THE EFFECTS OF PARKINSON'S DISEASE MIMETICS ON THE PROTEASOMAL AND NEUROFILAMENT SYSTEMS IN SH-SY5Y CELLS

Begoña Caneda-Ferrón

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DECLARATION

This work has not been accepted for any other degree and is not concurrently being submitted for any other degree.

We certify that the work submitted was carried out by the author. Due acknowledgement has been made of any assistance received.

Signed……(candidate)

Signed……(Director of studies)

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ABSTRACT

The effects of Parkinson's Disease mimetics on the proteasomal and the neurofilament systems in SH-SY5Y cells

Mitochondrial impairment, glutathione depletion and oxidative stress have been implicated in the pathogenesis of Parkinson's disease, linked recently to proteasomal dysfunction. This study analyses how these factors influence the various activities of the proteasome in SH-SY5Y human neuroblastoma cells treated with the PD mimetics MPP^+ (a complex I inhibitor) or dopamine. Treatment with these toxins led to dose and time dependent reductions in ATP and glutathione levels and also chymotrypsin-like and postacidic-like activities; however, trypsin-like activity was unaffected. Antioxidants blocked the effects of dopamine but not MPP^+, suggesting that oxidative stress was more important in the dopamine-mediated effects. With MPP^+, ATP depletion was a pre-requisite for loss of proteasomal function.

This study also shows that addition of MPP^+ or dopamine to purified samples of the human 20S proteasome also reduced proteasomal activities; with dopamine being most damaging. As was the case with toxin-treated cells chymotrypsin-like activity was the most sensitive and trypsin-like activity, the least sensitive. The direct effect of both compounds on proteasomal activity was, at least, partly due to oxidative damage to the proteasome, since the antioxidant vitamin C could partially alleviate the proteasomal impairment. Indeed, Western blot analyses showed that some of the β- and α-subunits of the proteasome were modified by dopamine treatment.

One of the hallmarks of Parkinson's disease is the appearance of Lewy bodies, which are protein inclusions containing α-synuclein, neurofilament proteins and ubiquitinated proteins. A growing body of evidence suggests that the UPS might be involved in the formation of these aggregates. This thesis, reports that neurofilaments can undergo proteasomal degradation and that MPP^+ and dopamine alter the expression/phosphorylation and distribution of these cytoskeletal proteins in SH-SY5Y cells. Therefore aberrant changes in both neurofilament profiles and proteasomal degradation may influence inclusion formation in dopaminergic neurons.

LIST OF ABBREVIATIONS

AD: Alzheimer's disease

AOEs: Antioxidant enzymes

AP: Alkaline phosphatase

ATP: Adenosine 5'triphosphate

BCIP: 5-bromo-4-chloro-3-indolyl-phosphate (di-sodium salt)

BSA: Bovine serum albumin

BSO: $_{L}$- buthionine-[S,R]-sulfoximine

CLA: Chymotrypsin-like activity

CDK-5: cyclin-dependent kinase 5

CNS: central nervous system

DA: Dopamine

DCHDF: 2,7-dichlorodihydrofluorescein diacetate

DHF: dichlorodihydrofluorescein

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl sulfoxide

E1: Ubiquitin-activating enzyme

E2: Ubiquitin-conjugating enzyme

E3: Ubiquitin ligase

EDTA: Ethylenediamine

EGTA: Ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid

ERK: Extracellular signal related kinase

FBS: Foetal bovine serum

FITC: Fluorescein Isothiocyanate

GDR: Glutathione reductase

GSH: Reduced glutathione

GSH Px: Glutathione peroxidase

GSSG: Oxidised glutathione

GTP: Guanosine 5'triphosphate

HRP: Horseradish peroxidase

JNK: c-jun N-terminal kinase

LB: Lewy Body

MAO: monoamine oxidase

MAPs: Microtubule associated proteins

MF: Microfilament

$MPDP^+$: 1-methyl-4-penyl-2,3 dihydropyridinium

MPP^+: 1-methyl-4-phenylpyridinium ion

MTP: Mitochondrial transition pore

MPTP: 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine

MT: microtubule

mtDNA: mitochondrial DNA

MTOC: Microtubule organizing centre

MTP: Mitochondrial transition pore

MTT: 3-[4-5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NAC: N-acetylcysteine

