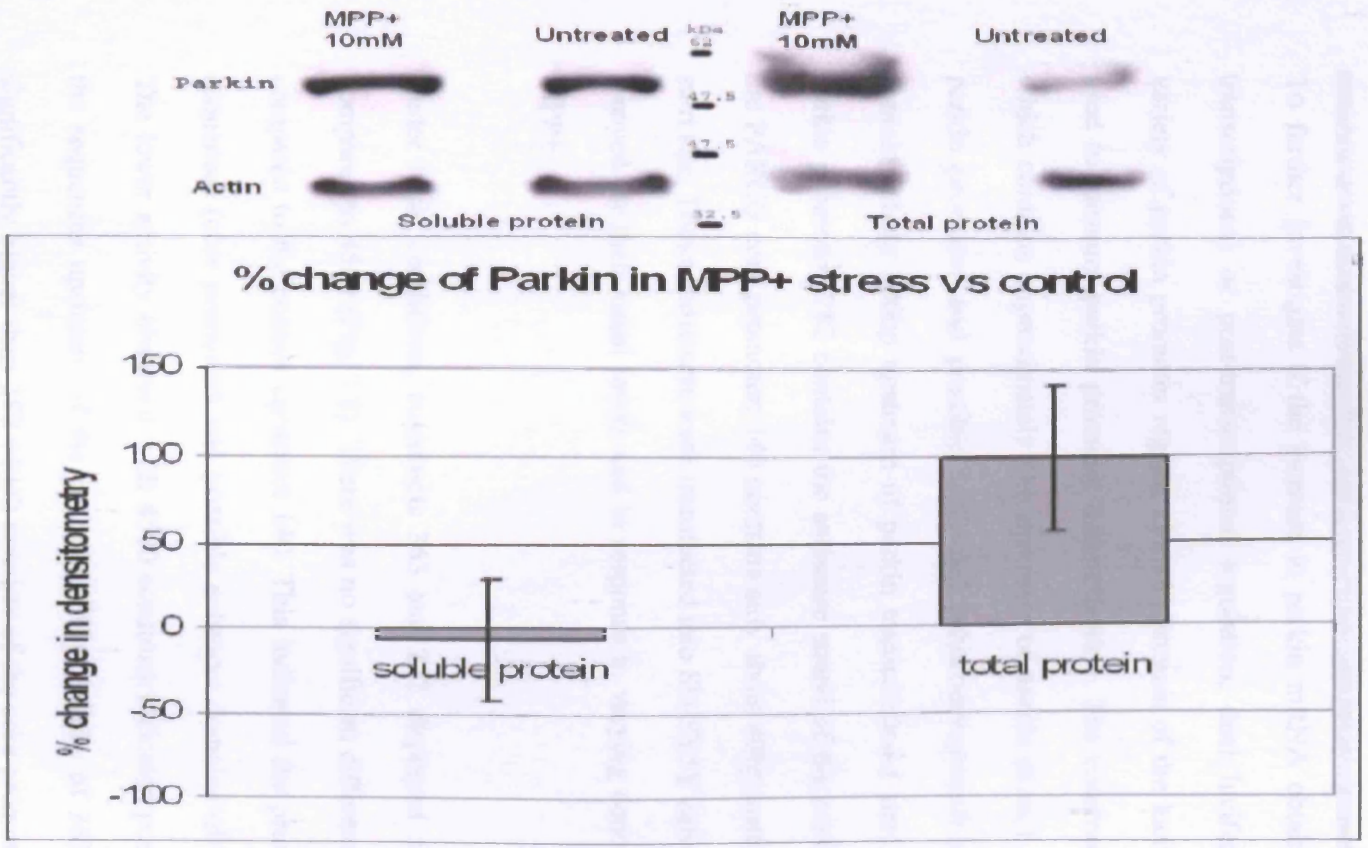


Figure 7.6 Effects of MPP+ on parkin protein levels

Cells were incubated for 6hrs in 0mM and 10mM MPP+ in full media culture media before lysing in either RIPA buffer or Laemlli buffer to extract soluble and total protein respectively. The exposed bands were analysed using GS-800 BioRad densitometer and the results were normalised onto β-actin protein and shown as a percentage change compared to untreated cells. There was a strong trend for an increase in total parkin protein expression compared to untreated control.

7.2.3 Parkin promoter activity

To further investigate if the increase in parkin mRNA obtained previously was due to transcriptional or post-transcriptional regulation, dual luciferase assays were used. A variety of parkin promoter regions cloned upstream of the luciferase gene (Fig. 7.7) were used to measure parkin promoter transactivation. The constructs include 4500 construct, which contains approximately 4kb upstream of parkin exon 1 and thus includes the core parkin promoter and possible additional enhancer/repressor sites; 363 construct which contains about 300bp upstream of parkin transcriptional start site and includes the core parkin promoter; 282 contains the antisense strand of the parkin promoter and represents the PARCG core promoter; 140 contains only about 40bp upstream of parkin transcription start site. These constructs were transfected into SH-SY5Y cells and luciferase activity was assayed for their basal levels and in response to varying concentrations of dopamine and MPP+.

Under basal conditions, constructs 363 and 282 displayed significantly higher activity compared to 4500 (Fig, 7.8). There was no significant difference in activity between 4500 compared to the control construct 140. This indicated the presence of essential promoter sequences (core promoter) and possible enhancer domains of the parkin promoter (363). The lower activity observed with 4500 construct indicated possible repressor domains in the sequences upstream of the promoter. The activity of 363 was also observed to be significantly higher than 282 which consists of the core promoter of the co-regulated anti-sense gene, PACRG.

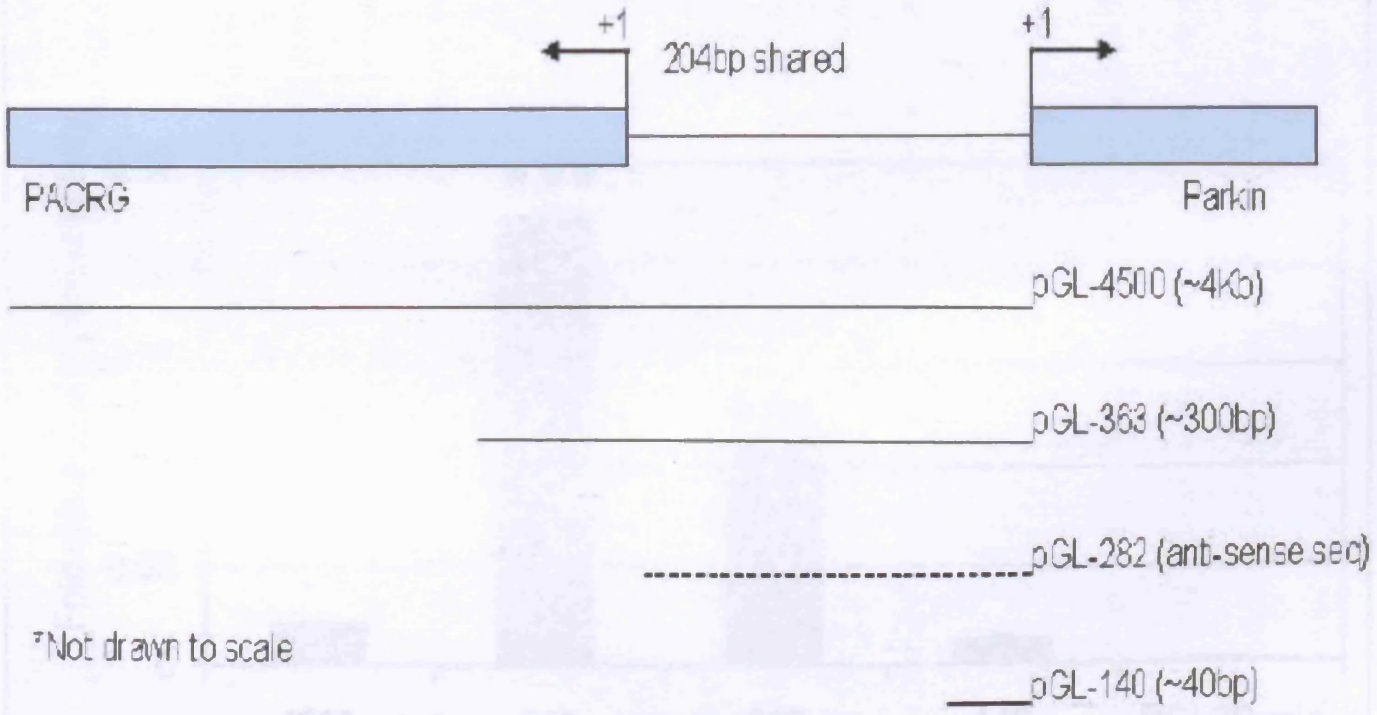


Figure 7.7 Parkin luciferase promoter constructs
Constructs used for dual luciferase assays. Each of the inserts was cloned in pGL-basic plasmid upstream of the firefly luciferase reporter gene. In all the transfections, they were co-transfected with pRL-SV40, which produces *Renilla* luciferase and thus corrects for transfection efficiency when measuring for the overall luciferase activity. 4500 encodes for approximately 4kb upstream of parkin exon 1 and thus contains the core parkin promoter and possible additional enhancer/repressor sites; 363 encodes for about 300bp upstream of parkin transcriptional start site and contains the core parkin promoter; 282 contains the antisense of the parkin promoter and codes for the PARCG core promoter; 140 contains only about 40bp upstream of parkin and PGL3basic indicates the empty vector.

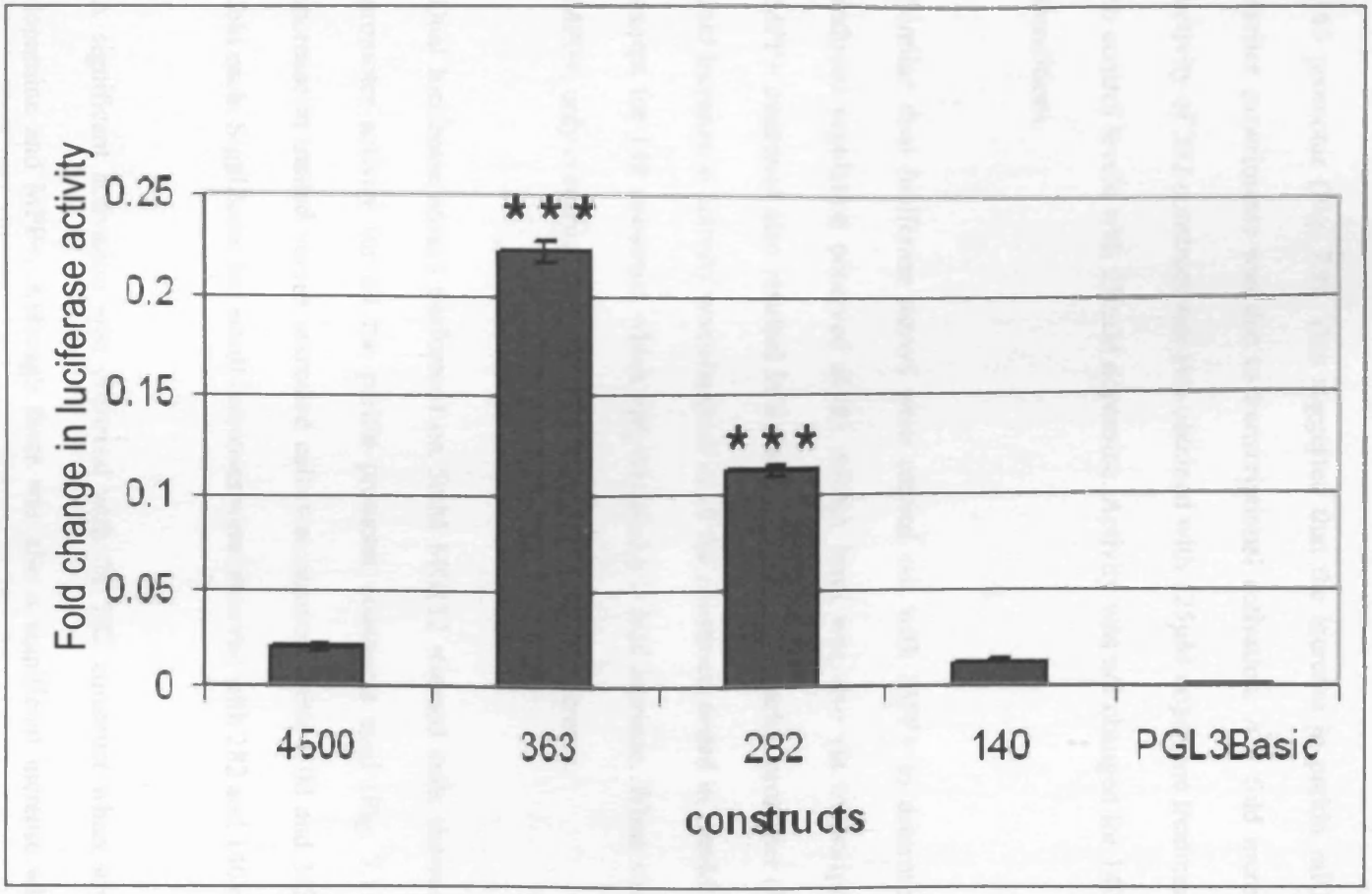


Figure 7.8 Basal parkin promoter activity
Cells were transfected with the different parkin promoter constructs and incubated in full culture media for 24hrs. Data was analysed by ANOVA with post hoc Bonferroni correction. Constructs 363 and 282 expressed significantly higher luciferase activity compared to the other constructs (***$p<0.001$) 363, in turn, also expressed significantly higher luciferase than 282

Administration of dopamine resulted in a significant increase in luciferase activity for the full length parkin promoter construct 4500 at 125μM and dose-dependent increase for the 363 promoter (Fig. 7.9). This suggested that the increase in parkin mRNA observed in earlier experiments was due to transcriptional activation. A 2 fold increase in promoter activity of 282 construct was also obtained with 125μM dopamine treatment, but decreased to control levels with 250μM dopamine. Activity was not changed for 140 construct in all conditions.

Similar dual luciferase assays were carried out with MPP+ to determine if the MPP+ induced regulation observed at the mRNA level was also via transcriptional activation. MPP+ treatment also resulted in a similar activation of parkin promoter (Fig. 7.10). A 3-4 fold increase in activity was observed in all the constructs tested in 10mM MPP+ treatment except for 140 construct which only showed a 2 fold increase. When stressed with 5mM MPP+, only construct 363 showed a significant activity increase.

Dual luciferase assays performed on 5mM MG132 stressed cells showed an increase in promoter activity for all the parkin promoter constructs used (Fig. 7.11). The greatest increase in treated versus untreated cells was observed with 4500 and 363 constructs at 2 fold each. Significant but small increases were observed with 282 and 140 constructs.

A significant activation was observed with the 282 construct when stressed with both dopamine and MPP+. Although there was also a significant increase when treated with MG132, the increase is negligible compared to the other two stresses. 282 construct

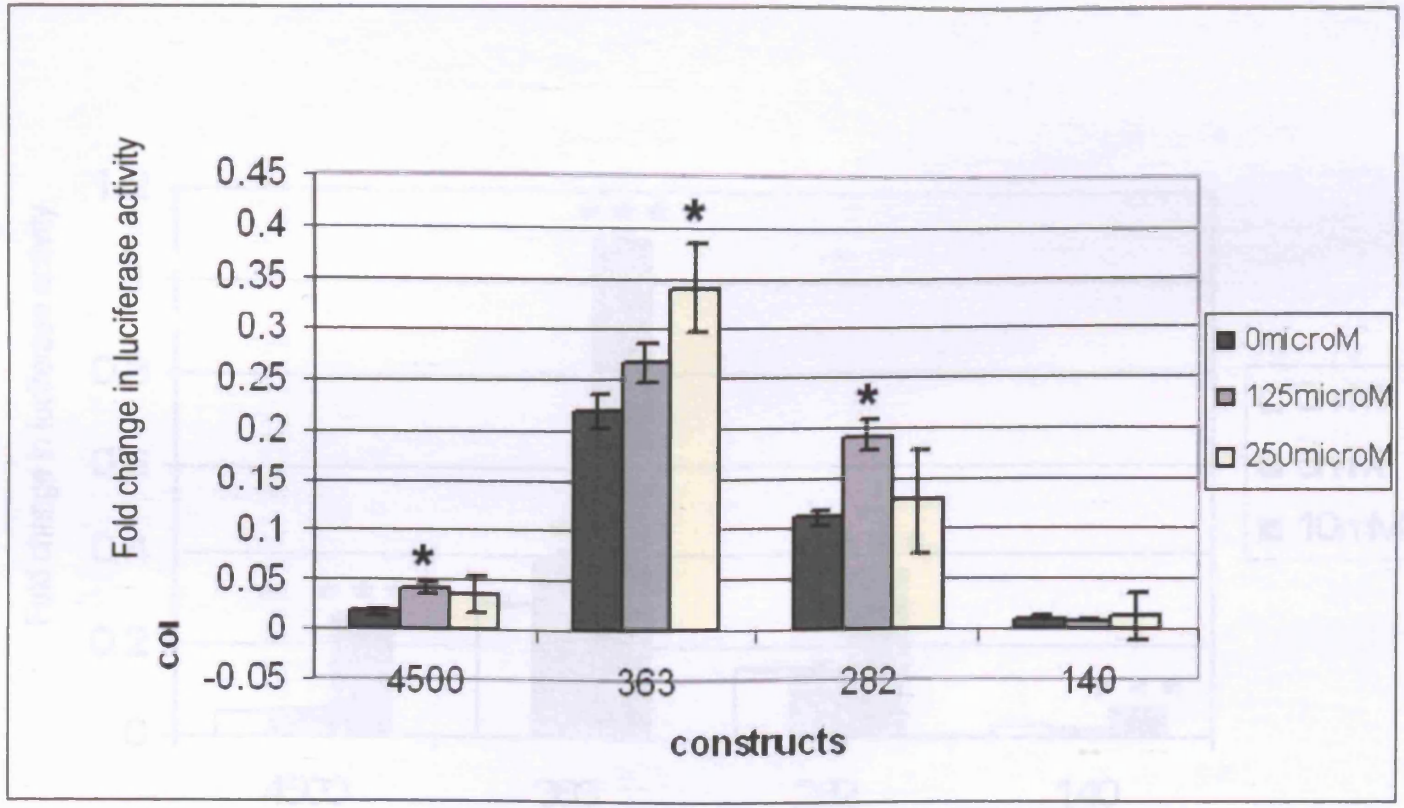


Figure 7.9 Effects of dopamine on parkin promoter activity

Cells were transfected with the different parkin promoter constructs and incubated for 24hrs in 0mM, 125mM and 250mM dopamine in full culture media. Constructs 4500 and 282 in 125mM dopamine were significantly (*$p<0.05$) different from untreated cells. The luciferase activity in cells transfected with construct 363, in 250mM dopamine, was also significantly higher than untreated cells (*$p<0.05$). Luciferase activity for 140 was not significantly altered in the different dosages of dopamine. The luciferase values were corrected for PGL3basic vector activity. The error bars indicate the standard error of the mean of 3 experiments, each performed in triplicates.