NTB: Nitro blue tetrazolium

NF: Neurofilaments

NF-H: Neurofilament heavy-chain

NF-L: Neurofilament light-chain

NF-M: Neurofilament medium-chain

NADH: Nicotinamide dehydrogenase

NO: Nitric oxide

NOS: Nitric oxide synthase

6-OHDA: 6-hydroxydopamine

PBS: Phosphate buffered saline

PD: Parkinson’s disease

PLA: Postacidic-like activity

pNF-H: Phosphorylated neurofilament heavy chain

pNF-M: Phosphorylated neurofilament medium chain

ROS: Reactive oxygen species

SDS-PAGE: Sodium dodecylsulphate polyacrilamide gel electrophoresis

SEM: Standard error of the mean

SN: Substantia nigra

SNpc: Substantia nigra pars compacta

SOD: Superoxide dismutase

TBS: Tris buffered saline

Thr: Threonine

TLA: Trypsin-like activity

UCH-L1: Ubiquitin carboxyl-terminal hydrolase L1

UPS: Ubiquitin proteasomal system

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CHAPTER I

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 PARKINSON'S DISEASE

Parkinson's disease (PD) is a severe, progressive motor disorder of the central nervous system, which was first described by the English physician, James Parkinson in 1817. It is reported to be the second most common neurodegenerative disease, the occurrence of which increases with age, affecting about 1 % of the population over 65. PD is mostly presented as a sporadic form, although in rare instances genetic forms of the disease also exist (5-10 % of all cases; reviewed by Wood-Kaczmar *et al.,* 2006; see section 1.1.2.4 for further information). Whilst the aetiology of sporadic PD remains unresolved, its pathogenesis is understood to be a consequence of a multifactorial cascade of deleterious events (Prezedborsky, 2005).

1.1.1 Disease pathology and clinical features

It is widely accepted that PD primary pathology is due to the degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SNpc). This results in low levels of brain dopamine (DA) in the striatum, responsible for the motor symptoms. Although it is commonly thought that the neuropathology of PD is characterised solely by dopaminergic neuron loss, the neurodegeneration extends well beyond dopaminergic neurons. Indeed, non-nigral lesions lead to cognitive and psychological impairments ie. dementia, sometimes seen in PD (reviewed by Blum *et al.* 2001 and Dauer and Przedborsky, 2003).

Another neuropathological feature of the disease is the appearance of Lewy bodies (LB), which are prominent intracytoplasmic inclusions of proteinaceous material containing mainly lipids, neurofilaments, α-synuclein, ubiquitinated proteins and proteasome subunits (Forno, 1996; Floor and Wetzel, 1998; Good *et al.* 1998; Kowal *et al* 2000; Lopiano *et al.* 2000; McNaught and Ollanow, 2006). However, the role of Lewy bodies in the parkinsonian brain remains controversial (Chung *et al.,* 2001a; Mc Naught et al., 2001; Barzilai and Melamed, 2003; Dauer and Pzredborsky, 2003; Shoesmith and Paulson, 2003; see section 1.1.3 for further details).

The mechanisms responsible for the specific death of dopaminergic neurons remain unresolved; however, age, genetic factors and the action of environmental and intrinsic

The clinical manifestations of PD include bradykinesia (slowness of movement), gait abnormalities, resting tremor, postural instability and muscular rigidity (reviewed by Beal M.F. 2001 and Wood-Kaczmar *et al.,* 2006). Symptoms appear only after loss of 50-70% of nigral DA neurons; this can be explained by the fact that the brain contains an excess of DA fibers (reviewed by Blum *et al.* 2001 and Barzilai and Melamed, 2003).

Disease is normally treated by administration of the DA precursor $_{L}$-dopa ($_{L}$-3, 4-dihydroxyphenylalanine) which can cross the blood-brain barrier boosting DA synthesis in the cells that remain alive in the substantia nigra, thus alleviating most of the symptoms (Beal, 2001). Unfortunately, over the years $_{L}$-dopa provokes involuntary movements (termed “dyskinesias”) in patients, which significantly impair their quality of life. All current treatments for PD address the symptoms, although present research is focused on the prevention of DA neuron degeneration. However, it is a difficult goal due to the ignorance of the specific molecular events that provoke neurodegeneration in PD (Dauer and Przedborsky, 2003).