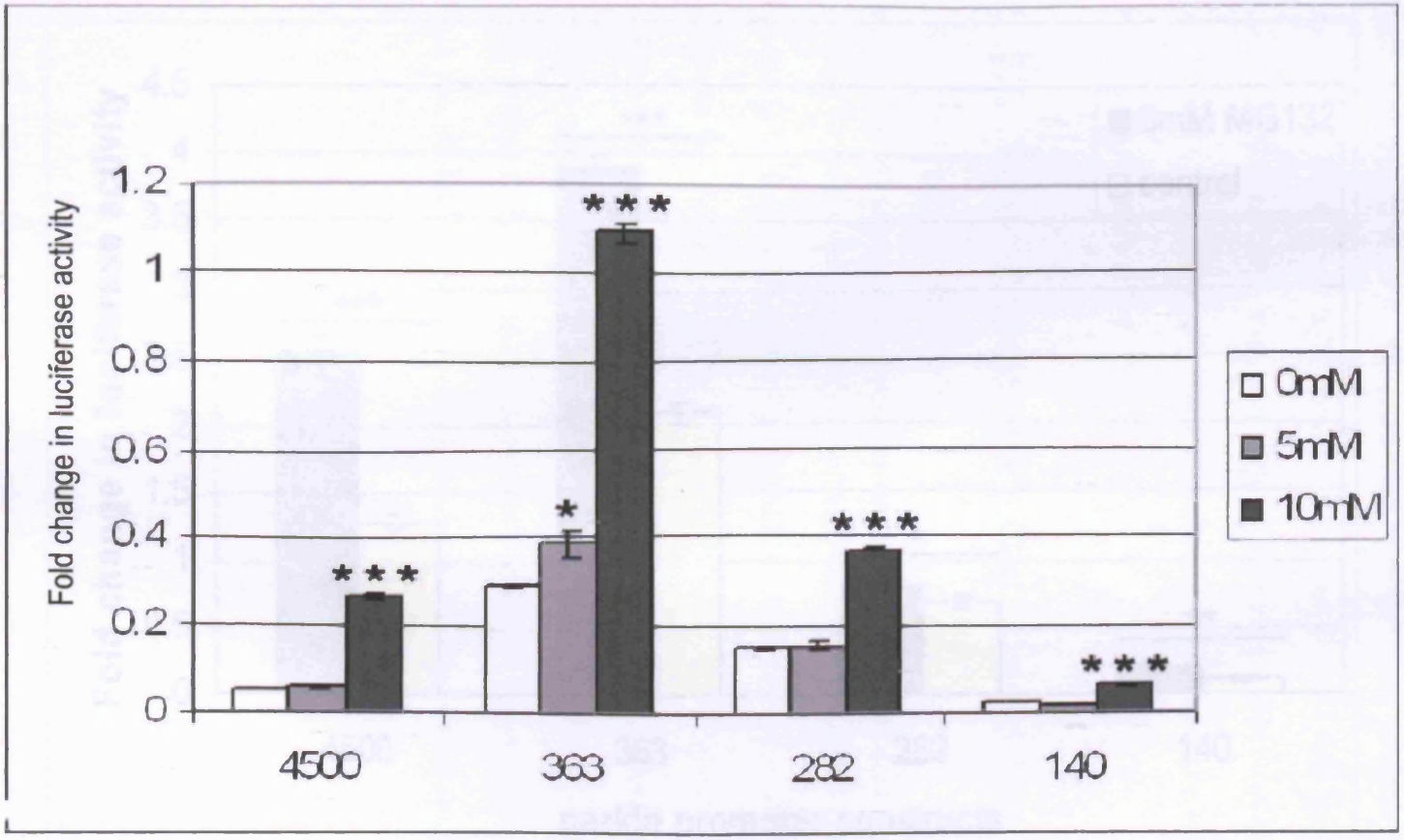


Figure 7.10 Effects of MPP+ on parkin promoter activity
Transfected cells were incubated for 6hrs in 0mM, 5mM & 10mM MPP+ in full culture media. There was a significant increase in luciferase activity for all the constructs tested in 10mM MPP+ (***$p<0.001$) and a less significant increase in activity of construct 363 in 5mM (*<0.05). The luciferase values were corrected for PGL3basic vector activity. The error bars indicate the standard error of the mean of 3 experiments, each performed in triplicates.

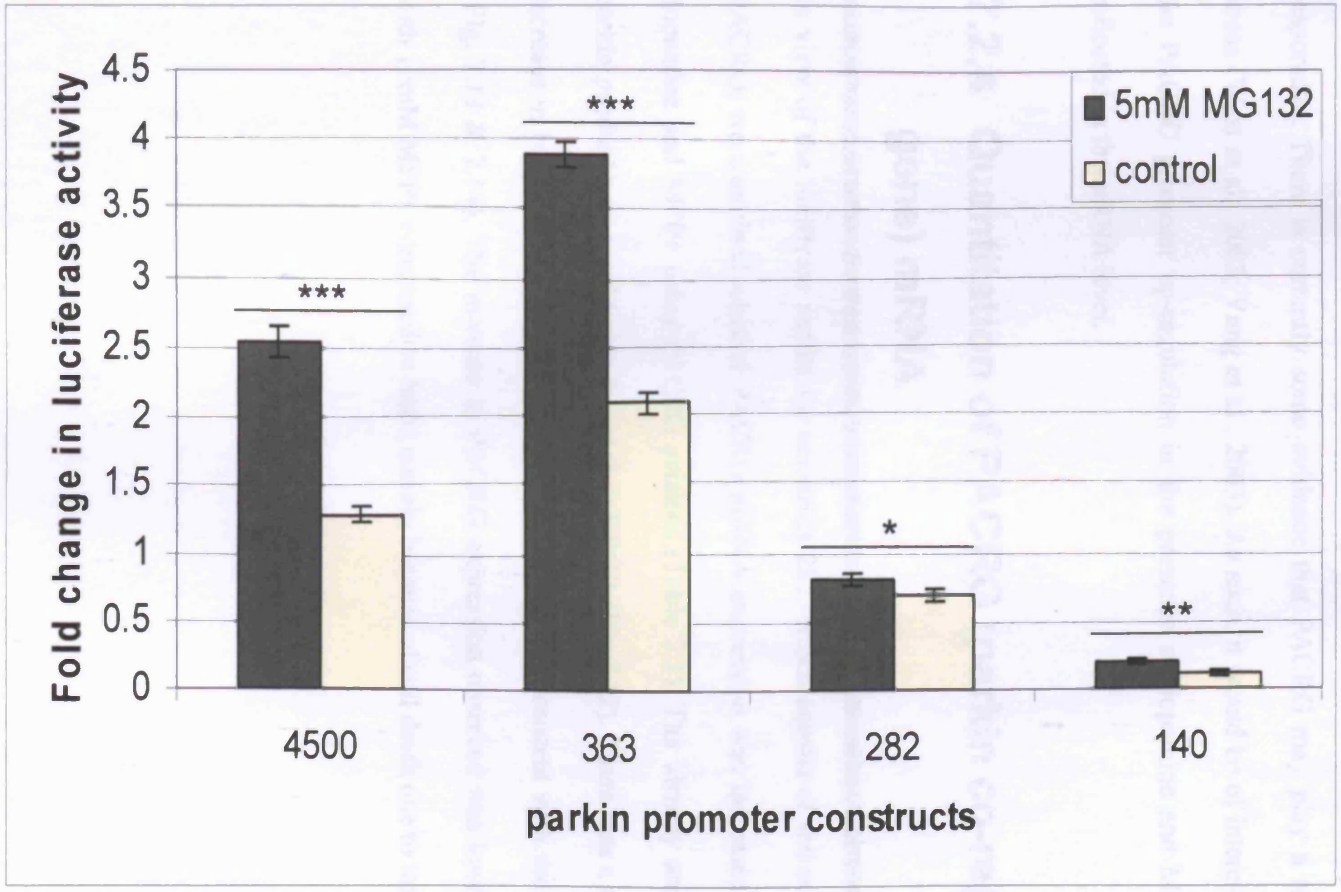


Figure 7.11 Effects of MG132 on parkin promoter activity
Transfected cells were incubated for 24hrs in 0mM and 5mM MG132 in full culture media. There were a significant increases in luciferase activity for all the constructs tested in stressed cells compared to untreated cells (*p<0.05; **p<0.01; ***p<0.001). The luciferase values were corrected for PGL3basic vector activity. Differences was analysed by ANOVA + Bonferroni post hoc test.

consists of the core promoter of the co-regulated anti-sense gene, PACRG, which suggests that activation of the PACRG (parkin co-regulated gene) promoter may also be stress responsive. There is currently some evidence that PACRG may play a role during cell stress (West et al., 2003; Yang et al., 2003). As such, it would be of interest to examine if the PACRG promoter up-regulation in the presence of dopamine and MPP+ would be reflected in the mRNA level.

7.2.4 Quantitation of PACRG (parkin co-regulated gene) mRNA

In view of the luciferase results for construct 282, which consist of the core promoter of PACRG, we examined whether PACRG mRNA expression was increased in response to dopamine and MPP+ using PACRG primers (Table 2.1). The identity and specificity of parkin product was confirmed after each RT PCR (Fig 7.12). There was a dose-dependent increase in both dopamine and MPP+ stressed samples consistent with the luciferase data (Fig. 7.13 & 7.14). The increase in PACRG expression observed was lower when treated with 10mM MPP+ compared to 5mM mainly because of cell death due to toxicity.

Figure 7.12 RT PCR PACRG product agarose gel check
Agarose gel check on PACRG RT PCR products to confirm product identity and PCR specificity. Specific product of the correct size was obtained. Amplified product identity was confirmed by sequencing to be PACRG

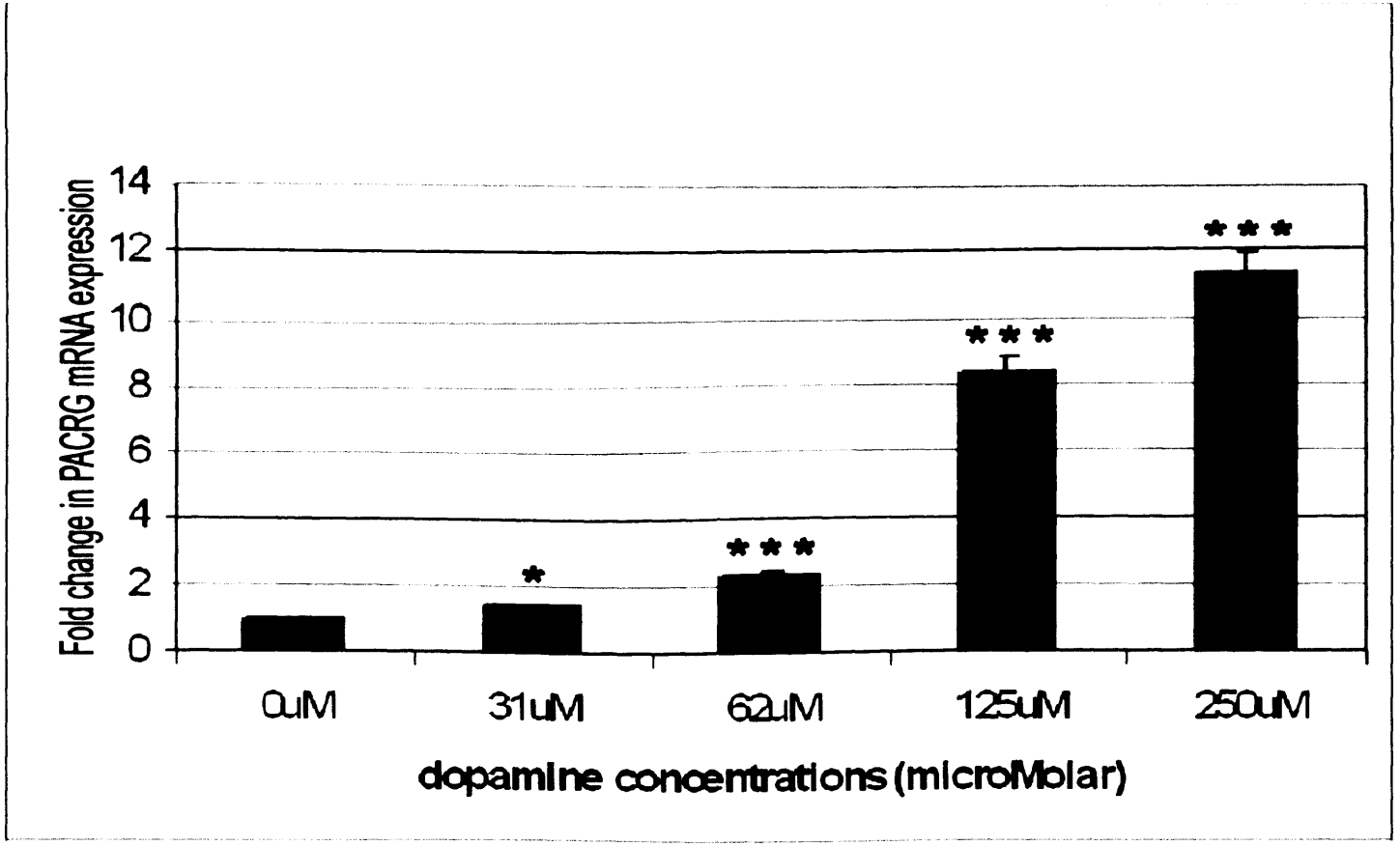


Figure 7.13 Effects of dopamine in PACRG mRNA expression
Cells were incubated for 24hrs in 0mM, 31.25mM, 62.5mM, 125mM and 250mM dopamine in full culture media, before mRNA was retrieved. The results of the RT PCR was normalised onto results obtained using housekeeping gene primers – b2-microglobulin. The error bars indicate the standard error of the mean of 3 experiments. Differences was analysed by ANOVA + Bonferroni post hoc test. The results indicated a significant dose-dependent increase in PACRG mRNA (*$p<0.05$ for 31.25mM, ***$p<0.001$ for 62.5mM, 125mM and 250mM).

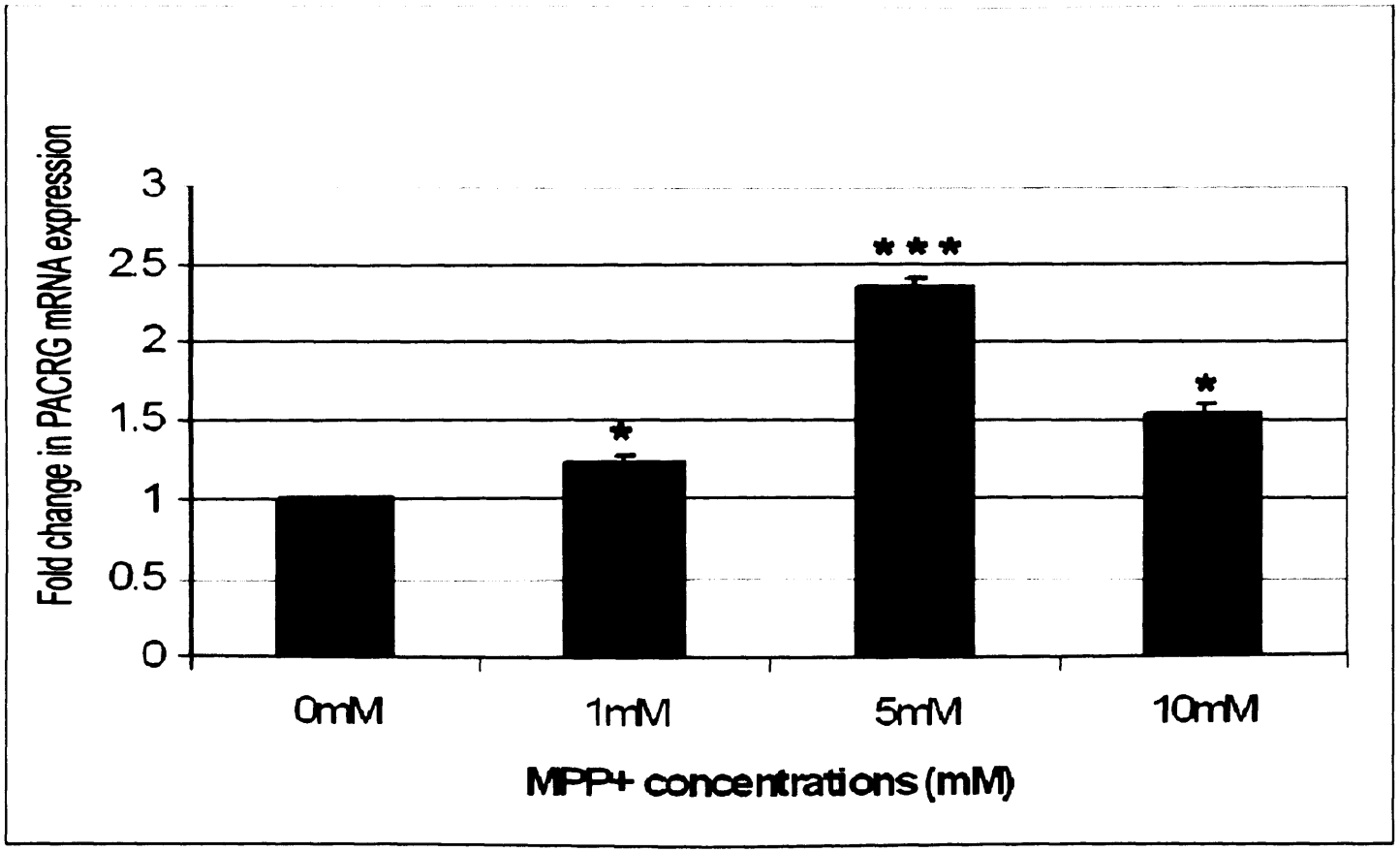


Figure 7.14 Effects of MPP+ on PACRG mRNA expression
Cells were incubated for 6hrs in 0mM, 1mM, 5mM and 10mM MPP+ in full media culture media before mRNA retrieval. Similarly, the results of the RT PCR was normalised onto results obtained using housekeeping gene primers – b2-microglobulin. The error bars indicate the standard error of the mean of 3 experiments. Differences was analysed by ANOVA + Bonferroni post hoc test. All the MPP+ stressed cells showed significant increases in PACRG mRNA with those stressed with 5mM MPP+ showing the highest increase (*$p<0.05$ for 1mM and 10mM, ***$p<0.001$ for 5mM).

7.3 Discussion

Parkin is generally thought to be neuro-protective due to its role in the ubiquitin proteasome system (UPS), facilitating the transfer of ubiquitin to damaged, or misfolded target substrates leading ultimately to their degradation. Mutations of the parkin gene have been associated with a large proportion of familial PD especially early-onset PD. As the age of onset of PD is usually higher in sporadic cases, the role of ageing and environmental stress in these cases becomes more important. There is also accumulating evidence for interplay between oxidative stress and parkin and recently parkin has shown to become more insoluble and inactivated by dopamine (LaVoie et al., 2005). One previous study has also shown that over-expression of parkin in cultured cells may confer protection against dopaminergic stress (Jiang et al., 2004) although we did not observe this in our system (Muqit et al., 2004). However, we have shown that parkin knock-down in cell lines renders them more susceptible to dopaminergic stress (MacCormac et al., 2004). However, no studies to date have focused on the regulation of endogenous parkin at the transcriptional level in the presence of oxidative stress.

In our studies, we have used dopamine, MPP+ and MG132 to determine if they might result in any alterations in the transcription of parkin. These stressors were used as dopamine has been shown to inactivate parkin, and MPP+/MPTP is another potent neurotoxin that generates free radical induced oxidative stress *in vivo* and *in vitro* (Javitch et al., 1985; Javitch and Snyder, 1984; Mizuno et al., 1987; Ramsay et al., 1986). Both these stresses have been reported previously to increase the transcription levels of another PD related gene, α-synuclein (Gomez-Santos et al., 2003). In the case of dopamine stressed cells, it is believed that stress-response kinases such as

SAPK/JNK and p38, as well as factors involved in autophagy, are activated which subsequently activate the expression of α-synuclein. For MPP+ stress, however, the mechanism of up-regulation is not clear. MG132 is a proteasomal inhibitor. As parkin is an E3 ligase involved in the ubiquitin proteasomal system, it would be interesting to observe the effects of MG132 on parkin regulation.

We show here that dopamine treatment results in up-regulation of parkin at both the mRNA and protein level. Moreover, this appears to be due to transcriptional activation since luciferase assays confirmed that specific parkin promoter constructs could confer transcriptional activation in response to dopamine. It is surprising that we did not see induction of luciferase for the 4500 construct (full length promoter) in light of our mRNA findings. However, this suggests that there may be additional enhancer sequences in the endogenous parkin promoter of SH-SY5Y cells. The possible role of alteration in chromatin structure as a regulation control also cannot be over-looked. The parkin protein cellular distribution also appeared to be altered during dopamine treatment. In spite of an overall increase in total parkin protein, there was a decrease in soluble parkin protein. This indicated a shift in the solubility of parkin and is in line with previous reports of increased insolubility of parkin in the presence of dopamine (LaVoie et al., 2005; Petit et al., 2005). Whether the solubility shift in this case was due to the molecular alterations caused by dopamine or to the increase in parkin is not known.

Up-regulation of parkin also appears to be a general response to oxidative stress since MPP+ also caused up-regulation in parkin mRNA and activation of parkin promoter constructs. In addition, we also showed that possible repressor domains might be

present in the promoter region upstream of the core parkin promoter, since the activation of the promoter present in construct 4500 is less than that of the core promoter.

Treatment with proteasomal inhibitor, MG132, up-regulated both the promoter activity and mRNA expression of parkin in SH-SY5Y cells. Therefore, like dopamine and MPP+, MG132 is able to stimulate parkin expression. However at this stage such conclusions are tentative and follow-on studies are required for confirmation. This is of special significance due to a contemporary publication reporting that MG132 repressed parkin expression (Koch et al., 2008).

The strength of the Koch *et. al.* result is difficult to judge since the relevant MG132 result was not shown in their report. Furthermore, the experimental design differed in that rat adrenal medulla PC12 cells were used for the study. The differences in the origin of the cell line used, PC12 versus SH-SY5Y, may account for the differences in results on the experiments. Results on using an alternative proteasomal inhibitor, epoxomicin, were shown for both PC12 and SH-SY5Y cells. However, it is not clear whether the displayed results can be used to support the interpretation since the number of experimental replicates was not specified, there was no statistical testing and some results appear skewed. For example, the figure 1c graph showing parkin mRNA expression in SH-SY5Y cells treated with epoxomicin, showed increasing parkin mRNA in untreated cells over time that was interpreted as a decrease in parkin mRNA in treated cells. In addition, although both MG132 and epoxomicin are proteasomal inhibitors, they may act differently thus leading to different regulational responses. In fact, even Koch *et. al.* claimed that they were surprised by own results.

Publications on the effects of proteasomal inhibition on parkin expression are limited in number to support either our present study or the Koch *et. al.* paper. Indeed, the only other mention in literature, Biasini *et. al.* showed that there was no significant change in parkin expression under proteasomal inhibition (Biasini et al., 2004). Despite the differences in the results and interpretations regarding proteasomal inhibition and parkin, the findings described in the present study follow the rationale that with proteasomal inhibition, protein degradation and the elimination of damaged proteins would cease. Consequently, parkin and other proteasomal associated genes may be up-regulated by positive feed-back due to excessive build of parkin. This will be discussed in more detail in chapter 9.

It remains unknown whether our findings in undifferentiated SH-SY5Y cells occur in human brain *in vivo*. Further studies will therefore be required to be performed in differentiated SH-SY5Y cells, primary neurons as well as human post-mortem brain *in vivo* to confirm our findings.

Our results also show that PACRG was upregulated in both dopamine and MPP+ stressed cells. PACRG (parkin co-regulated gene) is a recently identified gene whose function is not yet clearly known. PACRG gene is 0.6Mb long and transcriptionally starts at 204bp upstream and in antisense to parkin (West et al., 2003). There is some evidence that PACRG may play a role in protection against stress-induced cell death (West et al., 2003; Yang et al., 2003). However, the significance of PACRG in PD is unclear – notably, the PACRG/parkin knockout mouse mutant, *Quaking*, does not exhibit nigral degeneration (Farrer et al., 2001b; Lorenzetti et al., 2004).

It is still unclear how oxidative stress causes transcriptional activation of the parkin gene. It is likely that certain transcriptional factors that are stress-related are up-regulated and activate specific parkin promoter domains. Therefore, we analyzed the parkin core promoter sequence for putative stress-related transcription factor sites. Using MatInspector, version 7.4, (Cartharius et al., 2005; Cross et al., 1994) several potential transcription factor sites of oxidative-stress-induced transcription factors were found in the parkin core promoter. These transcription factors include stimulating protein 1 (SP1) and AP-1/CREB. They are known to be oxidative stress inducible and are increased or activated upon such stresses to enhance the transcription of prosurvival genes, which subsequently prevent cell death in response to the stress, DNA damage or both (Ryu et al., 2003; Shi et al., 2004). Nrf1, is also another bZIP family transcription factor, whose potential binding site was predicted to be present within the parkin promoter. This transcription factor has also been shown to respond to oxidative stresses (Kwong et al., 1999). It would be of interest to determine if these factors play any role in the regulation of parkin.

In conclusion, we have shown that when subjected to oxidative stresses such as dopamine and MPP+, the transcription of endogenous parkin increases. This may be a compensatory up-regulation in response to decreased parkin activity and decreased parkin solubility. The next step will be to investigate stress-specific transcription factors that may regulate parkin and determine whether these are altered in PD.

Chapter 8: Regulation of PINK1 in oxidative stress & other analysis

8.1 Introduction

As previously discussed, PINK1 is considered a Parkinson's disease associated gene. In this chapter regulational, functional and structural characteristics of PINK1 will be studied. The importance of stress conditions in Parkinson's disease has already been explained in previous chapters; therefore the response of PINK1 expression to these conditions was studied. Mutation of the protein is also a critical factor in Parkinson's disease, a variety of mutated forms of PINK1 expressed by patients were studied to reveal their effects in cell culture. Protein cleavage is also believed to be very important for its function, for this reason, random mutations in predicted region of PINK1 cleavage site were tested for cleavage properties. These studies combined should give a significant insight into PINK function and regulation in relation to Parkinson's disease.

8.1.1 Regulation of PINK1 in stress

The function of PINK1 is still currently unknown; however, studies have given a degree of insight into possible actions of this protein. It is produced as a precursor protein that is targeted to the mitochondria where it is then cleaved (Muqit et al., 2006). The cleaved forms of PINK1, in turn, localises mainly in the cytosol and to a smaller extent, in the mitochondria (Takatori et al., 2008; Weihofen et al., 2008). Unlike other mitochondrial proteins, the active, cleaved forms of PINK1 are subsequently degraded by the proteasome (Lin and Kang, 2008).

PINK1 is mainly known to be a cytoprotective protein. Wild type cytosolic PINK1 have been shown to protect neuronal cells against mitochondrial and oxidative stress caused by MPTP (Haque et al., 2008). Overexpression of PINK1 was also found to prevent

staurosprine-induced apoptosis (Petit et al., 2005) and its knock-down increased cell vulnerability to rotenone as well as MPP+ (Deng et al., 2005). Intact PINK1 kinase activity was also found to be crucial in activating its substrate TRAP1, which subsequently protects cells against oxidative stresses (Pridgeon et al., 2007). Lastly, exposure to the apoptotic inducing proteasome inhibitor, MG132, was shown to result in PINK1 aggregation (Muqit et al., 2006). It is evident that PINK1 is functionally involved in cellular pathways linked to stress; however, these studies have so far been limited to the protein level and no evidence of whether PINK1 regulation may be affected by the presence of these stresses has been obtained.

8.1.2 PINK1 heterozygous mutations

The majority of mutations of the PINK1 gene have been reported within the kinase domain (Abou-Sleiman et al., 2006b; Bonifati et al., 2005; Hatano et al., 2004; Li et al., 2005; Rogaeva et al., 2004; Rohe et al., 2004; Valente et al., 2004a; Valente et al., 2004b). The reason for this higher rate of detection in this region is because kinase domain is a functional domain of PINK1; hence mutations in this area are more readily detectable. This also indicates that disruption of this kinase activity is deemed the most probable disease mechanism.

All the mutations found previously were detected in homozygous situations in rare familial Parkinson's disease patients and until recently, have been believed to only be pathogenic when occurring homozygously. Nonetheless, heterozygous mutations have recently been discovered by Abou-Sleiman *et.al.* (Abou-Sleiman et al., 2006a) in 1.2% of the tested patients (9 in 768) who all apparently suffered from typical late onset idiopathic Parkinson's disease. Seven of these heterozygous PINK1 mutations found

were missense mutations in the kinase domain, a further missense in the C-terminal hydrophilic region, and a nonsense mutation at codon 456 which resulted in truncation of the last 125 amino acids were also discovered (Abou-Sleiman et al., 2006a). Despite extensive sequencing and assessment of gene arrangements, a second mutation was not found in any of these nine patients. Another three PINK1 heterozygous mutations were subsequently found, in this same study, in 1,536 control subjects. However, a much greater incidence of mutations was detected in the Parkinson's disease cohort, showing that heterozygous PINK1 mutations posed a risk factor in the development of late onset Parkinson's disease (Abou-Sleiman et al., 2006a). Three of the nine patient mutations were selected for *in vitro* functional analysis and were found to adversely affect PINK1 function and thus impaired mitochondrial membrane potential leading to an increase in cellular susceptibility to stress.

8.1.3 Mitochondrial membrane potential $\Delta\psi m$

To determine the functional consequences of PINK1 mutations, the effect of the mutant protein on mitochondrial function was examined.

To examine mitochondrial function, biochemical studies have been used to examine various parameters of bioenergetic function such as electron transport chain activity in mitochondrial preparations (Merlo-Pich et al., 2004). Fluorometric assays and fluorescence imaging techniques have also been used in the study of mitochondrial membrane potential ($\Delta\psi m$) in single cells or populations of cells (Abou-Sleiman et al., 2006b). The $\Delta\psi m$ is the main electrical component of the protomotive force and is central to mitochondrial function since it provides the force that drives the influx of protons (crucial for ATP synthase) or of calcium into the mitochondria as well as

determining the generation of the free radical superoxide (O_2^-). Depolarisation of $\Delta\psi m$ usually reflects opening of the mitochondrial permeability transition pore (mPTP).

Measurement of the $\Delta\psi m$ has become established as a sensitive indicator for the energetic state of the mitochondria and the cell (Brand et al., 1994) and previously cationic probes have been used to measure $\Delta\psi m$ isolated mitochondria. More recent techniques utilize fluorescent membrane permeable lipophilic cationic dyes that can cross the plasma membrane and be taken up by mitochondria in response to the electrochemical gradient and $\Delta\psi m$. These compounds include tetramethylrhodamine ethyl and methyl esters (TMRE and TMRM respectively). The 'redistribution method' to study the dyes is used in our studies (Duchen, 2004). This method is described below.

The principle of the 'redistribution method' is that the fluorescence signal of the compound is directly proportional to the dye concentration which represents the distribution of dye in response to the potential difference across membranes. Thus mitochondria with a higher membrane potential (-150mV) will result in a greater concentration of the dye from the cytosol (400-800 fold) compared to that conferred by the plasma membrane (-60mV) from extracellular space (10 fold). Therefore the available fluorescent probes are relatively selective for mitochondrial function. Measurements are normally taken after the dye has reached steady-state equilibrium.

In the following studies into PINK1 mutations, the redistribution of TMRM in SH-SY5Y cells was examined and populations of cells with altered PINK1 levels exposed to different conditions namely vehicle or proteasomal stress were compared. It was assumed, in these studies, that after equilibrium has been reached, the mitochondrial

fluorescence signal would reflect the mitochondrial dye concentration that would be a direct function of the $\Delta\psi m$. Thus in the presence of proteasomal stress, $\Delta\psi m$ would decrease, and less dye would accumulate in the mitochondria. Therefore, the mean fluorescent signal of the dye in stressed cells would be less than control. The signal can thus be determined using methods such as confocal microscopy. Fluorescence activated cell sorting (FACs) was selected for quantitative analysis due to the efficiency and high throughput of the method, allowing a large number of cells to be analysed for inter-group comparison of different samples. It is possible to measure by FACS the relative reduction in mean fluorescence intensity (MFI), which can be interpreted as an indication of the $\Delta\psi m$ dissipation (Zoratti and Szabo, 1995).

8.1.4 PINK1 cleavage

Mutations in the PINK1 gene leading to familial Parkinson's disease as well as increased susceptibility to the disease in the case of heterozygous occurrence have brought PINK1 to the attention of many Parkinson's disease researchers. Although the majority of the disease causing mutations are located in the kinase domain, which is the only functional domain found in PINK1, a subset of mutations were found outside, mainly in the N-terminal region between the mitochondrial targeting motif and the kinase domain. It is currently still not clear how these mutations may affect PINK1 function. Findings that PINK1 is cleaved to produce a 10kDa N-terminal fragment, approximately 100 amino acids long (Beilina et al., 2005; Muqit et al., 2006), raised the possibility that some of these mutations may act to disrupt the processing of precursor PINK1 to its active form.

8.1.5 Apoptosis assay

Cell death via apoptosis and necrosis differ in that cell membranes maintain their integrity during the early stages of apoptosis, while they become leaky during necrotic cell death. Several methods of identifying apoptotic cells are available. In our studies, we utilised the detection of early apoptosis by measurement of AnnexinV-PI.

During cell death by apoptosis or necrosis, phosphatidylserine, a protein that interacts with the inner cell membrane is translocated to the outer layer of the membrane. This serves as one of several signals by which cells destined for death are recognised by phagocytes. To detect early apoptosis, Annexin V may be used. Annexin V is a member of a highly conserved family that bind acidic phospholipids in a calcium-dependent manner. Annexin V binds to the phosphatidylserine present on the cell surface in the initial stages of cell death. To differentiate apoptotic cell death from necrotic cell death, a dye exclusion test using propidium iodide (PI), which establishes whether the cell membrane intergrity is conserved or leaky, is included. The measurement of Annexin V-PI by various means such as flow cytometry, light or electron microscopy may be used to identify early apoptotic cells. Fluorescence activated cell sorting (FACs) was used in our studies to calculate the percentage of early apoptotic cells in total cells. This system was used as it allows large quantities of cells to be analysed in a short space of time, and is therefore a robust method for inter-group comparisons.

8.1.6 Hypothesis & Aims

Much *in vitro* and *in vivo* evidence have shown the protective function of PINK1 in presence of various stresses. For example, PINK1 prevents mitochondria cytochrome C release, and thus cell death, in the presence of oxidative stress by activating TRAP1 via phosphorylation through the kinase domain in PINK1. In this study of PINK1, in conjuction with previous chapters, we sought to determine if the increased protection produced by PINK1 during such conditions might partly, also be due to alterations in PINK1 regulation in the presence of such stresses.

To better understand the function of PINK1, mutation and protein analysis of PINK1 were also carried out. Three heterozygous PINK1 mutations were discovered in control subjects by Abou-Sleiman et.al. 2006. Two of these mutations were examined in vitro, to find out if they affect PINK1 function, similar to other heterozygous mutations reported in the paper. As there is strong evidence that proteasomal inhibition induces mitochondrial depolarisation and apoptosis, MG132 was selected as a stress agent for the experiments.

Considering the possible importance of PINK1 cleavage to its function, cleavage site was studied to find out how mutations in the vicinity of this site affect the processing and function of PINK1.

Aims of Project:

15. To find out if regulation of PINK1 is altered in the presence of different stresses.

16. To investigate if the two PINK1 mutations found in control patients affect PINK1 function using the measure of mitochondria membrane potential in the presence of proteasomal stress.

17a. To investigate the location of PINK1 cleavage site by utilising PINK1 expressing constructs with point mutations in and surrounding the predicted cleavage site.

17b. To examine the functional consequences when PINK1 cleavage site is disrupted.

8.2 Results

8.2.1 Regulation of PINK1 in stress

PINK1 expression was examined in SH-SY5Y cells subjected to various stresses, oxidative stress: dopamine, mitochondrial complex I inhibitor, MPP+ and proteasomal inhibitor, MG132. RT-PCR was used to quantify PINK1 mRNA in these cells and expression was compared to at least two other housekeeping genes to ensure consistency in results (one representative graph for each stress is shown). The identity and specificity of PINK1 product was confirmed after each RT PCR (Fig. 8.1).

All the stresses caused a strong response on PINK1. Dopamine and MG132 both resulted in a decrease in PINK1 mRNA expression, however, the down-regulation induced by MG132 was much more substantial than that of dopamine with a 60% decrease compared to 35% (Fig. 8.2 and 8.3). The profile of the response exerted by MPP+ was opposite to that of the other stresses. MPP+ treatment caused a 70% increase in PINK1 mRNA expression (Fig. 8.4).

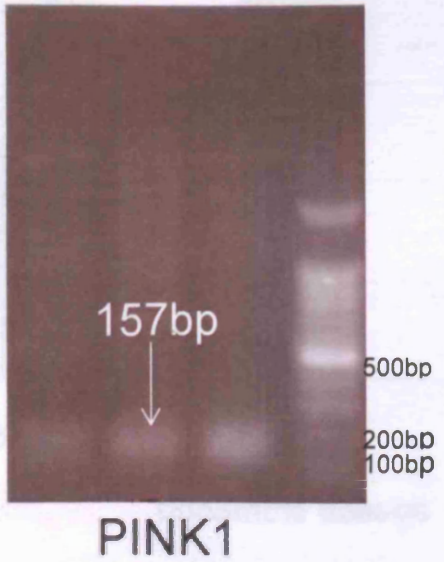


Figure 8.1 RT PCR PINK1 product agarose gel check
Agarose gel check on RT PCR PINK1 product to confirm product identity and PCR specificity. Specific product of the correct size was obtained. Amplified product was confirmed to be PINK1 by sequencing.