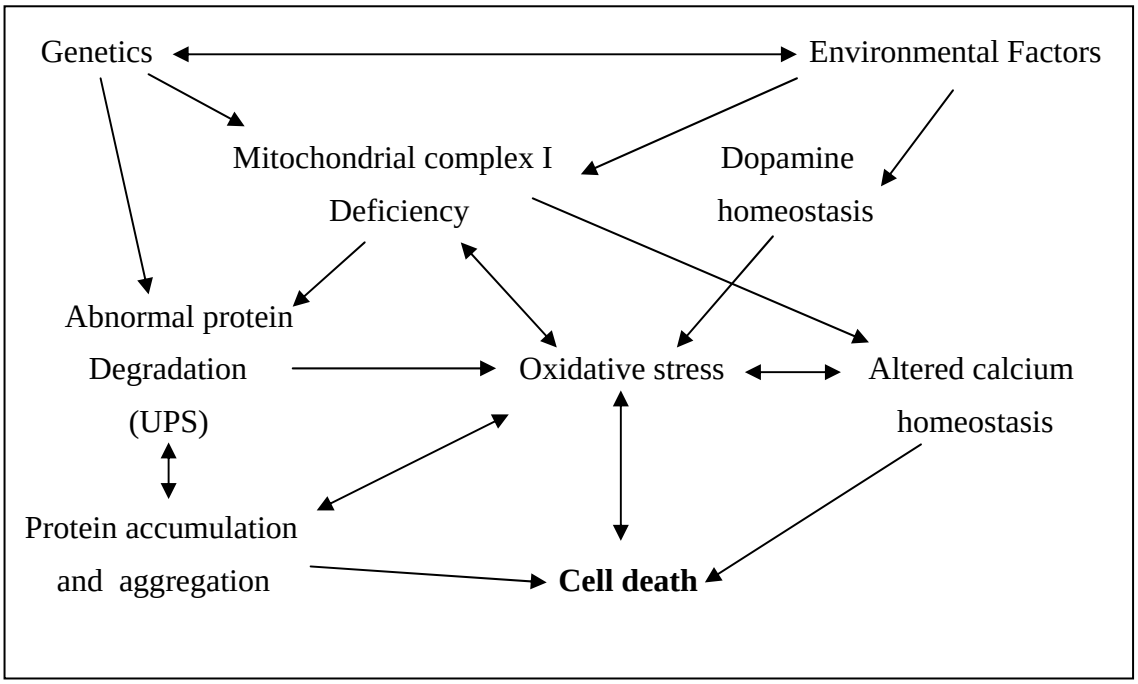


Figure 1.1. Overview of the potential mechanisms involved in the development of PD. *Based on Betarbet et al., (2002) with modifications.*

1.1.2 Factors involved in the disease

1.1.2.1 Mitochondrial dysfunction

Mitochondria are central to the life of eukaryotic cells (Bernardi *et al.,* 1999). Their primary function is to support aerobic respiration and to provide energy substrates (such as ATP) for intracellular metabolic pathways. Mitochondria also play an important role in the pathways to cell death, mainly apoptotic cell death (Bernardi *et al.,* 1999; Shapira, 2006).

The link between PD and mitochondria was first established with the identification of a deficiency in the activity of complex I (NADH: ubiquinone oxidoreductase) in PD SNpc and subsequently in peripheral tissues of patients (Schapira *et al.* 1989; Parker *et al.* 1989). Indeed, a 25-30 % loss in complex I activity has been reported in PD brains (Schapira *et al.* 1989; Parker *et al.* 1989; Gu *et al.* 1998; Conn et al. 2001).

Further evidence has strengthened the hypothesis of mitochondrial dysfunction playing a key role in the pathogenesis of PD since exposure to neurotoxins linked to complex I

inhibition, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or rotenone, can lead to parkinsonism in humans or animal models (Beal, 2001, Betarbet *et al.*, 2000).

Complex I is the largest of the electron transport chain macrocomplexes and consists of 46 subunits, seven of which are coded for by mitochondrial DNA (mtDNA). The remaining 39 subunits are coded by nuclear genes, imported into mitochondria, and assembled with the mtDNA-encoded subunits in a complicated process (Keeney *et al.*, 2006). Decreased complex I activity primarily results in ATP depletion in cells but also in an increase of free radicals which may be responsible for the oxidative mediated damage observed in PD. Reciprocally, free radicals may also damage the mitochondria (reviewed by Schapira, 2006).

Recent studies from Keeney and co-workers (2006) provide further understanding of the nature of mitochondrial dysfunction in PD since they report that reduced complex I activity in PD brain mitochondria appears to arise from oxidation of its catalytic subunits from internal processes (reproduced by using NADH to drive electrons through complex I and blocking the transfer of electrons with rotenone at the quinone reduction site), not from external oxidative stress (reproduced by exposure to H_2O_2), and this correlates with complex I misassembly. They argue that complex I auto-oxidation may derive from abnormalities in mitochondrial or nuclear encoded subunits, assembly factors, rotenone-like complex I toxins or some combination (Keeney *et al.*, 2006).

1.1.2.2 Oxidative stress: role of DA and loss of glutathione

As introduced earlier, oxidative stress accompanied by a reduction in glutathione content are also believed to contribute to PD pathogenesis (Jenner and Olanow, 1996; Schulz *et al.*, 2000; Jha *et al.*, 2002; Mytilineou *et al.*, 2002). Floor and Wetzel (1998) found that oxidative stress is elevated in SNpc in comparison with other regions resulting in elevated oxidative damage which may contribute to the degeneration of nigral dopaminergic neurons in ageing and in PD.

1.1.2.2.1 Antioxidant defence systems in the brain

ROS are generated in the brain as products of normal cell metabolism (eg. normal 0_2 intake, aerobic respiration and oxidative metabolism of DA and other substrates) but also in response to internal and external toxins (reviewed by Prasad *et al.*, 2000). Particularly, the electron transport chain is an important source of ROS derived from oxidative phosphorylation (Hughes *et al.*, 2005).