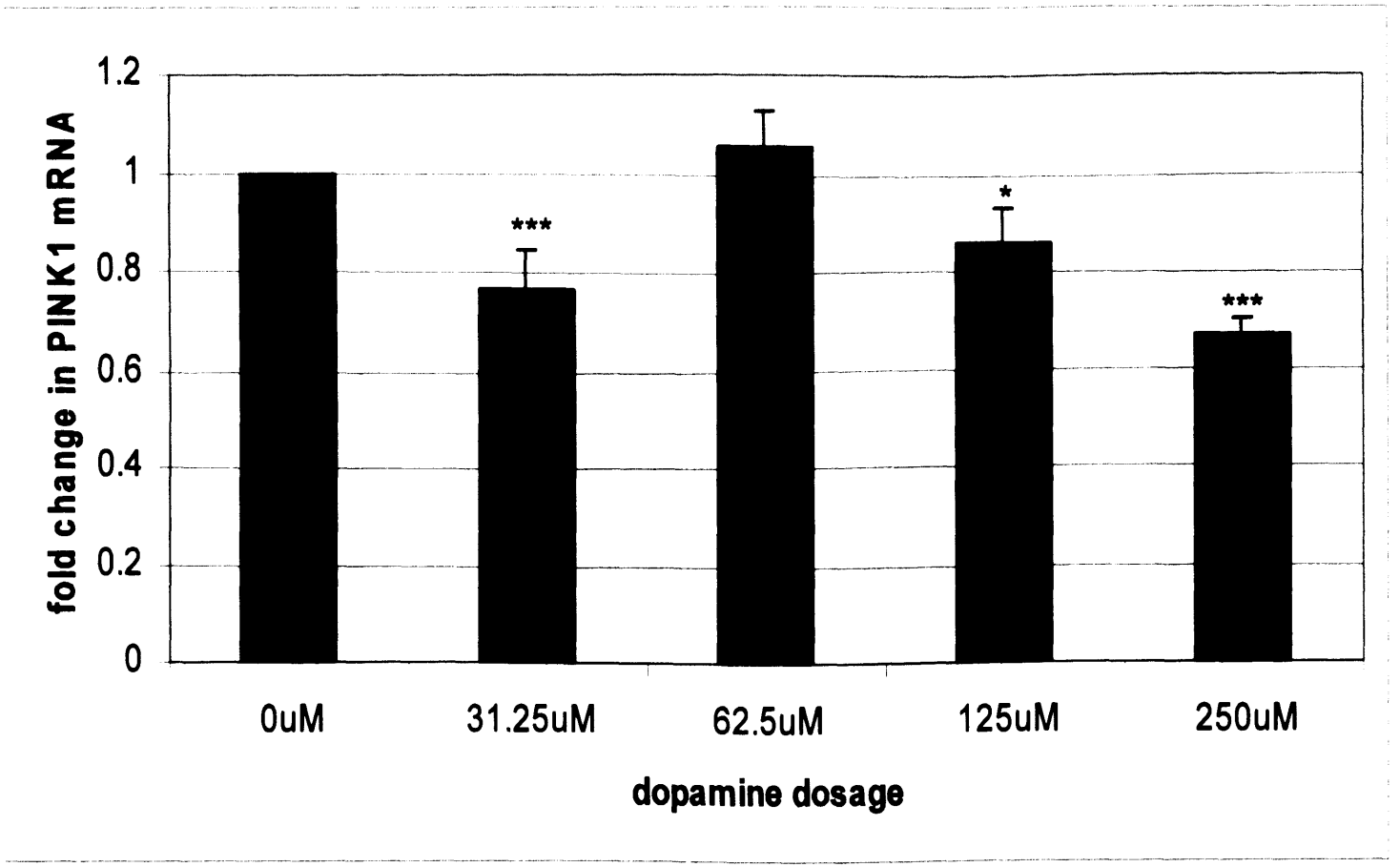


Figure 8.2 Effects of dopamine on PINK1 mRNA expression
SH-SY5Y cells were incubated with 0mM, 31.25mM, 62.5mM, 125mM and 250mM dopamine for 24hrs before RNA was extracted for analysis. The error bars indicate the standard error of the mean of 3 experiments performed in triplicate. Differences was analysed by ANOVA + Bonferroni post hoc test. There is an overall decrease in PINK1 expression with the increase in dopamine dosage (*$p<0.05$; ***$p<0.001$).

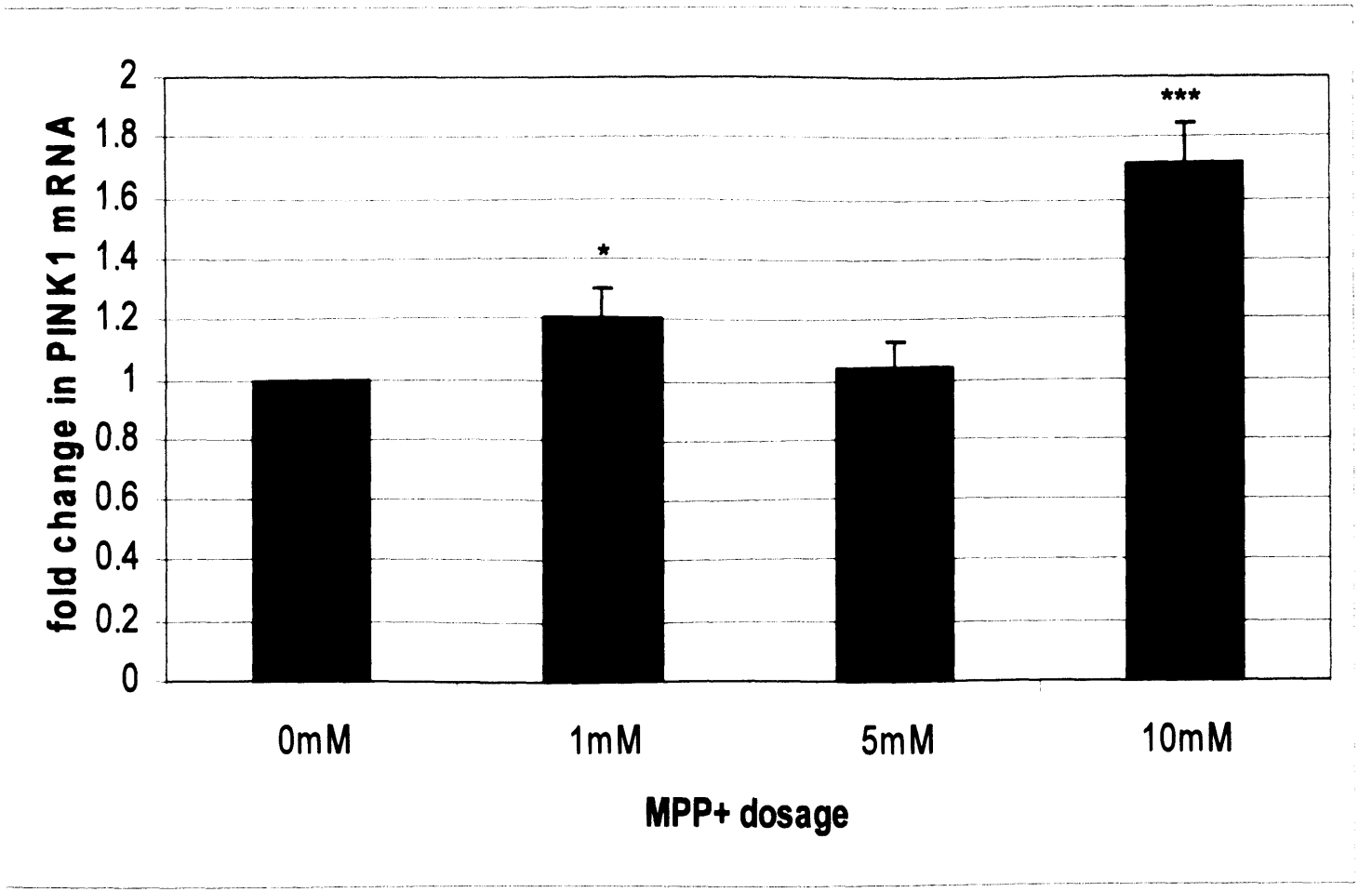


Figure 8.3 Effects of MPP+ on PINK1 mRNA expression
SH-SY5Y cells were incubated with 0mM, 1mM, 5mM and 10mM MPP+ for 6hrs before RNA was extracted for analysis. The error bars indicate the standard error of the mean of 3 experiments performed in triplicate. Differences was analysed by ANOVA + Bonferroni post hoc test. There is an overall increase in PINK1 expression with the increase in MPP+ dosage (*$p<0.05$; ***$p<0.001$).

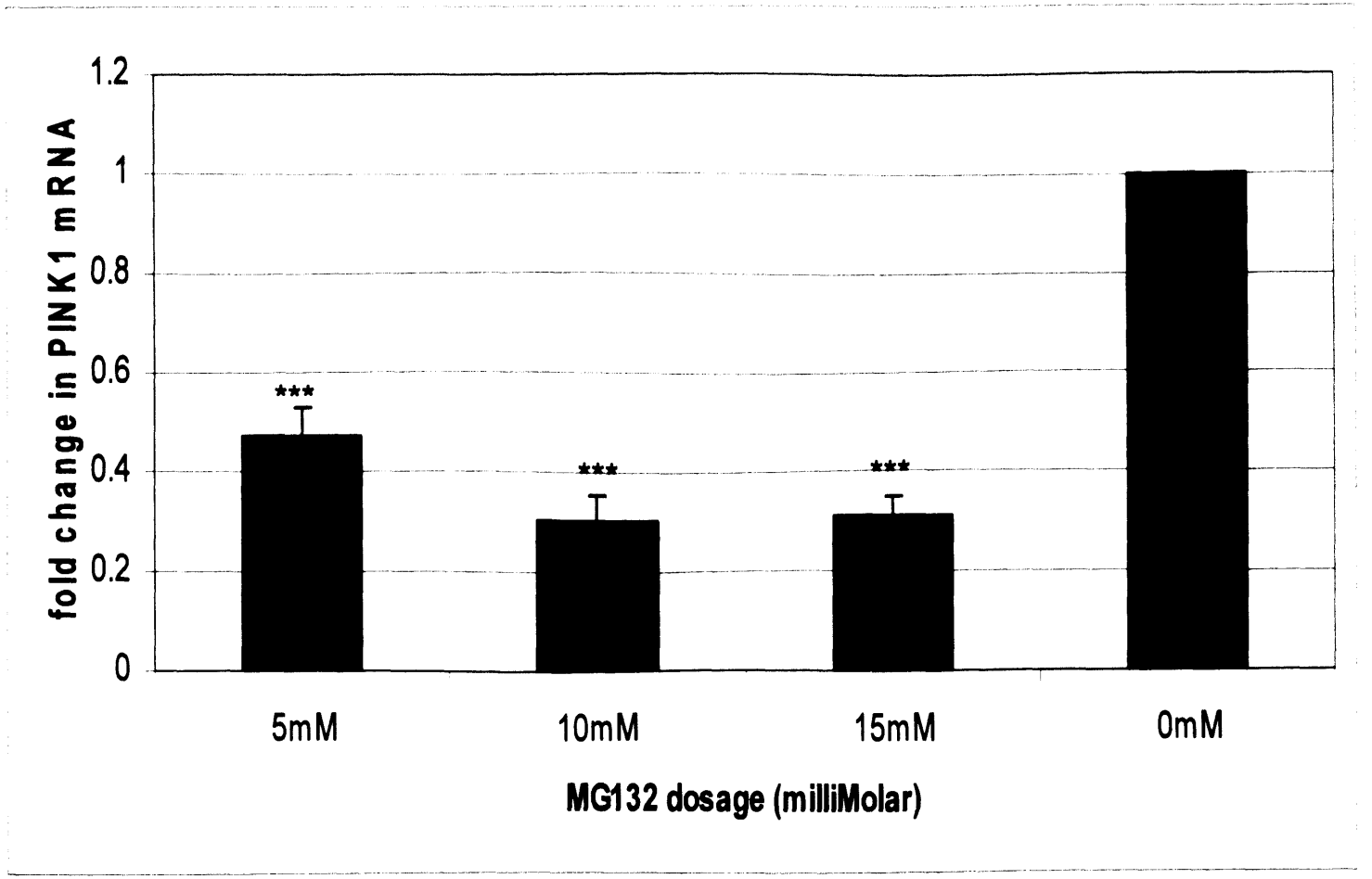


Figure 8.4 Effects of MG132 on PINK1 mRNA expression
SH-SY5Y cells were incubated with 0mM, 5mM, 10mM and 15mM MG132 for 24hrs before RNA was extracted for analysis. The error bars indicate the standard error of the mean of 3 experiments performed in triplicate. Differences was analysed by ANOVA + Bonferroni post hoc test. There is significant decrease in PINK1 expression with MG132 stress (***$p<0.001$).

8.2.2 Pathogenecity of PINK1 heterozygous mutants

It has been shown previously that the G309D *PINK1* homozygous mutation impairs $\Delta\psi m$ following cellular stress rendering cells more susceptible to the stress (Valente et al., 2004a; Valente et al., 2004b). Homozygous mutants Y431H, N451S and C575R which were identified in PD patients were also recently found to similarly impair $\Delta\psi m$ following cellular stress (Abou-Sleiman et al., 2006a), thus making them potentially pathogenic and a possible candidate for the onset of PD. E476K and R501P are heterozygous mutants identified in normal controls.

To investigate if these heterozygous mutations found in control patients were potentially pathogenic especially in the presence of stress, they were subjected to stress induced alterations in mitochondrial membrane potential ($\Delta\psi m$) and examined. The addition of the proteosomal inhibitor, MG-132 which induces apoptosis via mitochondrial injury, to native SH-SY5Y cells resulted in a 30% decrease in TMRM fluorescence from basal values (data shown in (Abou-Sleiman et al., 2006a)). It has been shown that decreases in TMRM fluorescence reflected depolarization of the $\Delta\psi m$ (Martin and Koshland, 1991). This was confirmed by Abou-Sleiman *et.al.* (Abou-Sleiman et al., 2006a), showing that native SH-SY5Y cells treated with the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), dissipated the $\Delta\psi m$ by abolishing the linkage between the respiratory chain and the phosphorylation system. Abou-Sleiman *et.al* also showed that following the addition of

CCCP, TMRM values were reduced by 70% compared to basal values (data shown in (Abou-Sleiman et al., 2006a)).

SH-SY5Y cells were transiently co-transfected with wild-type or mutant *PINK1* plasmid and a green fluorescent protein (GFP) reporter plasmid and then treated with vehicle or MG-132 for 24hrs. Analysis of TMRM fluorescence in GFP-positive cells revealed that none of the *PINK1* mutations had a significant effect on $\Delta\psi m$ under basal conditions (Fig. 8.5). However, following stress with MG-132 there was a significant decrease in $\Delta\psi m$ from basal levels in cells transfected with E476K and R501P PINK1 compared with wild-type PINK1 (Fig. 8.6: E476K, -54.7% ± 10.1; R501P, -58.1% ± 10.9 vs. wt PINK1 –16.3% ± 6.0; $^*p < 0.01$; n= 12). In contrast, transfection of wt PINK1 resulted in approximately 50% less reduction in $\Delta\psi m$ compared to vector, following stress suggesting that PINK1 is able to protect against some of the damaging effect of the mutants on $\Delta\psi m$ following stress (Fig. 8.6). Transfection *per se* did not alter $\Delta\psi m$ in our system, whereby following stress, the reduction in $\Delta\psi m$ in vector transfected cells was similar to that of native SH-SY5Y (data shown in (Abou-Sleiman et al., 2006a)).

8.2.3 PINK1 cleavage

From previous evidence of PINK1 cleavage to produce a 10kDa cleaved product, the cleavage site of precursor PINK1 is estimated to be around 100 amino acids from the N-terminal. To locate the precise site of PINK1 cleavage, various PINK1 expressing constructs with random point mutations around the predicted cleavage site, including one known pathogenic PINK1 mutation, were used in western blots to examine if the

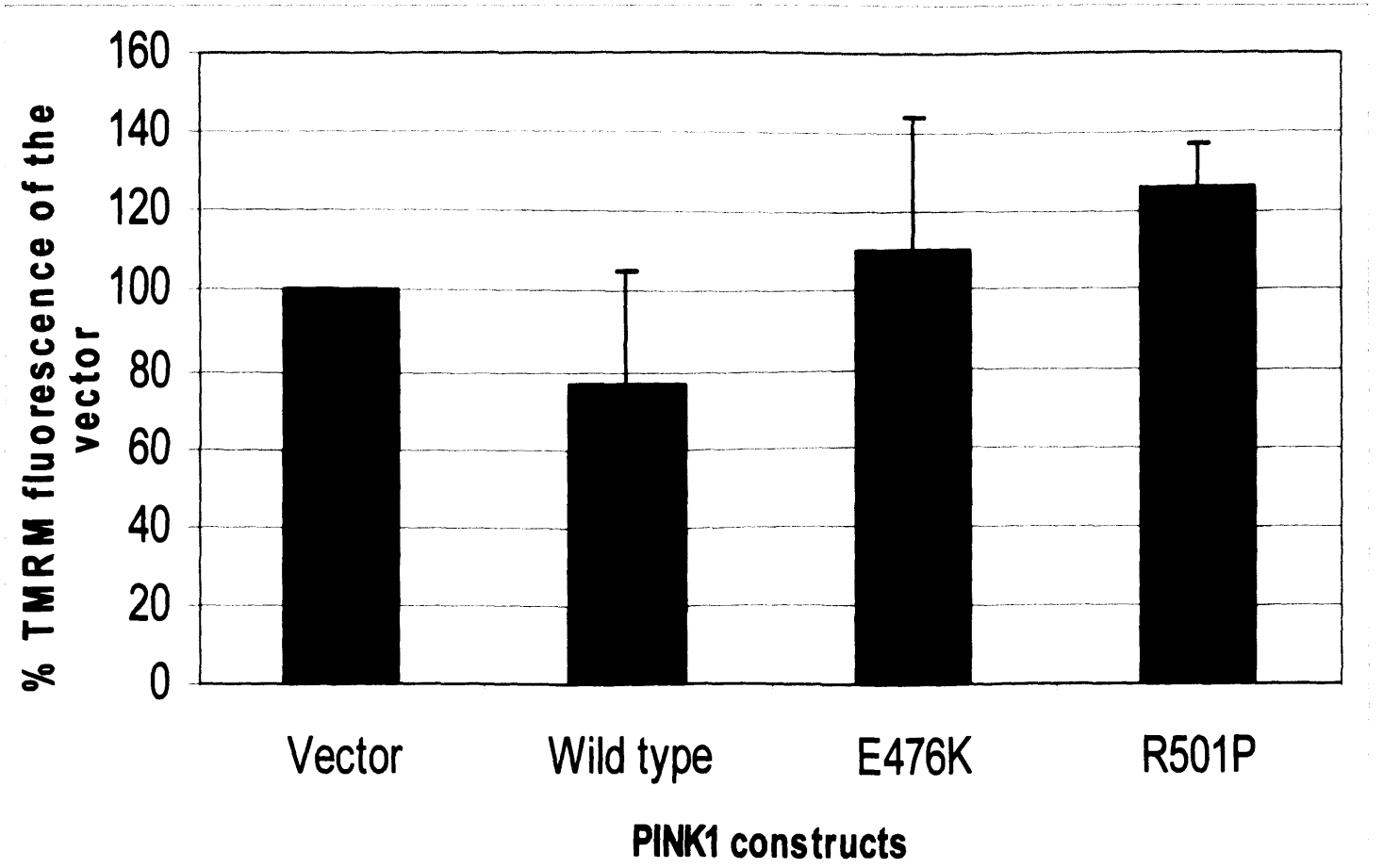


Figure 8.5 Basal Δψm of PINK1 constructs
Median Δψm of cells co-transfected with WT PINK1 construct, E476K or R501P PINK1 mutant constructs and GFP shown as a percentage to empty vector transfected cells (100%). Results were not significantly different