Cellular damage occurs when production of ROS exceeds the available antioxidant defence system. The antioxidant defence system of cells include the enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH Px) and glutathione reductase (GRD). An overview of the generation of ROS and the action of the antioxidant enzymes (AOEs) is included in Figure 1.2. Since energy production in neurons (ie. ATP) depends mainly on oxidative phosphorylation, and the brain contains lower amounts of antioxidant molecules and AOEs than other tissues, neurons are usually more exposed to oxidative damage (reviewed by Mytilineou *et al.*, 2002).

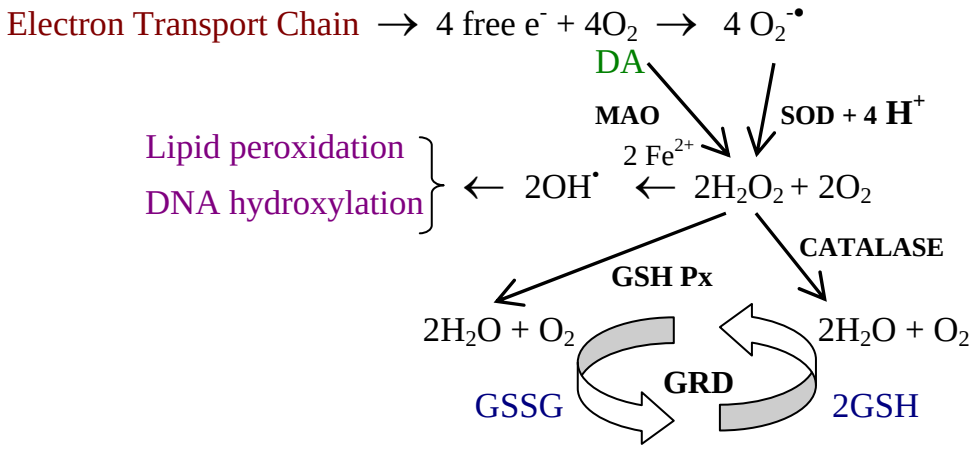


Figure 1.2. Reactive oxygen species produced by the mitochondria and associated antioxidant defence systems (taken from Cassarino and Bennett, 1999 with modifications). *Normal aerobic respiration produces ROS, some of it is caused by leakage of partially reduced O_2 within the electron transport chain. Leakage of electrons (e^-) onto molecular O_2 produces the superoxide anion ($O_2^{-\bullet}$). Superoxide dismutase (SOD) can react with $O_2^{-\bullet}$ to form H_2O_2 and O_2. H_2O_2 can also be formed from the oxidation of DA via the enzyme monoamine oxidase (MAO; see 1.1.2.2.2). Iron can react with H_2O_2 to give a highly reactive hydroxyl radical ($OH^\bullet$). Glutathione peroxidase (GSH Px) detoxifies H_2O_2 produced from $O_2^{-\bullet}$ by oxidising reduced glutathione (GSH), which also acts as an antioxidant enzyme. Oxidised glutathione (GSSG) is then reduced back to GSH by glutathione reductase (GRD). Alternatively H_2O_2 is detoxified by catalase, a peroxisomal enzyme.*

1.1.2.2.2 Role of DA in ROS formation in PD

Increased oxidative stress in PD may be initiated by a decline in the antioxidant defence system (mainly a depletion in glutathione within SNpc; see next section) but may also be due to the fact that DA-containing neurons are believed to be more prone to oxidative stress due to DA oxidation (either through its auto-oxidation or via the enzyme monoamine oxidase (MAO)), which subsequently generates free radicals and other ROS within SNpc (Jha *et al.,* 2000; Schulz *et al.,* 2000; Stokes *et al.,* 2000; Yoo *et al.,* 2003).

Indeed, auto-oxidation of DA leads to the formation of neuromelanin and can generate quinone and semiquinone species and other ROS, whilst MAO-mediated DA oxidation can generate H_2O_2, as well as deaminated DA metabolites such as 3,4-dihydroxybenzoic acid (DOPAC) and homovanillic acid (HVA). DA-quinone formation can also be formed through the action of enzymes like tyrosinase, cyclooxygenase or peroxidase, as well as by metals (Mn or Fe; Stokes *et al.,* 2000). Detoxification of cytosolic and mitochondrial peroxides depends predominantly on glutathione peroxidase and reductase since catalase is compartmentalised into peroxisomes (reviewed by Schulz *et al.,* 2000; Mytilineou *et al.,* 2002). H_2O_2 can react with ferrous iron (Fe^{2+}) to form highly reactive hydroxyl radical (OH•) via the fenton reaction. Interestingly, iron levels are increased in the SN of PD patients along with elevations in various indices of oxidative damage (Jha *et al.,* 2000; Berg *et al.,* 2001). Hydroxyl radicals can damage proteins, nucleic acids and membrane phospholipids (Jha *et al.,* 2000; Schulz *et al.,* 2000). Indeed, Schulz *et al.* (2000) discussed that in PD, the loss of dopaminergic neurons, which results in decreased DA levels, may lead to a compensatory increase in DA turnover, with increased formation of H_2O_2 and increased demands on glutathione synthesis in the remaining neurons. This hypothesis is supported by experimental studies showing that enhanced DA turnover is associated with increased formation of oxidised glutathione (GSSG) which in turn can be prevented by inhibitors of DA metabolism (Spina *et al.,* 1989).

1.1.2.2.3 Glutathione level is reduced in PD brains

The most significant alteration in the antioxidant defence in PD is a reduction in GSH levels in SNPc of patients (30-40% reduction compared to controls; Sofic *et al.,* 1992; Dringen, 2000; Schulz et al. 2000) and it has been reported that the degree of the disease correlates with the extent of GSH loss (Riederer *et al.,* 1989). Since glutathione is an important natural antioxidant, a deficiency of GSH in the SN could make this region more vulnerable to oxidative injury (Perry *et al.,* 1982). However, in the brain most of the glutathione is localised in the glia, so the decrease in GSH levels in SNpc may not only occur in dopaminergic neurons, which only make up 1-2 % of the total cell population, but also in glial cells (Schulz *et al.,* 2000).

Depletion of GSH in PD substantia nigra, as for the complex I deficiency, appears to be selective for this brain area (Gu *et al.* 1998) and may occur early in the development of PD causing a cascade of events, which ultimately result in cell death (Dringen, 2000; Schulz et al. 2000). The cause of the loss of GSH in PD is not clear but since it is not accompanied by the corresponding increase in GSSG it may not be wholly the consequence of oxidative stress (Sofic *et al.,* 1992). The activity of γ–glutamyl-cysteine synthetase (an enzyme involved in glutathione synthesis; see Figure 1.3) is normal in PD, thus it would seem that no failure of glutathione synthesis occurs. Indeed, an increase in the activity of this synthetase has been reported in PD brains; this may be an attempt of dopaminergic neurons to compensate GSH depletion (Sian *et al.,* 1994; Schulz et al. 2000).

Studies show that an early event following glutathione depletion in mesencephalic cell cultures is the release of arachidonic acid (AA), whose metabolism may contribute to cell damage and death via the generation of oxygen free radicals (Mytilineou et al. 2002; Kramer *et al.;* 2004). Indeed, Kramer *et al.* (2004) suggested that the release of AA is phospholipase-2-dependent, thus involving inflammation processes.

Furthermore, decreases in glutathione availability and oxidative stress in the brain are also believed to promote mitochondrial damage via increased ROS and produce a toxic cellular environment capable of attacking a variety of biomolecules as well as inhibiting energy production (Materson *et al.,* 1991; Schapira *et al.,* 1994). Indeed, Jha *et al.* (2000) found that in PC12 cells depletion of glutathione levels results in selective inhibition of mitochondrial complex I.

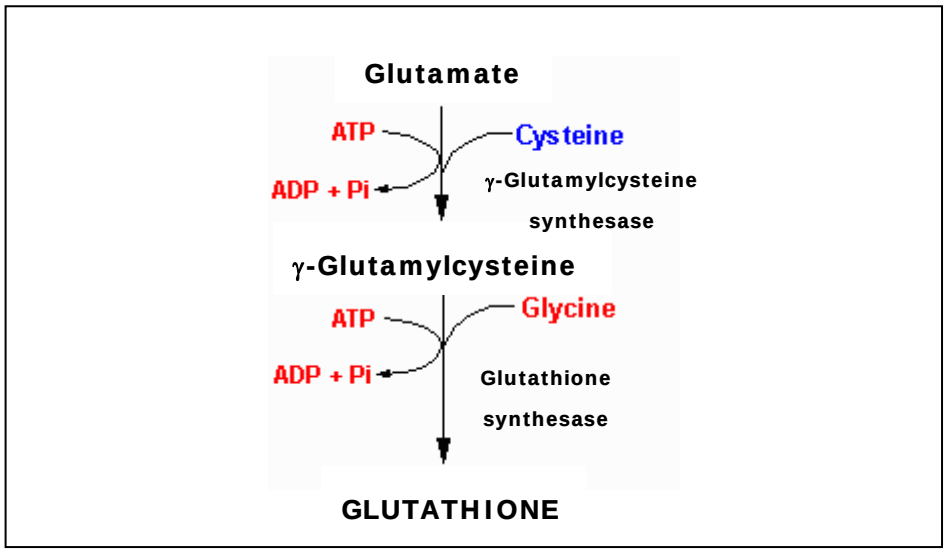


Figure 1.3. Synthesis of glutathione. *Glutathione is a tripeptide synthesised in vivo by the consecutive action of the enzymes: γ-glutamylcysteine synthetase which uses glutamate and cysteine as substrates to form γ-glutamylcystein and glutathione synthetase which catalyses the addition of glycine to the dipeptide γ-glutamylcystein to form glutathione. ATP hydrolyses is required in both enzymatic steps (Dringen. 2000).*