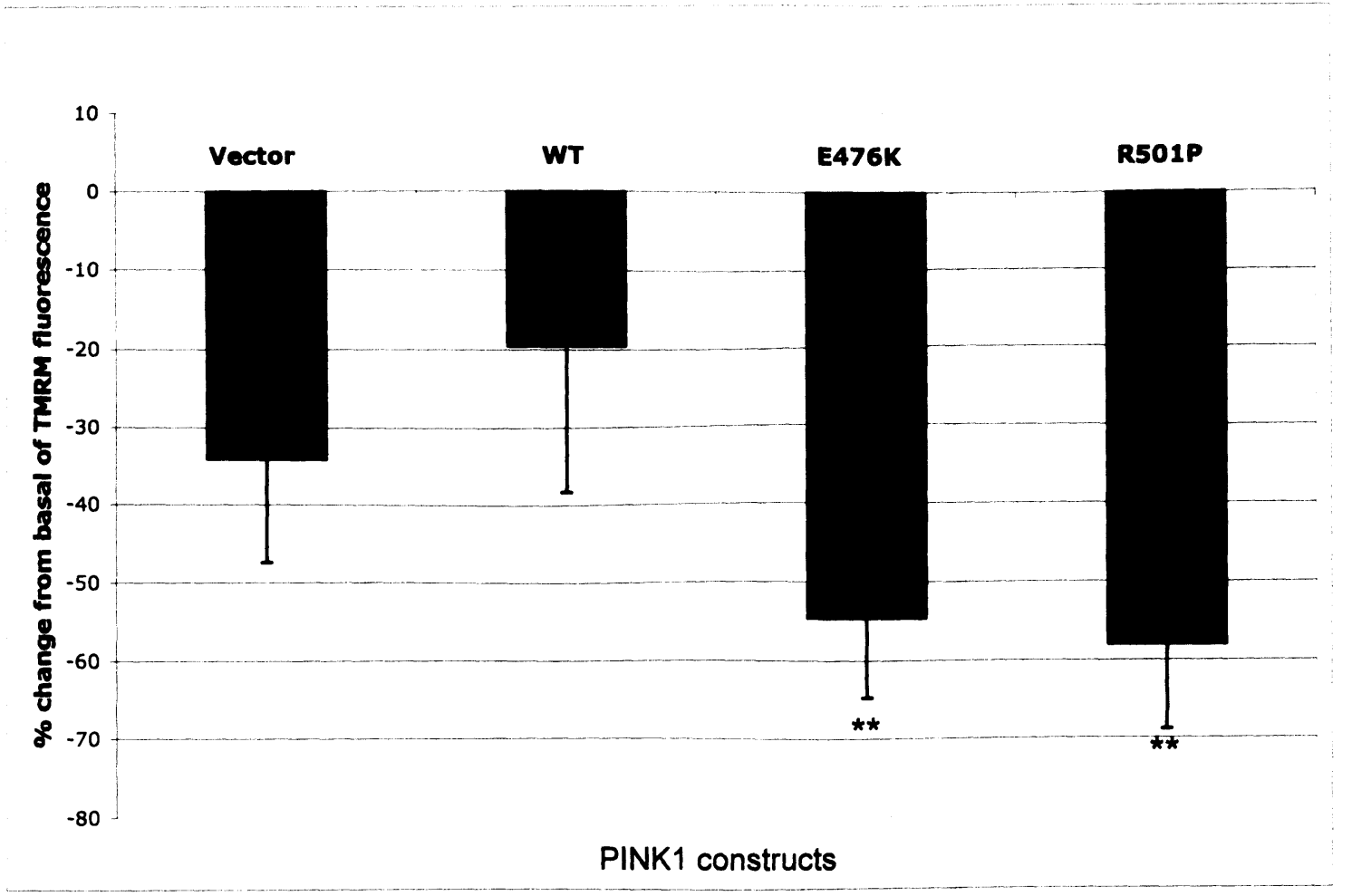


Figure 8.6 Change in Δψm of PINK1 constructs in MG132
Percentage change of median Δψm in GFP positive cells in basal conditions transfected with WT, E476K, R501P and empty vector and those stressed with 15mM MG132 for 24hrs. E476K and R501P fluorescence decreased significantly compared to WT (**$p<0.01$)

presence of these mutations might affect the cleavage process. One of the mutants studied, L102Q PINK1, showed significantly lowered cleavage from full length (Fig. 8.7). It could be deduced that the L102Q mutation either changed the PINK1 cleavage site or altered the structural configuration of PINK1 such that cleavage was blocked (Fig. 8.8). However, the L102Q mutation might also alter the localisation of PINK1, changing the environment, thus, cleavage could not occur. L102Q is a random and not a pathogenic mutation.

To ensure that the localisation of L102Q PINK1 was not changed, and that the lack of cleavage was mainly due to sequence or configuration modifications, fluorescence microscopy was used. L102Q PINK1 was found to localise as normal to the mitochondria, together with MitoTracker (Fig. 8.9).

Cells transfected with wild-type or L02Q mutant PINK1 expressing constructs were exposed to MG132 stress and analysed, using FACs, for the percentage of early apoptotic cells. This allows the investigation of whether cleaved PINK1 is required for the protection of cells against MG132 stress. More L102Q mutant PINK1 transfected cells were apoptotic compared to wild-type in the presence of MG132 stress (Fig. 8.10b). The percentages of apoptotic cells in stress free basal conditions were similar (Fig. 8.10a).

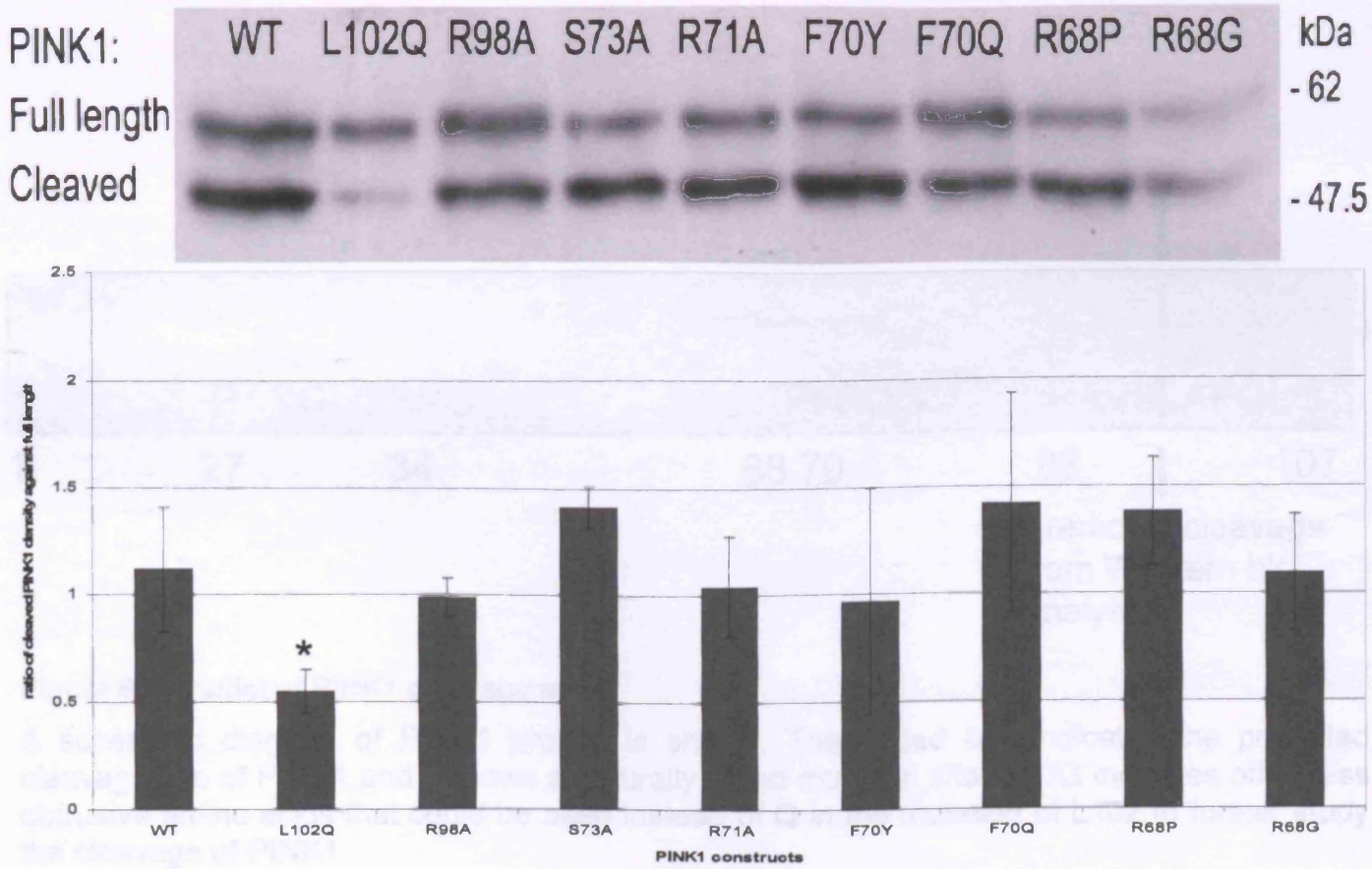


Figure 8.7 Protein cleavage of PINK1 mutants
Protein extract was obtained from cells transfected with wt PINK1 and various PINK1 homozygous mutants. The exposed bands were analysed using GS-800 BioRad densitometer and shown as a ratio of cleaved vs full length PINK1. The error bars indicate the standard error of the mean of 3 experiments. Differences were analysed by paired T-test. The amount of cleaved L102Q PINK1 was significantly lower than its full length product (*$p<0.05$).

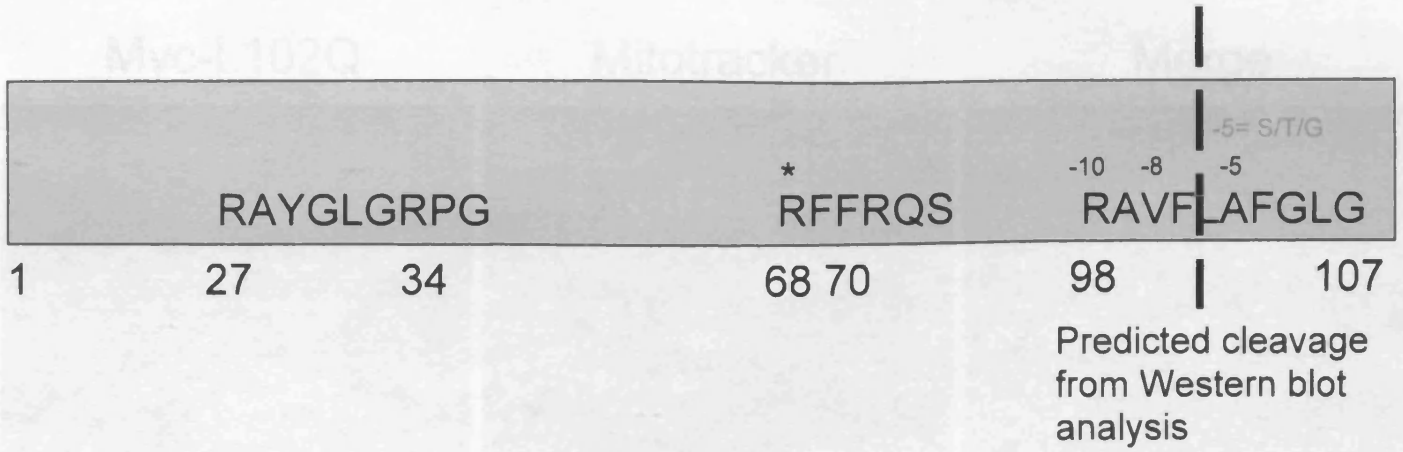


Figure 8.8 Predicted PINK1 cleavage site

A schematic diagram of PINK1 protein is shown. The dotted line indicates the predicted cleavage site of PINK1 and * shows a naturally found mutation site. S/T/G indicates other less obtrusive amino acids that could be used instead of Q in the mutation of L102 to further study the cleavage of PINK1

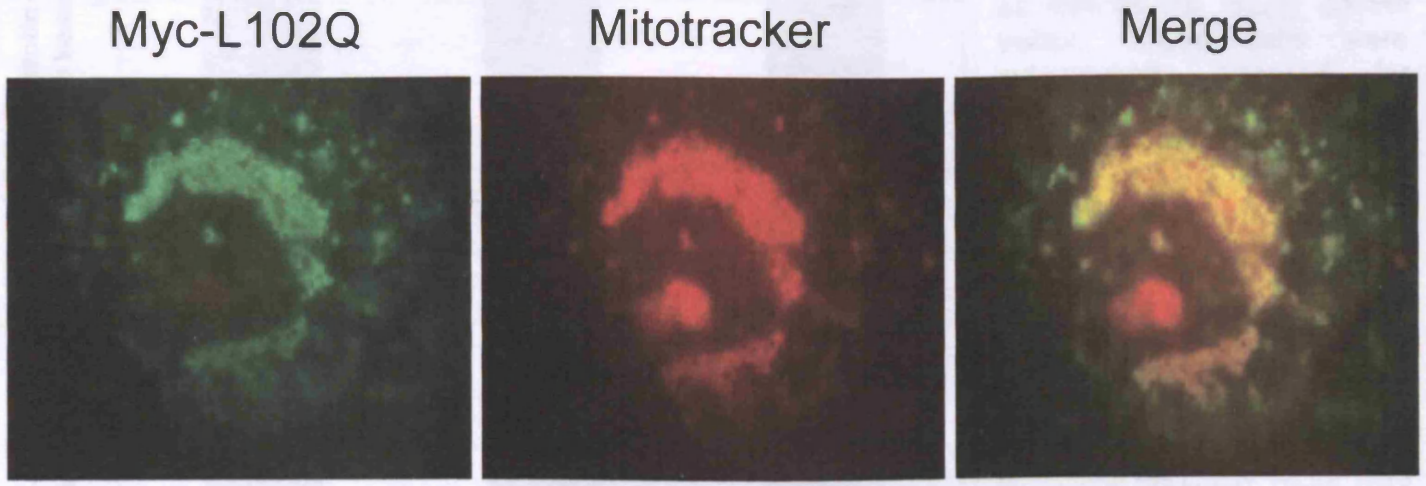


Figure 8.9 Fluorescence microscopy localization of L102Q PINK1
Fluorescence microscope view of fixed SH-SY5Y cell transfected with Fitz-Myc-L102Q PINK1 and treated with MitoTracker-red. L102Q PINK1 localised in the mitochondria, together with MitoTracker.

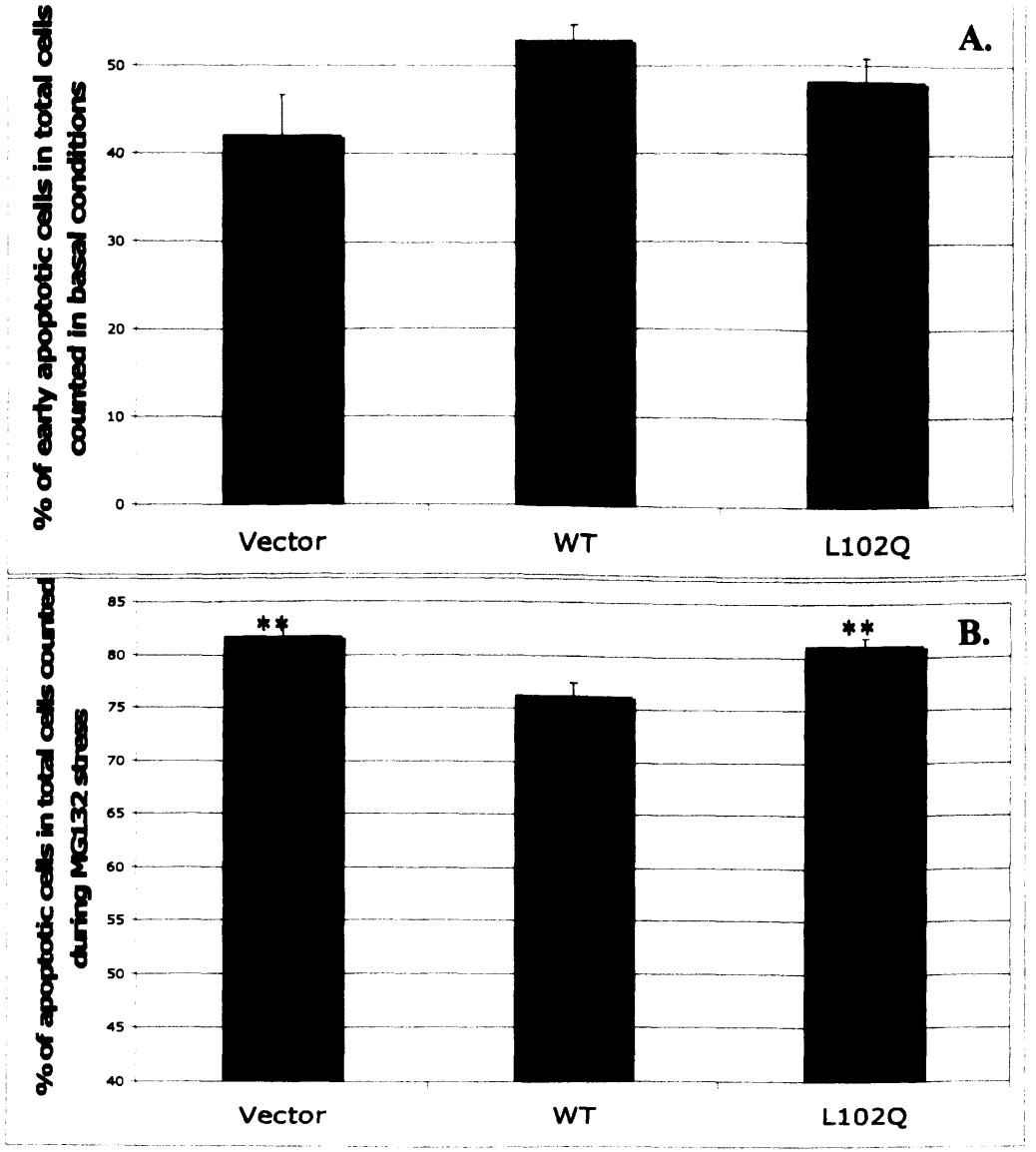


Figure 8.10 Apoptotic effect of L102Q PINK1

SH-SY5Y cells were transiently transfected with wild-type and L102Q mutant PINK1 expressing constructs as well as the empty control vector. These cells were subsequently stressed for 24hrs with 15mM MG132 and counted using FACs, in the presence of Annexin V, for the percentage of early apoptotic cells.