1.1.2.3 Environmental factors

Environmental factors have also been linked to PD aetiology, combined with genetic susceptibility (reviewed by Moyal-Segal and Soreq, 2006; Betarbet *et al.*, 2006) but the relative contributions of environmental versus genetics factors are still being debated (Langston, 2002; Moyal-Segal and Soreq, 2006). Despite the increase in the number of genes implicated in PD, recent twin studies suggest that genetics do not have a major role in PD aetiology and that non-genetic risk factors, like prolonged exposure to environmental toxins, are more important (discussed by Di Monte, 2003). Data from epidemiologic studies linked residence in rural areas and thus, exposure to pesticides to higher risk of developing PD (Di Monte 2001, 2003; Dauer and Przedborsky, 2003). Indeed, most of these studies agree that pesticides have a dose and time dependent effect, with a higher risk of developing PD in agricultural workers exposed to the pesticides over the long term (reviewed by Di Monte, 2003). Paraquat, a commonly used pesticide has been strongly associated with PD risk in a study performed in Taiwan (Liou *et al.*, 1997). However, epidemiologic studies are controversial and report different degrees of association between pesticide exposure and risk in developing PD, probably due to the different methodology utilised (Di Monte, 2003). The environmental hypothesis for PD is supported by *in vitro* studies which show that

certain pesticides including paraquat, dieldrin and maneb can cause degeneration of the DA neurons (Mc Cornmack *et al.,* 2002; Uversky, 2004). Moreover, exposure to transition metals (e.g. iron and copper) can also cause nigrostriatal damage and subsequently, contribute to PD. These metals can accumulate in the SNpc and catalyse harmful chemical reactions which can generate free radicals (Dexter *et al.,* 1989; Di Monte, 2001, 2003).

However, not all environmental factors increase the risk of developing PD. Coffee drinking and smoking are inversely associated with the risk of developing PD (Ascherio *et al.,* 2001; Hernan *et al.,* 2002; Tanner *et al.,* 2002). Indeed, several compounds present in the tobacco might inhibit MAO reducing both the formation of hydrogen peroxide, a product formed from MAO-mediated DA oxidation, and the metabolic activation of toxicants like MPTP (Fowler *et al.,* 1996).

1.1.2.4 Genetic factors

Although PD is generally idiopathic the discovery of some rare familial cases linked to genetic mutations has revealed novel clues about the aetiology of PD (Vila *et al.,* 2001; McNaught *et al.,* 2001; Chung *et al.,* 2001a; Shimura *et al.,* 2001).

To date, ten genetic markers have been identified in familial cases of PD (named PARK1-10). Some of these mutations have been extensively characterised and are associated with protein aggregation and degradation (reviewed by Huang *et al.,* 2004 and Le and Appel, 2004). Other mutations are associated with mitochondrial components including, phosphatase and tensin homologue (PTEN)-induced kinase-2 (PINK1) and Leucine-rich repeat kinase-2 (LRRK2), or with proteins involved in oxidative stress response (eg. DJ-1 and HtrA serine peptidase 2 (HTRA or OMI)). Table 1.1 summarises some of the most relevant PD- linked mutations for this study.

For example, mutations in the gene coding for α-synuclein, one of the major components of Lewy bodies, lead to an autosomal dominant form of PD. Three point mutations A53T, A30P and E46K have been reported (Polymeropoulos *et al.,* 1997; Kruger *et al.,* 1998, 2002; Zarranz *et al.,* 2004) alongside duplications and triplications of the gene in familial PD (reviewed by Abou-Sleiman *et al.,* 2006). The function of α-synuclein is still uncertain; however it has been reported to be involved in synaptic vesicle formation (Abou-Sleiman *et al.,* 2006). Several proteins have been described as