A.) Cells transfected with wild-type and L102Q mutant PINK1 showed similar percentage of early apoptotic cells. Cells containing the empty vector were slightly less apoptotic.

B.) In the presence of MG132, cells transfected with L102Q mutant PINK1 and empty vector had more early apoptotic cells compared to wild-type PINK1 transfected cells (**$p<0.01$).

8.3 Discussion

8.3.1 Regulation of PINK1 expression in stress

PINK1 was found to display protective effects in the event of cellular stress (Deng et al., 2005; Haque et al., 2008; Petit et al., 2005; Pridgeon et al., 2007). However, these studies into the functional consequences of PINK1 in the presence of stresses have, until now, been restricted solely to the study of PINK1 protein. In view of this, we show for the first time that PINK1 expression was altered in the presence of different stresses. Proteasomal inhibitor, MG132, and oxidative stress, dopamine, were found to decrease PINK1 expression while oxidative and mitochondria complex I inhibitor, MPP+, increased PINK1 expression. As MG132 has been shown prevent PINK1 degradation and cause PINK1 aggregation (Muqit et al., 2006), it is therefore possible that the decrease in PINK1 expression occurred as a response to the increase in cellular PINK1 resulting from its reduced degradation and aggregation.

MPP+ is a mitochondrial toxin and can also cause oxidative stress by producing free oxide radicals. The over-expression of PINK1 hs been shown to produce protection during exposure to MPP+ (Haque et al., 2008). This protection from oxidative stress was believed to be through the prevention of mitochondria cytochrome C release which leads to cell death via apoptosis. The mechanism of this action is through phospho-activation of TRAP1 by the kinase domain in PINK1 (Pridgeon et al., 2007). Activated TRAP1 is then responsible for maintaining mitochondria integrity and prevention of cytochrome C release. As such, the up-regulation of PINK1 could be due to a positive feed-back loop, with the result of increasing cellular PINK1 leading to increased

activation of TRAP1 and preventing cell death by MPP+. The mechanism of down-regulation of PINK1 expression by dopamine is not clear, although the difference in PINK1 regulation between the oxidative dopamine and MPP+ could be due to MPP+ being a mitochondria complex I inhibitor as well as induce oxidative stress.

It would be interesting to identify the factors responsible for the alteration in PINK1 expression during the stresses studied, and subsequently study the pathways involved. However, due to the lack of PINK1 promoter constructs, we were not able to better study the effects of these stresses on the regulation of PINK1 promoter. The ability to activate such pathways is of clinical interest with the potential to develop novel therapeutic approaches to protect and prevent neuronal cell death.

8.3.2 Pathogenecity of PINK1 heterozygous mutants

Two mutations found in control subjects were assayed, E476K and R501P. Neuroblastoma cells over-expressing the mutants or wild-type PINK1 were stressed with the proteasome inhibitor MG-132 and their mitochondrial membrane potential ($\Delta\psi m$) recorded. Both mutants resulted in a statistically significant decrease in $\Delta\psi m$ when compared to wild type PINK1. This result was similar to that found in 3 pathogenic mutations found in patients, N451S, Y431H and C575R, which indicated that these mutants adversely affected PINK1 function (Abou-Sleiman et al., 2006a).

While the pathogenecity of heterozygous PINK1 mutations remained to be definitively proven, the results of this study add significant weight to the previously published PET data which suggested that heterozygous PINK1 mutations result in nigrostriatal

dysfunction (Binkofski et al., 2007). Abou-Sleiman *et.al.* showed a three-fold enrichment of heterozygous PINK1 mutations in Parkinson's disease patients compared to controls (Abou-Sleiman et al., 2006a). They further demonstrated, using an in vitro assay similar to that performed here, that the patient mutations adversely affected PINK1 function, implying they were not simply neutral polymorphisms. Although the mutations examined here were obtained from control subjects, the decreased tolerance to stress contributed by these mutants implied that either these controls were also susceptible to developing Parkinson's disease or that other factors may also be required before the onset of the disease. As such, we conclude that haploinsufficency of PINK1 may render the dopaminergic cells of the substantia nigra more susceptible to cellular stress. However, the identification of heterozygous mutations in individuals apparently unaffected by Parkinson's disease leads us to suggest that the susceptibility to cellular stress is probably insufficient within their lifespan to manifest as clinical Parkinson's disease; rather a second factor is required. This second factor may take the form of an additional mutation in another Parkinson's disease gene or related pathway or exposure to exogenous toxins. Indeed, digenic inheritance, whereby characteristics or traits controlled by the integrated action of two genes, of Parkinson's disease, has recently been demonstrated for heterozygous mutations of PINK1 and DJ-1.

Nevertheless, having identified heterozygous mutations in approximately 1.2% of Parkinson's disease patients indicates that haploinsufficency at PINK1 is a significant risk factor for the development of Parkinson's disease.

8.3.3 PINK1 cleavage

A L102Q mutation of the PINK1 gene was found to block the cleavage of precursor PINK1 to its active form. This mutation was not found to affect the targeting and translocation of PINK1 to the mitochondria, showing that the mitochondrial targeting motif of precursor PINK1 is not affected. Whether the site at amino acid 102 is the precise cleavage location is not yet clear. Other explanations might account for the significant decrease in PINK1 cleavage with L102Q mutation. The change of non-polar residue, leucine, to a larger and polar glutamine residue could have altered the protein configuration of PINK1, enough to hide the true cleavage site and block its cleavage. The ability of PINK1 to cleave was found to be important in its protection of cells against MG132 stress.

More experiments, including structural homology comparisons with other mitochondria targeted proteins and performing experiments using a more similar residue substitution at L102, would have to be performed to conclusively pinpoint the exact PINK1 cleavage site. In the meantime, functional experiments could also be done to further investigate the extent of functional consequences when the PINK1 cleavage is blocked.

Chapter 9: Conclusion

9.1 Conclusions & Future Prospects

The causes of idiopathic Parkinson's disease remain unknown. Several factors may contribute to its onset, including: susceptibility genes, environmental stress and aging. Among these factors, genetic and environmental factors have been given the foremost attention in research, as reviewed earlier in chapter 1, sections 1.1 and 1.2.

Studies in this thesis aimed to characterize the influence of environmental factors, in particular oxidative stresses, on the regulation of genes associated with Parkinson's disease. The expression of all the genes studied, α-synuclein, parkin and PINK1, were responsive to stresses (Table 9.1; Fig. 9.1). Levels of α-synuclein increased following exposure of cells to dopamine or 1-methyl-4-phenylpyridinium (MPP+). Parkin was also up-regulated when cells were treated with either dopamine, MPP+, or the proteasomal inhibitor, MG132. In contrast, PINK1 levels were up-regulated only by MPP+, but down-regulated in both dopamine and MG132 treatments. The different responses to different stresses were worthy of note due to the different functions, localization and protective nature of these proteins studied.

Gene	Stress exposed	Gene expression	Cellular location	Cellular function
α-synuclein	Dopamine	↑	Vesicle membrane associated, cytoplasmic	Synaptic vesicle transport
	MPP+	↑		
parkin	Dopamine	↑	cytoplasmic	Ubiquitination, Protective
	MPP+	↑		
	MG132	↑		
PINK1	Dopamine	↓	Mitochondria, small portion in cytoplasm	Phosphorylation, Protective
	MPP+	↑		
	MG132	↓		

Table 9.1

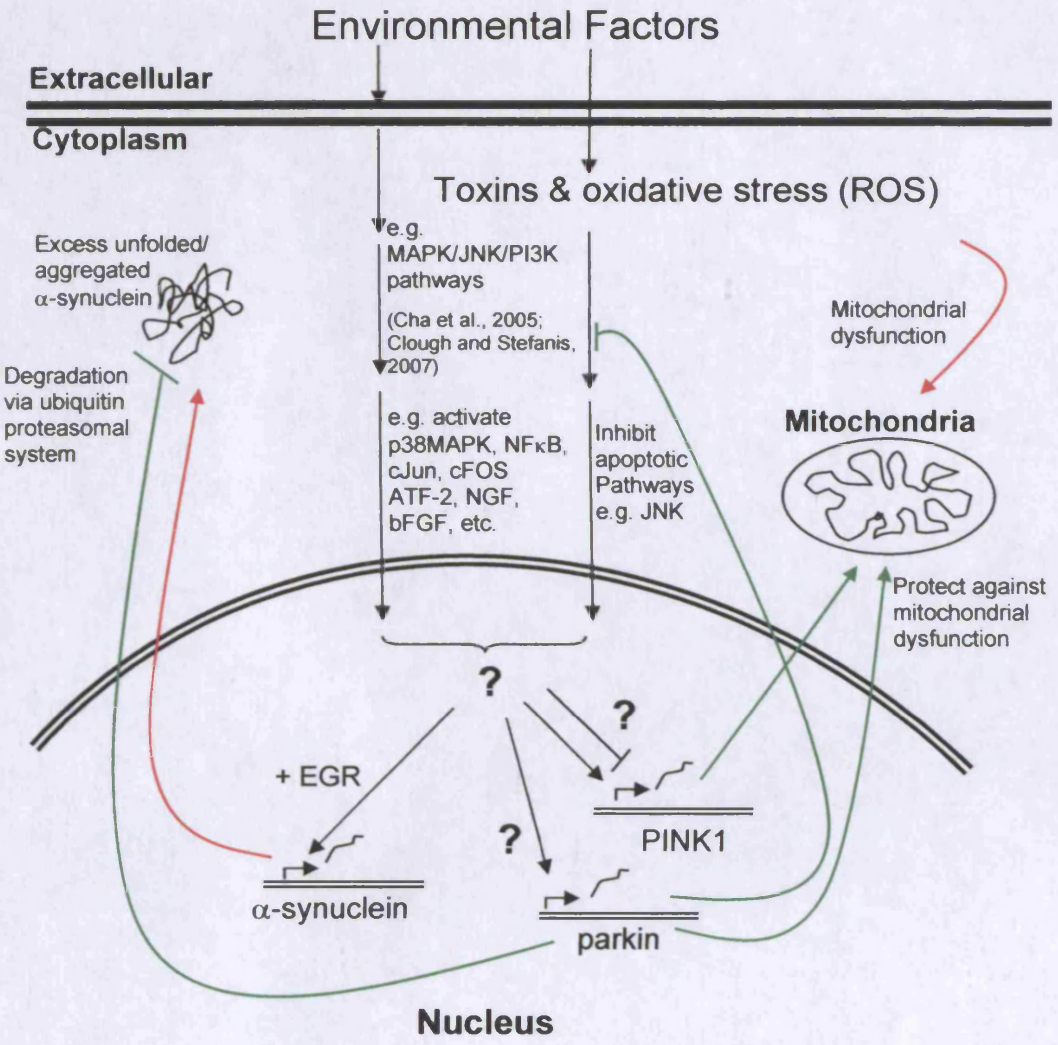


Figure 9.1 Regulation of Parkinson's disease-associated genes by environmental factors

ROS refers to reactive oxygen species. The red arrows indicate pathways that may lead to toxicity and/or cell death, while the green arrows indicate protective pathways. The question marks indicate areas which require more research to fully understand the mechanisms to neuronal cell death induced by harmful environmental factors and protective pathways which may be utilized in therapy to prevent cell deaths.

As described in the first chapter, α-synuclein is a cytoplasmic protein of yet unknown function. The over-expression of α-synuclein, as a result of the duplications and triplications of the α-synuclein locus, was sufficient to lead to neuronal cell death and Parkinson's disease (Chartier-Harlin et al., 2004; Farrer et al., 2004; Ibanez et al., 2004a; Singleton et al., 2003). Certain polymorphisms of the α-synuclein promoter could also lead to subtle up-regulations and have been associated with the disease (Chiba-Falek and Nussbaum, 2001). It may be postulated that aberrant up-regulation of α-synuclein could lead to excess amounts and accumulation of α-synuclein, leading to its aggregation and to the formation of Lewy bodies. Although it is not clear whether Lewy bodies are toxic to cells, they are the main pathologic characteristic of Parkinson's disease. Our results showing that oxidative stresses up-regulate α-synuclein supports the hypothesis that these stresses lead to Parkinson's disease by causing aberration in gene regulation as mentioned in chapter 1, section 1.2.1.

Both parkin and PINK1 are known to be protective against cell death. PINK1 is predominantly localized within the mitochondria and is involved in maintaining mitochondrial integrity. Parkin, on the other hand, is a cytoplasmic E3 ligase involved in ubiquitin proteasomal function. However, an association between the two genes relating to mitochondria has described in a Drosophila model. It showed PINK1 as an upstream activator of parkin function in maintaining mitochondrial integrity. The up-regulational response of both genes when exposed to MPP+ is of added interest since it is not only an oxidative stress but also a toxin targeting mitochondrial complex I. Therefore, the increase in PINK1 and parkin levels in response to MPP+ may be explained as a mechanism to further prevent mitochondrial dysfunction.

Although MG132 has been observed to lead to mitochondrial dysfunction, due to the generation of reactive oxygen species (Goldbaum et al., 2006), the primary target of the inhibitor is the proteasome. This might thus explain the difference in regulation of parkin and of PINK1. Certain transcription factors, for example Rpn4 (Dohmen et al., 2007), which are usually degraded by the proteasome, accumulate due to its inhibition. It may be postulated that such factors could then transverse into the nucleus to activate genes associated with the proteasomal system, resulting in the activation of parkin expression.

The stress caused by the increase of reactive oxygen species leads to the oxidative damage of cellular proteins. This results in the misfolding and aggregation of the damaged proteins, which require removal by proteasomal degradation or autophagy (Petropoulos and Friguet, 2006). Parkin was up-regulated when exposed to each of the oxidative stresses used in our experiments. This increase in parkin expression may be a component of mechanisms involved in the removal of oxidatively damaged α-synuclein and other denatured proteins. Parkin ubiquitinates damaged proteins, targeting them for degradation via the proteasomal or autophagy pathways (Olzmann and Chin, 2008; Tanaka et al., 2004). An increase in parkin could therefore lead to more rapid clearance of these oxidatively damaged proteins. Moreover, the accumulation of parkin following the use of proteasomal inhibitors, as mentioned in the previous paragraph, may also be a response to compensate for the proteosomal malfunction.

Other than merely targeting proteins for proteasomal degradation, ubiquitination has also been known to stimulate the activity of some transcription factors, for example Myc, and also activate certain signaling pathways, such as c-Jun kinase and NFκB

pathways (Depraetere, 2001; Lipford and Deshaies, 2003). Many of these pathways and transcription factors are involved in protective or apoptotic responses to oxidative stresses; in particular, parkin has been shown to inhibit the apoptotic JNK (c-Jun N-terminal kinase) pathway (Cha et al., 2005) (Fig. 9.1). This may contribute another explanation towards the purpose of increased parkin in oxidative conditions, as activation of parkin expression could subsequently lead to activation of protective signalling or deactivation of deleterious pathways via altered ubiquitination.

The study described above outlines an interesting interaction between stresses and the expression levels of the selected genes. However such regulation can involve a number of mechanisms at different stages of gene expression. The in depth elucidation of the intricate control mechanisms regulating these genes is of great interest and worthy of further analysis. Follow-on studies to further clarify the effect of stresses should include research into the level of regulation targeted by these stresses, for example promoter regulation or post-transcriptional modification, and other factors that mediate the response. We established that stress-induced regulation for both α-synuclein and parkin operated at the promoter level. A further study into the transcription factors responsible for the regulation of α-synuclein showed a novel interaction between the α-synuclein promoter and an Early Response Gene transcription factor family member in oxidative stress conditions (Fig. 9.1).

The discovery of α-synuclein promoter regulation by an Early Response Gene transcription factor is a novel finding. Literature search reveals two other transcription factors previously found to be involved in the regulation of α-synuclein, although not in

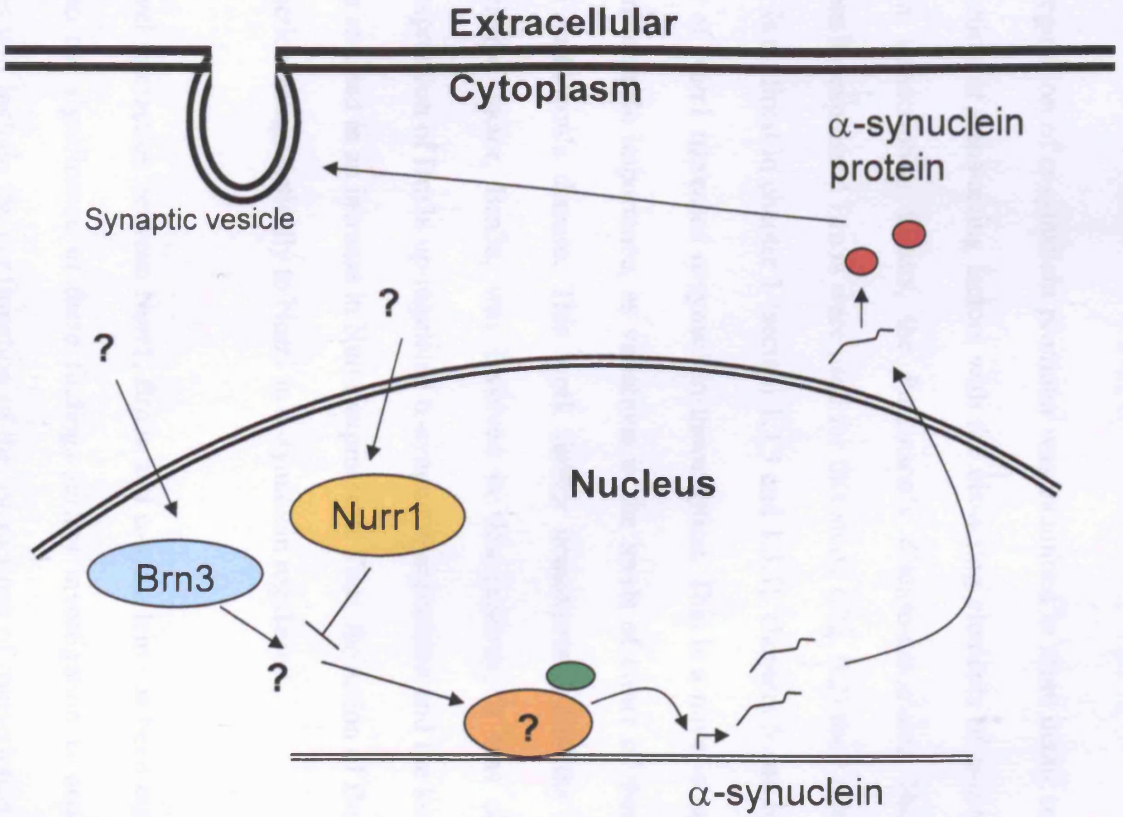


Figure 9.2 Regulation of α-synuclein by Brn3 and Nurr1.
Our research shows that Brn3a and Nurr1 may regulate may α-synuclein transcription in an antagonistic manner. The mechanism of the regulation is not yet clear. The question marks indicate areas where further research is required to elucidate the mechanisms of normal regulation of α-synuclein. This would allow us to better understand the cellular functions of α-synuclein and the general workings in a neuronal cell.

oxidative stress conditions. These include poly-ADP-ribose polymerase-1, which binds to the polymorphic Rep1 region of the α-synuclein promoter and the other is GATA. The interaction between GATA and the α-synuclein promoter is not known (Chiba-Falek et al., 2005; Scherzer et al., 2008).