At least ten mutations have been reported in the gene that encodes parkin resulting in an autosomal recessive form of early-onset PD referred as autosomal recessive juvenile Parkinsonism (AR-JP; reviewed by Wood-Kaczmar *et al.,* 2006). Except for one positive case (Pramstaller *et al.,* 2005), the consensus is that parkin mutations do not lead to LB formation (Hyun et al., 2002; Shimura et al., 2001, McNaught et al., 2002a). Parkin is expressed primarily in the nervous system and has been reported to function as an ubiquitin protein ligase (E3) within the UPS (Kitada *et al.,* 1998; Zhang *et al.,* 2000; Shimura *et al.,* 2001; Barzilai and Melamed, 2003; Huang *et al.,* 2002). Several proteins have been described to act as regular substrates for parkin, including parkin-associated endothelial-receptor-like receptor called Pael receptor (Imai *et al.,* 2001), a synaptic vesicle-associated protein termed CDCrel-1 (Zhag *et al.,* 2000), a 22-KD glycosylated form of α-synuclein referred as αSp22 (Shimura et al., 2001) and synphilin-1 (Chung *et al.,* 2001b) both of which are proteins that are involved in PD (Wood-Kaczmar *et al.,* 2006). Of interest is that parkin is found in LBs and has been reported to colocalise with α-synuclein in the brain (Schlossmacher *et al.,* 2002). Moreover, parkin is reported to undergo proteasomal degradation, so parkin also ubiquitinates itself and promotes its own proteasomal degradation, and the turnovers of mutant-parkins are slower than the wild-type equivalent (Hyun *et al.,* 2002). Therefore, parkin mutations may impair

proteasomal degradation and lead to a dysfunction in neuronal homeostasis. Interestingly, *in vivo* recent studies have shown that parkin protects against α-synuclein-induced toxicity in drosophila (Haywood and Staveley, 2004), rats (Yamada *et al.,* 2005) and also against 6-hydroxyldopamine (6-OHDA) in an *in vivo* rat model (Vercammen *et al.,* 2006).

Another autosomic dominantly inherited case of PD is due to a missense mutation (Ile93Met) in the gene coding for ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1; Leroy *et al.,* 1998). UCH-L1 is involved in the conversion of polyubiquitin chains back into ubiquitin monomers within the ubiquitin-dependent protein degradation pathway. This mutation has been found to decrease the activity of the enzyme. How this is linked to PD is not clear but decreased UCH-L1 activity may impair the ubiquitin UPS and, as in mutant α-synuclein cases, may provoke a reduction in protein metabolism (Barzilai and Melamed, 2003; Chung *et al.,* 2001b; McNaught *et al.,* 2001). UCH-L1 has been found to also have an ubiquitin ligase activity, which takes place when the enzyme is present as a dimer and attaches ubiquitin via K63 and not the typical K48 linkage that promotes ATP dependent proteasome degradation. The genetic evidence for its pathogenicity is weak as only a single mutation has been identified in one family (Abou-Sleiman *et al.,* 2006). A common polymorphism (S18Y) in the same gene was later found linked to a decreased susceptibility to PD (Maraganore *et al.,* 1999: Facheris *et al.,* 2005). This S18Y variant has reduced ligase activity but comparable hydrolase activity as the wild enzyme. The UCH-L1 S18Y polymorphism encodes a UCH-L1 which is unable to form dimers, thus favouring proteasomal degradation, event which might explain the fact that S18Y mutations reduce susceptibility to PD (Liu Y.C *et al.,* 2002).

In conclusion, although it is not clear how mutations in α-synuclein, parkin or UCH-L1 genes cause DA cell death, these mutants support the premise that the ubiquitin proteasomal system (UPS) may play an important role in the pathogenesis of idiopathic PD due to a reduction in protein catabolism (McNaught *et al.,* 2001; Alves-Rodrigues *et al.,* 1998; David *et al.,* 2002; Davies 2001; Ding *et al.,* 2001b; Le and Apple, 2004). In addition to this, it is evident that several of these mutations also point to the role of oxidative stress in PD pathogenesis (eg. PARK6 and PARK7, see Table 1.1).

Interestingly, an increasing number of mutations in several genes are being identified in sporadic PD cases. Some of these genes are associated with DA transmission and metabolism and with the metabolism of xenobiotics. However, the positive or negative linkage of these mutations with PD in these studies vary depending on the ethnic origin of the patients (reviewed by Moyal-Segal and Soreq, 2006). Also, several authors have discussed the possible relation between abnormalities in mtDNA and defects in mitochondrial function in at least a proportion of PD patients (Gu *et al.* 1998; review by Schapira 2006 and Abou-Sleiman et al 2006).

Locus	Location	Inheritance	Function	Onset	LB
PARK 1/ 4	α-synuclein	AD	Involved in synaptic vesicle formation	30-60 years	+ +
PARK 2	Parkin	AR	An E3 ligase	~ 30 years	- / + in one case
PARK 5	UCH-L1	AD	Ubiquitin hydrolase and ligase	Late	?
PARK 6	PINK1	AR	Mitochondrial kinase	30-50 years	?
PARK 7	DJ-1	AR	Involved in oxidative stress response	20-40 years	?
PARK 8	LRRK2	AD	A protein kinase	40-60 years	+ variable pathology
Unmapped	HTRA2 or OMI	AD? predisposition	A serine protease and/ or involved in stress response	44-70 years	?