The regulation of α-synuclein promoter was examined in more detail, to analyze the interaction of trans-acting factors with the cis-acting elements of α-synuclein. Two known transcription factors, the Parkinson's disease-associated Nurr1 and the neuronally expressed Brn3a were used for this study (Fig. 9.2) their relevance to the study is outlined in chapter 1 (section 1.3.3 and 1.3.4), chapters 5 and 6. The knock-down of Nurr1 increased α-synuclein transcription. This is a novel observation, and of considerable importance, as variations in the levels of either of these genes can cause Parkinson's disease. This work further demonstrated that the POU family trancription factor, Brn3a, was involved in this pathway, it was observed that overexpression of Brn3a up-regulated α-synuclein expression and the knock-down of Brn3a resulted in an increase in Nurr1 expression. Thus, the action of Brn3a appeared to function antagonistically to Nurr1 in α-synuclein regulation.

A novel interaction between Nurr1, Brn3a and α-synuclein has been outlined above. Due to the significance of these findings further investigation is necessary. Such studies will include the confirmation of the interactions of transcription factors with the α-synuclein promoter using CHIP (chromatin immuno-precipitation) assays to pull-down any interacting factors in the presence and absence of stresses. Having confirmed the trans-acting factors involved, the signal transduction pathways that mediate the stress response on α-synuclein can be identified. Similar studies may also

be extended to the other Parkinson's disease-associated genes to establish mechanisms that lead to the onset of disease.

The investigation described above details novel studies on Parkinson's disease-associated genes at the level of transcriptional regulation. Having initiated the study, it was also possible to study these factors at other levels. For example in the case of PINK1, a mutation in the PINK1 gene leading to the loss of a cleavage site in the protein resulted in decreased PINK1 protection from cell death. This short study highlighted the significance of protein function, to compliment research in regulation. Such studies will provide important insights into Parkinson's disease.

The three genes, α-synuclein, parkin and PINK1, have been succesfully profiled to different extents, in that α-synuclein regulation has been characterized to the greatest extent, at the level of mRNA expression, promoter regulation and identification of trans-acting factors. In contrast, studies on parkin involved analysis of the mRNA expression regulation and the identification of cis-acting elements on the promoter. The elucidation of PINK1 regulation focused on the transcriptional level.

Future progress in these projects would benefit from profiling parkin and PINK1 to the same extent as that achieved in the investigation of α-synuclein. These would involve performing EMSAs on the parkin promoter to identify trans-acting factors that may bind to the parkin promoter during stress and both promoter reporter assays and EMSAs on PINK1 promoter. These further studies may uncover commonalities between the three genes under stress; of particular interest is the similarity between

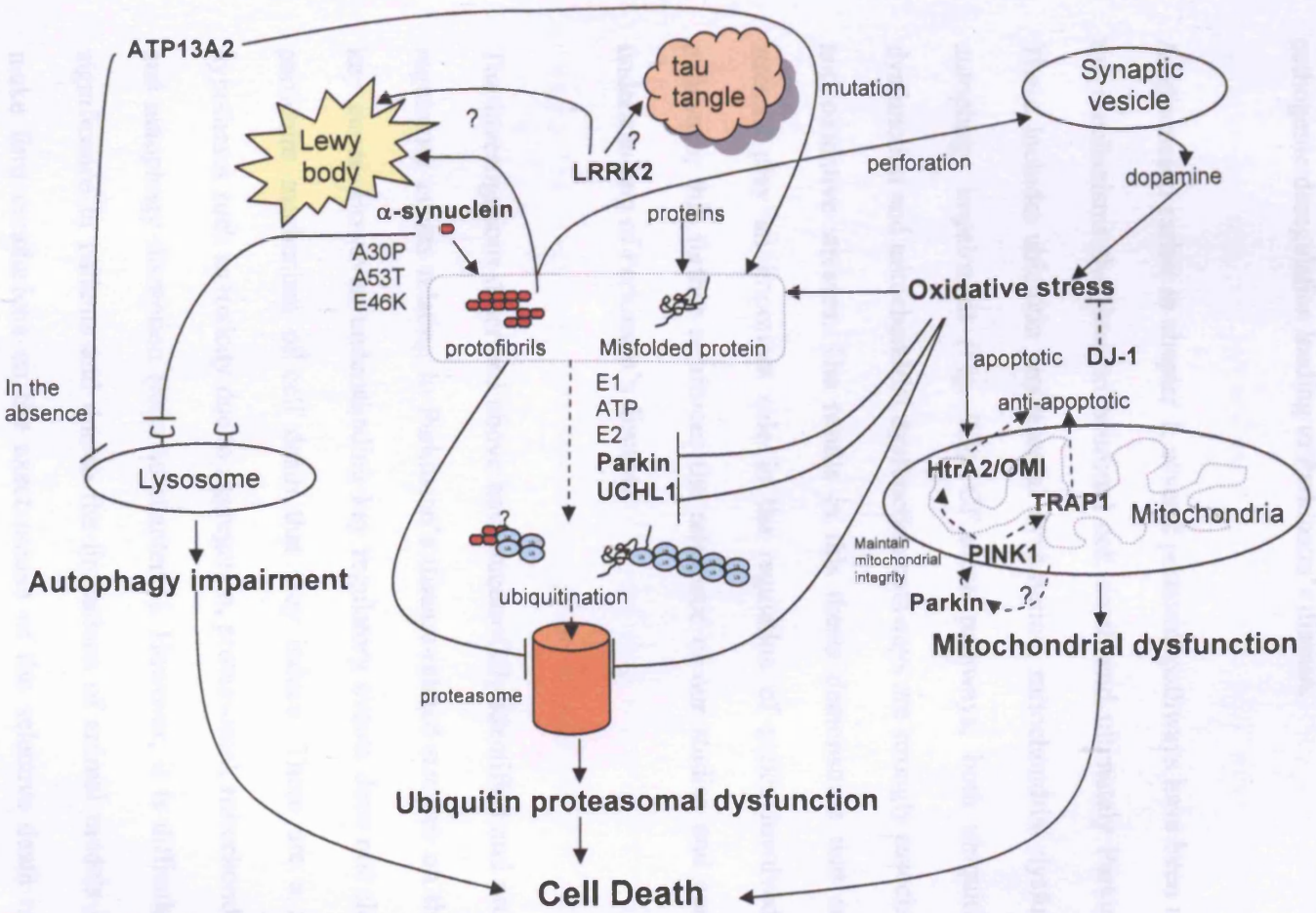


Figure 9.3 Pathways to parkinsonism.
A summary of the possible pathways to dopaminergic neuronal cell death (→) and potential protective mechanisms (- - →) are shown. The three major mechanisms of cell death are ubiquitin proteasomal dysfunction, mitochondrial dysfunction and autophagy impairment.

the interactions of transacting factors with the cis-acting elements on the three promoters. This data will provide an important insight to the molecular signaling mechanisms that may define the healthy regulation of these genes as compared to the pathogenic deregulation leading to Parkinson's disease.

As discussed earlier in chapter 1, several potential pathways have been used to explain the mechanisms that lead to neuronal cell death and ultimately Parkinson's disease. These include: ubiquitin proteasomal dysfunction, mitochondrial dysfunction and the autophagy impairment (Fig. 9.3). Of these pathways, both ubiquitin proteasomal dysfunction and mitochondrial dysfunction pathways are strongly associated with toxins and oxidative stresses. The results in this thesis demonstrate that such toxins and stresses play an important role in the regulation of genes involved in the above pathways; this further reinforces the relevance of our studies and contributes to the understanding of Parkinson's disease.

The investigations described above have successfully identified and profiled important regulatory events relating to Parkinson's disease-related stresses on the regulation of key genes. However, understanding key regulatory events does not clearly define the particular mechanism of cell death that they induce. There are a number of key hypotheses such as toxicity due to aggregation, proteasomal, mitochondrial dysfunction and autophagy disruption (refer to chapter 1). However, it is difficult to assess their significance in patients and due to the limitations of animal models it is difficult to make firm conclusions on the exact course of the selective death of dopaminergic neurons. A myriad of cellular systems are known to be affected, however, which of these is the predominant factor in the onset of Parkinson's disease is still unknown.

The lack of resources to study the development of the disease in patients or the presence of a strong model, presents limitations to the further study of Parkinson's disease, which would otherwise allow greater understanding of the pathology. For example, the nature of Lewy bodies is still yet unknown. Determination of whether the production and aggregation of insoluble proteins to form Lewy bodies is a protective mechanism or a source of toxicity to the dopaminergic neurons would be important in therapy. Once known, they could be regarded as therapeutically beneficial or as a target for intervention.

Regarding the consequences of the presented research in the context of therapeutic intervention, to date, the exogenous replacement of dopamine to the striatum by the administration of levo-dopa is the mainstay of Parkinson's disease therapy. However, this only improves the symptoms present and does not alter the progression of the disease. There has been some research into the identification of either new or re-purposed drugs that exhibit benefit in slowing the age-dependent neuronal damage that occurs in Parkinson's disease (Casper et al., 2000; Hirohata et al., 2008; Maharaj et al., 2004). These mainly include anti-inflammatory drugs, however, their mode of action is still unclear.

The advantage of our studies into factors responsible for regulation of genes associated with Parkinson's disease allows these factors to be exploited in therapy. As mentioned above, these studies described here can be expanded to clarify the difference between normal gene regulation and that associated with disease development. Genes may be artificially activated or repressed according to needs for the survival of the neuronal

cells. Molecular interventions such as gene therapy or introducing trophic factors to stimulate pathways that activate these transcription factors could be used. Similar methods have already been utilized with limited success.

Gene therapy and restorative treatments such as stem cell transplantation and growth factor delivery have shown promising results in Parkinson's disease therapy (Deierborg et al., 2008; Mochizuki et al., 2008). For example, GDNF (gial-derived neurotrophic factor) and Neurturin are trophic factors produced naturally in the brain and are important for the development and maintenance of dopaminergic neurons. They have been shown to have both protective and neuro-restorative effects on these neurons *in vitro* and *in vivo* and therefore have been the subject of clinical trials (Peterson and Nutt, 2008).. Unfortunately, there was limited success in the trials. The problem lay mainly in the delivery of the factors across the blood-brain barrier due to their large size and chemical structure (Peterson and Nutt, 2008).

Many solutions have been put forward to solve the problem of targeted delivery. They include camouflaging their structure using nanoparticles and direct injection into the brain (Peterson and Nutt, 2008). One other solution was to use gene therapy. This involved mainly viral delivery of genes in the signaling pathway of these trophic factors. For example, Akt/PKB, an oncoprotein which is activated by GDNF, when delivered into the striatum of mice and not only protected them from 6-hydroxydopamine, but also promoted the regeneration of the neurons after exposure (Ries et al., 2006).

Parkinson's disease involves multiple causations that lead to a very similar outcome – death of dopaminergic neurons. Research at a molecular level may identify the link, association or commonality between each cause. Such studies, as outlined in this thesis, are critical to gain the understanding for effective intervention of the condition. Hopefully, with more knowledge gained from on-going research, more focus could be put on the development of therapies to curtail the death, regenerate or restore function to these neurons and improve the quality of life for approximately 6.3 million sufferers worldwide (Baker and Graham, 2004).

The End

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Appendix

Molecular basis of Parkinson's disease

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Parkinson's disease is the second most common neurodegenerative disorder and remains incurable. Considerable progress has been made in understanding the molecular mechanisms of this disease, in particular, a distinct set of genes have emerged, whose dysfunctional regulation is strongly associated with the condition. These genes include α-synuclein, parkin, PTEN induced Putative Kinase 1 (PINK1), DJ-1, Leucine Rich Repeat Kinase 2 (LRRK2) and ATP13A2. Here we discuss what has been learnt in the study of these genes and how these genes may contribute to the pathogenesis of Parkinson's disease through different molecular pathways and consider how these pathways might converge to lead to the onset of Parkinson's disease. *NeuroReport* 00:000–000 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease. It is pathologically characterized by the presence of Lewy bodies and cell loss in various brain regions, in particular dopaminergic neurons in the substantia nigra pas compacta [1]. Lewy bodies are eosinophilic cytoplasmic inclusions that have a fibrillar structure and a dense core, surrounded by a halo. They are often associated with Lewy neurites.

The average age of onset of Parkinson's disease is between 60 and 80 years, and about 1% of the general population above the age of 65 years is affected [2]. However, rare familial forms of Parkinson's disease, which account for less than 5% of the Parkinson's disease cases, may also occur, with a more variable age of onset depending on the mode of inheritance, but generally at a younger average age of below 45 years [3].

Linkage and positional cloning studies have led to the identification of six genes and four other genetic loci associated with familial Parkinson's disease. A strong genetic evidence supporting a causal role for the following genes in familial Parkinson's disease is available: α-synuclein (*PARK1; PARK4*), parkin (*PARK2*), phosphatase and tensin homologue (PTEN)-induced putative kinase 1 [PTEN-induced kinase 1 (PINK1); *PARK6*], DJ-1 (*PARK7*), leucine-rich repeat kinase 2 [leucine-rich repeat kinase 2 (LRRK2); *PARK8*]), and more recently, ATP13A2 (*PARK9*) (Table 1).

The discovery of these genes has aided the characterization of key factors and abnormalities in the protein pathways that lead to the neurological degeneration in Parkinson's disease. This review will focus on how the study of Parkinson's disease associated proteins has allowed the elucidation of these pathways. The genes involved are categorized on whether disease causation is by autosomal dominant or recessive behavior. Within these categories, the dysfunctions leading to Parkinson's disease have been characterized by association to mechanistic pathways; impairment in the ubiquitin-dependent proteasomal pathway, mitochondrial dysfunction, and, more recently described the degradative pathway, autophagy.

Autosomal dominant Parkinson's disease

Autosomal dominant Parkinson's disease results from gain of function mutations that lead to the clinical manifestation of Parkinsonism.

Alpha synuclein (*PARK1; PARK4*)

Alpha synuclein was the first gene identified to be associated with Parkinson's disease. Several α-synuclein mutations, A53T, A30P, and E46K, were found to result in familial Parkinson's disease in several kindreds [4]. More recently, genomic duplications and triplications were also discovered at the α-synuclein locus in families with autosomal-dominant Parkinson's disease. This amplification of the α-synuclein locus, although rarewas found to occur at a higher frequency in patients than α-synuclein mutations. The severity and age of onset of Parkinson's disease in these patients correlate with α-synuclein copy number suggesting a gene–dosage effect [5]. The exact function of α-synuclein is still unknown, although it is suspected to be involved in the regulation of neurotransmitter vesicle function at the presynaptic membrane because of its ability to bind and stabilize lipid bilayers and its apparent enrichment in presynaptic terminals [6].

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Table 1 Parkinson's disease-associated genes

Locus	Gene	Inheritance	Function	Neuropathology	Age of onset	References
PARK1/4	SNCA (α-synuclein)	Autosomal dominant	Involved in synaptic function	Lewy bodies, Lewy neurites; variable presence of glial inclusion, tau inclusion, and amyloid plaque	30–60	[41]
PARK2	Parkin	Autosomal recessive	E3 Ligase	Lewy bodies reported in a single case	~30	[42]
PARK6	PINK1	Autosomal recessive	Mitochondrial kinase	Unknown	30–50	[4]
PARK7	DJ-1	Autosomal recessive	Involved in oxidative stress response	Unknown	20–40	[4]
PARK8	LRRK2 (dardarin)	Autosomal dominant	Protein kinase	Variable presence of Lewy body, Lewy neurites, tau inclusion, and amyloid plague	40–80	[4]
PARK9	ATP13A2	Autosomal recessive	Possible ion pump	Unknown	<20	[5]

In addition, α-synuclein also rescues from neurodegeneration in cysteine-string protein-α knockout mice [4,6]. As such it is believed to be protective by maintaining levels of co-chaperones, which maintain the integrity of the nerve terminals, and may have partial action in place of a synaptic co-chaperone, cysteine-string protein-α.

There is consensus that any change in the levels of α-synuclein expression or the presence of mutations in α-synuclein has a toxic effect on dopaminergic neurons. Alpha synuclein monomers interact, under certain circumstances, to form protofibrils or fibrillar β-pleated sheets, which has brought about much debate as to which configuration is actually toxic. The leading suspect is thought to be α-synuclein protofibrils because both the A53T and A30P mutants promote protofibril formation, but only A53T promoted β-pleated fibril formation *in vitro* [4]. Phosphorylation of α-synuclein, especially at Ser 129, is important for the formation of fibrils, shown both *in vitro* and *in vivo*, using a Drosophila model and in brains of patients with Parkinson's disease and other related synucleinopathies [4]. Toxicity caused by the α-synuclein protofibrils may involve the leakage of dopamine from synaptic vesicles because of perforation of the vesicular membranes by these protofibrils [7]. This may account for the selective toxicity of α-synuclein in the dopamine-producing neurons of the substantia nigra. In contrast to the data relating to protofibril-related toxicity, it is uncertain whether α-synuclein fibrillar aggregates are actually toxic or protective. The formation of α-synuclein aggregates was shown to be protective in Drosophila and parkin, a ubiquitin E3 ligase involved in the ubiquitin-dependent proteasomal protein degradation pathway, was shown both *in vivo* and *in vitro* to enhance α-synuclein aggregation, which then resulted in reduction of its toxicity [4].

It is not clear whether α-synuclein is degraded through the ubiquitin proteasomal pathway [8]. The presence of proteasomal subunits and ubiquitin in Lewy bodies in association with α-synuclein, however, strongly supports its involvement in α-synuclein pathogenicity [9]. Nevertheless, α-synuclein may also be degraded by another pathway – chaperone-mediated autophagy (CMA) [10]. It has also been shown that disease-causing mutants of α-synuclein, although localized by CMA chaperones to lysosomes, remain bound to receptors on the lysosomes and are not internalized, thus blocking lysosomal function and preventing the clearance of other proteins [10]. Moreover, in a cell model, macroautophagy was found to be upregulated after impairment of CMA and acted as a compensatory pathway for protein clearance; however, these cells were nonetheless more sensitive to oxidative stresses than those with normal CMA function [11]. More evidence is accumulating for the pathogenic role of impaired cellular autophagy leading to Parkinson's disease, including the discovery of mutations in ATP13A2, which is implicated in *PARK9*.

LRRK2/dardarin (*PARK8*)

Mutations in LRRK2 have been found in a large number of Parkinson's disease patients. The G2019S mutation, in particular, was detected in 5–6% of autosomal dominant familial Parkinson's disease and 1–2% of sporadic cases [4]. This prevalence was found to be even higher for specific populations such as Ashkenazi Jews and North African Arabs [4].

LRRK2 is a large multidomain protein comprising, from the *N*-terminal, residues predicted to adopt the configuration of armadillo repeats and ankyrin repeats [12], a leucine-rich repeat domain, a Roc-GTPase domain and *C*-terminal of Ras domain, a kinase domain similar to both the receptor-interacting protein kinase and that of the tyrosine kinase-like subfamily, and a *C*-terminal WD40 domain [12]. The presence of multiple protein interaction and catalytic domains in LRRK2 highlights its cellular importance and postulates its role as a hub for multiprotein signaling.

Pathogenic mutations scatter throughout all functional domains of LRRK2 [13]. The predominance of the G2019S mutation in Parkinson's disease patients has, however, led it become the main focal point of research into LRRK2's role in Parkinson's disease. The LRRK2 G2019S mutation is situated in a highly conserved DYG

motif of the kinase domain. This mutation results in a two-fold to three-fold increase in the kinase activity of LRRK2, thus implicating a gain of function in its pathogenic mechanism [14]. Several studies showed that the kinase activity of the mutant LRRK2 was responsible for neuronal toxicity, and that this, in turn, was likely dependent on the activity of its GTPase Roc domain *N*-terminal to the kinase domain [14]. It was shown, using mutations in the Roc domain, that the binding of GTP to this domain regulates the kinase activity of LRRK2 as well as its phosphorylation by other kinases [15]. Furthermore, the lack of deletions or truncations of LRRK2, as well as the autosomal dominant pattern of inheritance, also point toward a gain-of-function pathology.

The cellular function of LRRK2 is still unknown. It is essentially a cytoplasmic protein that may associate with intracellular membranes, such as the outer mitochondria membrane, Golgi apparatus, and endoplasmic reticulum [14]. Although LRRK2 is not found to interact with either α-synuclein or tau, the identification of α-synuclein-positive Lewy body pathology or tau-positive neurofibrillary tangle pathology in LRRK2 patients suggests a possible common role of LRRK2 in the phosphorylation and processing of these two proteins [16]. More recently, LRRK2 was shown to be a phosphorylate moesin as well as ezrin and radixin at a previously known phosphorylation site encompassing Thr^{558} [17]. The phosphorylation of this site regulated the binding of moesin to actin. The GTPase, *C*-terminal of Roc, and kinase domains as well as the WD40 motif and *C*-terminal tail of LRRK2 were all found to be essential for this phosphorylation [18].

Autosomal recessive Parkinson's disease

Autosomal recessive Parkinson's disease (ARPD) occurs when both alleles of a gene are mutated resulting in the clinical manifestation of Parkinsonism. DJ-1, parkin, PINK1, and ATP13A2 are all associated with early onset ARPD and, interestingly, also all are linked to mitochondrial function.

Parkin (*PARK2*)

Kitada and colleagues [18] were the first to find mutations in the parkin gene in Japanese ARPD families. Parkin was then found to be the most common cause of early onset ARPD, accounting for almost 50% of cases [4].

Parkin is a 465 amino acid E2-dependent E3 ubiquitin ligase consisting of an ubiquitin-like domain at the *N*-terminus and two really interesting new gene domains separated by a linker region at the *C*-terminus [4]. The main cellular role of parkin is to ubiquitinate proteins, targeting them for proteasomal degradation. More recently, parkin has also been found to mediate K63-linked polyubiquitination that targets the ubiquitinated protein for clearance by autophagy [19]. Therefore, deregulation of parkin may lead to dysfunction of both ubiquitin proteasomal and autophagy pathways.

In contrast to other forms of Parkinson's disease, Lewy bodies are typically not found in parkin-related Parkinson's disease [4]. Nevertheless, relatively few postmortem studies have been performed in parkin-related Parkinson's disease to substantiate this assertion. A variety of homozygous and compound heterozygous mutations causing rearrangements and missense mutations in the parkin gene have been found [4]. Of these, missense mutations were mainly found within the parkin *C*-terminal, really interesting new gene finger domain. Most of these mutations were found to either impair its binding to putative substrates or render its ligase activity defective, thus resulting in loss-of-function [20]. This loss-of-function mechanism is postulated to lead to neurodegeneration and result in Parkinson's disease with a lack of Lewy bodies [4]. In one case, however, Lewy bodies were present in a Parkinson's disease brain because of compound heterozygote mutations that reduced but did not abolish the parkin activity [4].

Studies of parkin knockout mice have suggested that parkin loss-of-function may lead to mitochondrial dysfunction and oxidative stress [4]. Parkin knockout mice have been shown to develop mitochondrial deficits [4] and parkin knockdown in cell lines renders cells more vulnerable to oxidative stress [21]. Parkin's ubiquitin ligase activity was also shown to be modified by nitric oxide-mediated oxidative stress, and S-nitrosylated parkin was found in insoluble inclusions in Parkinson's disease brains [4,22]. Dopamine, another possible oxidative stressor especially in dopaminergic neurons, was shown to convalently modify parkin, resulting in the loss of its activity [22]. Our laboratory has previously shown that endogenous parkin localizes to aggregates after exposure to dopamine in neuroblastoma cells [23]. Furthermore, oxidative stresses such as dopamine and 1-methyl-4-phenylpyridinium (MPP+) were shown to transcriptionally upregulate parkin *in vitro* [24]. Parkin-associated endothelin-receptor-like receptor, an endoplasmic reticulum-associated substrate of parkin, was found to be aggregated when overexpressed, leading to endoplasmic reticulum-stress and dopaminergic neuronal cell death [25]. This was shown to be alleviated by the overexpression of parkin [25] and, interestingly, also by the introduction of dopamine synthesis inhibitor [26], strongly implicating dopamine's involvement in the aggregation process. Therefore, parkin may play an important role in cell protection against oxidative stresses.

PTEN-induced kinase 1 (*PARK6*)

PINK1 was first identified in cancer cell expression profile experiments and was shown to be transcriptionally activated by PTEN, thus its name PTEN-induced kinase 1 [4]. Mutations in PINK1 were subsequently discovered in three large consanguineous families with ARPD – one

Spanish and two Italian [27]. PINK1 is an eight-exon gene that encodes for a 581 amino acid long protein. Through sequence comparisons with other proteins, it was found to consist of an *N*-terminal mitochondrial targeting motif, a *C*-terminal autoregulatory domain and a highly conserved serine/threonine kinase domain [4,27]. Recently, this has been corroborated using a baculovirus-infected insect cell system showing that PINK1 preferentially phosphorylates serine/threonine residues on basic substrates compared with acidic subtrates, and not tyrosine residues [28]. This phosphorylation was further shown to be regulated and selected by PINK1's *C*-terminal domain [28].

Several mutations of the PINK1 gene have been found that are mostly distributed around the kinase domain [4,27,29]. As such, disruption of kinase activity is deemed to be the most probable disease mechanism. Some studies, using the measure of mitochondrial membrane potentials, have been performed, which suggest that the loss of PINK1 function adversely affects mitochondrial function thereby also increases cellular susceptibility to stress [27]. More definitive studies, however, have yet been performed to support this hypothesis. Nonetheless, the identification of TNF receptor-associated protein 1 (TRAP1) (also known as heat shock protein 75) as a substrate of PINK1, Omi/HtrA2 as an interactor with PINK1, as well as their association with mitochondrial protection strengthens the above hypothesis.

TRAP1 is a mitochondrial molecular chaperone associated with an antiapoptotic function preventing oxidative-stress-induced mitochondrial cytochrome *c* release. This protective function was found to be dependent on TRAP1's phosphorylation by PINK1 [30]. Pathogenic PINK1 mutations such as G309D, L347P and W437X were shown to have an impaired phosphorylation ability on TRAP1 [30].

Omi/HtrA2 is a mitochondrial, antiapoptotic, serine protease. It was shown that phosphorylation of Omi/HtrA2 during stress modulated its proteolytic activity and that this p38 pathway-mediated phosphorylation was dependent upon the presence of PINK1 [31]. This was especially showed when brains of Parkinson's disease patients with PINK1 mutations were found to have less phosphorylated Omi/HtrA2 compared with idiopathic patients. The direct interaction between PINK1 and Omi/HtrA2, however, has not been shown [31].

On account of the association of PINK1 and its interactors and DJ-1 with the mitochondria, this strengthened the hypothesis that mitochondrial dysfunction may play a role in Parkinson's disease pathogenesis. This idea, although having been around for some time because of post-mortem studies showing mitochondrial impairment and oxidative damage, was overshadowed by another theory given rise by α-synuclein and parkin, which focused on protein aggregation and proteasomal dysfunction [4]. The findings that the phenotypes in Drosophila with PINK1 loss-of-function mutation could be rescued by using transgenic expression of Parkin, thus showing that Parkin and PINK1 may act in a common pathway, have shown that the two theories might, in fact, act together in the pathogenesis of Parkinson's disease [32,33]. Both PINK1 and parkin mutant Drosophila models have similar phenotypes – male sterility, muscle and dopaminergic neuronal degeneration, and increased sensitivity to oxidative stresses. Furthermore, double knockouts of both genes resulted in an identical phenotype to the single mutants suggesting a linear relationship between them [32,33]. Parkin has been suggested to act downstream from PINK1 in this pathway, as some of the disease phenotypes seen in PINK1 knockdown Drosophila models could be rescued by the introduction of both wild-type Drosophila parkin and human parkin but not vice versa [33]. Interestingly, overexpressing parkin could restore normal mitochondrial morphology, DNA and protein content, but did not alleviate the PINK1 mutant Drosophila from its sensitivity to stressors, suggesting that the pathway involving PINK1 and parkin may only be involved in the maintenance of mitochondrial integrity [32]. The relevance of the Drosophila model to Parkinson's disease was questionable as PINK1 knockout mice failed to display any nigrostriatal neurodegeneration [34], however, Exner *et al.* [35] later showed, using human cell lines including primary cell lines from patients with two different PINK1 mutations, that PINK1 knockout or mutation resulted in mitochondrial morphology abnormalities that could only be reverted by enhanced expression of parkin or the reintroduction of wild-type PINK1. After this discovery that PINK1 and parkin act through the same pathway, it would be especially interesting to identify substrates that, when phosphorylated by PINK1 in the mitochondria, lead to the regulation of parkin, which predominantly resides in the cytoplasm.

DJ-1 (*PARK7*)

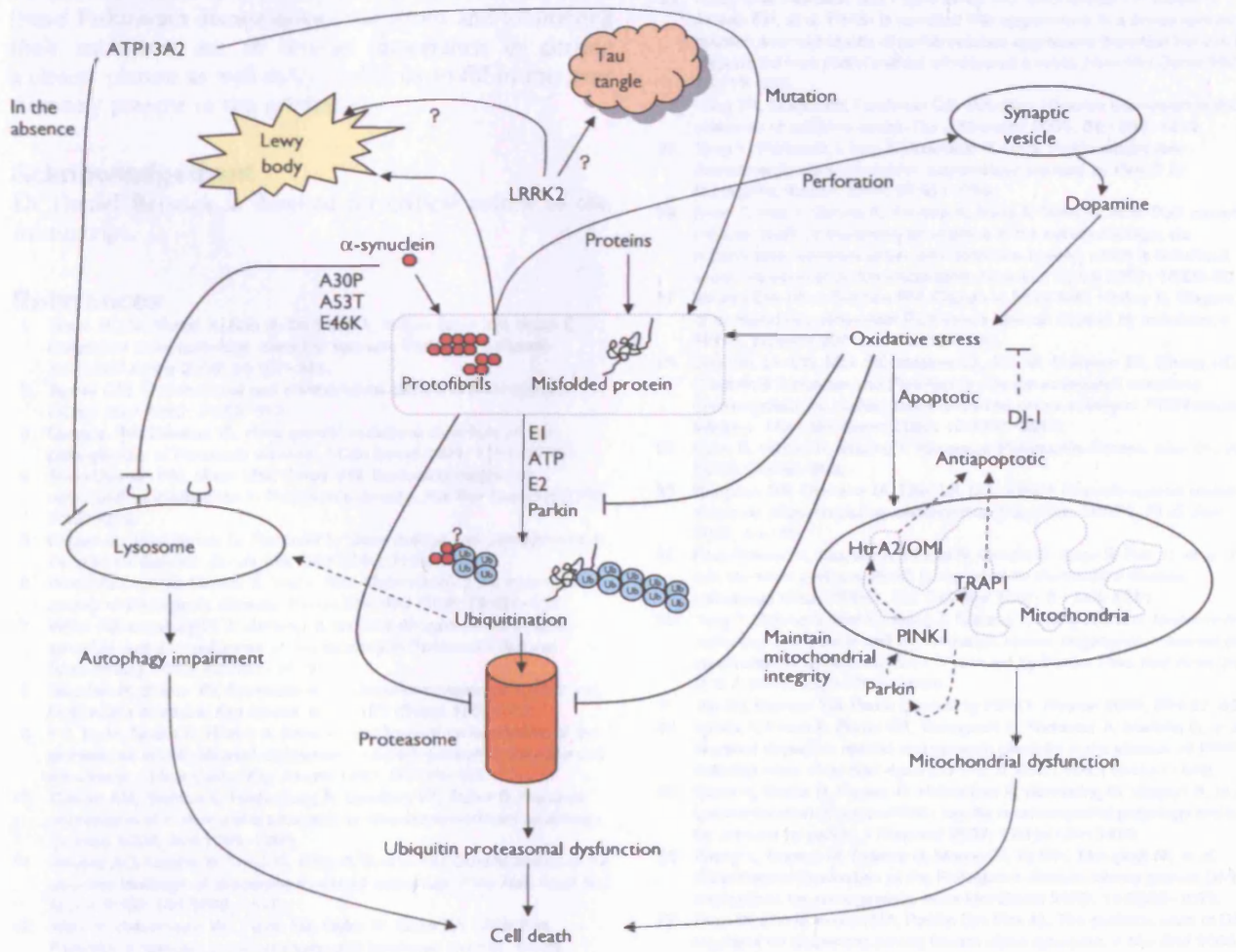
Parkinson's disease causing DJ-1 mutations is rare and account for only about 1–2% of early onset ARPD cases. Its cellular and subcellular localization is unclear, but it has been shown to be enriched in the brain and peripheral tissues and is primarily cytoplasmically localized, with a small pool associated with the mitochondria [36]. DJ-1 is a member of the THiJ/Pfpl/DJ1 superfamily and is thought to be involved in the oxidative stress response by acting as a redox-dependent chaperone [37]. It is capable of such a role because of the presence of several cysteine residues, which undergo an acidic shift in pI value when exposed to reactive oxidative species, thus potentially mopping them up [4,29]. As the quenching ability of DJ-1 is modest, however, it is suspected that other pathways are involved, of which much evidence points toward the

involvement of regulation of apoptosis through the phosphoinositide-3 kinase – AKT pathway [4,6]. This hypothesis is supported by evidence showing that RNAi knockdown of DJ-1α in Drosophila increased its sensitivity to oxidative stress that was reduced with increased PI3K/Akt-signaling capacity [6]. The mechanism of interaction between DJ-1 and the PI3K/Akt pathway, however, remains unknown.

ATP13A2 (*PARK9*)

ATP13A2 encodes for a member of the P_5-ATPases of the P-type ATPase superfamily. P-type ATPases generally make use of ATP to maintain an ion gradient across the cell membrane. As each step in this process is reversible, they, in turn, use the membrane potential to produce ATP [38]. The substrate specificities and functions of the P_5-ATPases are still relatively unknown, but because of their close homology to P_1-ATPases and P_4-ATPases, they are most probably ion pumps. It has been shown that ATP13A2 is expressed in most tissues in mice, but is especially abundant in the brain [39]. Mutations in ATP13A2 have been identified recently as the underlying cause of an autosomal recessive form of early onset parkinsonism with pyramidal degeneration and dementia, Kufor–Rakeb syndrome [40]. Much is unknown about how these loss-of-function mutations lead to the genesis of this parkinsonian syndrome and whether there is any interaction between this protein and the other Parkinson's disease-associated genes. In-vitro models, however, have shown that, although wild-type ATP13A2 is localized to the lysosomes, the mutated proteins were retained in the endoplasmic reticulum and degraded by the proteasome [40], it is suspected that an overload of such retained proteins may thus lead to neurodegeneration as seen in Kufor–Rakeb syndrome because of proteasomal dysfunction. Another theory is that such loss of functional

Fig. 1



Pathways to parkinsonism.

ATP13A2 from the lysosomes may lead to lysosomal dysfunction, which may, in turn, impair cellular autophagy. Currently, there is increasing evidence in the importance of autophagy impairment in Parkinson's disease.

Afterword

Parkinson's disease involves multiple causations that lead to a very similar outcome – death of dopaminergic neurons. Research at a molecular level may identify the link, association, or commonality between each cause. Such studies, as outlined above, are critical to gain the better understanding of the disease. Several potential pathways have been now uncovered – ubiquitin proteasomal dysfunction pathway, mitochondrial dysfunction pathway, and the autophagy impairment pathway (Fig. 1), each of that reveals a little more on the intricacies involved in the interactions among the known Parkinson's disease-associated genes, the environment, and cellular functions.

In the short-term future, delineating the functions of all these Parkinson's disease-associated genes and identifying their substrates are of utmost importance in getting a clearer picture as well as to enable us to fill in the gaps currently present in the pathways.

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Characterisation of a novel *NR4A2* mutation in Parkinson's disease brain

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Abstract

Following a mutation screen of *NR4A2* (also known as NURR1) in 409 Parkinson's disease (PD) patients we identified a novel single base substitution in the 5'UTR of the gene (c.-309C>T). Several mutations of the *NR4A2* 5'UTR have been previously described in PD, but pathogenecity remains controversial, partly due to low prevalence and partly due to a lack of functional data in human central nervous tissue. Here, we provide *in vitro* and *in vivo* expression data on the c.-309C>T mutation. *In vitro* data on *NR4A2* expression and the transactivation of downstream targets was corroborated, respectively, by allele specific real-time PCR from brain tissue and the assessment for enrichment of functional categories by modelling the hypergeometric distributions of data from a whole genome expression study. We also provide evidence from *in vitro* studies that show the c.-309C>T mutation increases neuronal vulnerability to stress.

Our findings indicate the c.-309C>T mutation reduces *NR4A2* expression resulting in the downregulation of genes involved in the development and maintenance of the nervous system and synaptic transmission. These downregulated pathways contained genes known to be transactivated by NR4A2 and were not disrupted in idiopathic PD brain suggesting causality of the c.-309C>T mutation in that patients parkinsonism.

Early growth response family of transcription factors mediate α-synuclein promoter up-regulation in response to oxidative stress

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Abstract

Parkinson's disease is a highly prevalent neurodegenerative disorder. Several factors may contribute to its onset, including: susceptibility genes, environmental stress and aging. α-synuclein is one of several genes shown to be mutated in cases of familial Parkinson's disease It has also been suggested that variations in the expression of the wild type genes may also lead to Parkinson's disease. We show that α-synuclein is up-regulated when neuroblastoma cells were exposed to stresses such as dopamine and 1-methyl-4-phenylpyridinium (MPP+). Moreover, we identify a novel interaction between the α-synuclein promoter and dopamine-induced early growth response transcription factor family members in these oxidative stress conditions. This work confirms and extends previous reports that oxidative stresses are implicated in Parkinson's disease, and enhances our understanding of the effect of oxidative stress on a Parkinson's disease-associated gene.