Table 1.1. Summary of the PD-associated genes. The table shows some of the most studied mutations associated with familial PD. *AD=autosomal dominant; AR= autosomal recesive ++= fulminant LB pathology; += LB are present; ?= unknown.*

1.1.2.5 Defects in protein degradation and neurodegeneration

Normal cellular functions generate significant levels of abnormal proteins. This is a particularly important event within the central nervous system (CNS), where high levels ROS and other free radicals are generated due to the enzymatic and auto-oxidation of several neurotransmitters, including DA, which is relevant to PD (McNaught and Olanow, 2006). It is essential to the cell to tightly regulate the production and clearance of these abnormal proteins, since they are prone to misfold and aggregate and are detrimental to the cell (Kopito, 2000). Indeed, proteolysis declines with age (Szweda *et al.,* 2002; Terman and Brunk, 2004) and in certain neurodegenerative disorders (Carrard *et al.,* 2002; Szweda *et al.,* 2002).

Mammalian cells possess two major systems for general protein degradation, lysosomal proteases and the UPS (Ding and Keller, 2001; Ciechanover, 2005). The UPS is involved in the degradation of abnormal and soluble intracellular proteins within the cytosol, nucleus or endoplasmic reticulum (for further details see section 1.1.3). This system has been reported to be impaired in SNpc from PD patients (Mc Naught and Jenner, 2001; Mc Naught *et al.,* 2001). Indeed, as earlier introduced, mutations in several genes encoding for components of the UPS are linked to several forms of inherited PD (Le and Appel, 2003; Krüger *et al.,* 2002) and several toxins or pesticides which induce parkinsonism have been reported to impair proteasomal function (Keller *et al.,* 2000; Elkon *et al.,* 2004; Höglinger *et al.,* 2003; Shamoto-Nagai *et al.,* 2003; Betarbet *et al.,* 2006; Wang *et al.,* 2006; Zeng *et al.,* 2006). All these findings suggest the involvement of the UPS in PD (further discussed in sections 3.1.1 and 3.1.2).

On the other hand, lysosomal degradation involves cathepsins, which degrade membrane and extracellular components following endocytosis into the lysosome (Nakanishi, 2003; Ciechanover, 2005; McNaught and Olanow, 2006). Cathepsins are also involved in cellular destruction during cell death (necrosis and apoptosis). Aspartyl (cathepsin D) and cysteinyl (cathepsin B, H and L) proteases are the most implicated lysosomal enzymes in neurodegeneration (reviewed by Artal-Sanz and Tavernarakis, 2005).

Other proteases have also been linked to neurodegeneration, for instance caspases and calpains (Artal-Sanz and Tavernarakis, 2005). The latter are cytosolic calcium-activated cysteine proteases, which exist mainly as two isoforms: μ-calpain (also known as

calpain I) or m-calpain (calpain II). These isoforms are respectively activated by low (micro-molar) and high (milli-molar) levels of calcium (Mendhi, 1991; Goll *et al.,* 2003; Costelli *et al.,* 2005). Calpains are involved in cell proliferation, differentiation, migration and apoptosis (Nixon, 2003) and in neurons in growth cone motility and guidance (Artal-Sanz and Tavernarakis, 2005). They activate or alter the regulation of certain enzymes, including key protein kinases and phosphatases, and regulate cytoskeleton organisation (Saido *et al.,* 1994; Gryspan *et al.,* 1997). Indeed, calpains can degrade several cytoskeletal proteins (e.g. spectrin, tau or NFs) and several constituents of myelin (Stys and Jiang, 2002). Both calpain activity and intracellular calcium levels have been reported to increase with ageing (Costelli *et al.,* 2005). Moreover, calpains have been linked to several neurodegenerative conditions (Chard *et al.,* 1995; Moldoveanu *et al.,* 2002). For example, calpains have been reported to be increased in Alzheimer's disease (AD; Gryspan *et al.,* 1997) and in animal models of Huntinton´s disease (Bizat *et al.,* 2003 a, b) and PD (Crocker *et al.,* 2003). Interestingly, α-synuclein, the major component of LBs, has recently been reported to be a substrate for calpains (Mishizen-Eberz *et al.,* 2005). In addition to this, the implication of calpains in PD is further supported by the fact that overexpression of m-calpain has been detected in brain of PD patients (Mouatt-Prigent *et al.,* 1996).

1.1.3 Lewy bodies

One of the pathological hallmarks of PD is the presence of neuronal cytoplasmic filamentous inclusions known as LBs. However, LBs are not specific for PD and are also found in AD, dementia with LBs and even in normal individuals of advanced age at a higher frequency than the prevalence of PD (Dauer and Przedborsky, 2003). These inclusions were first described by the German neuropathologist Frederich H. Lewy in 1912 (Wakabayashi *et al.,* 2005). LBs are widely distributed in the CNS, and in cases of idiopathic PD are found within the brain stem nuclei, which includes SN, locus coeruleus and dorsal motor vagal glanglus, and to a lesser extent within the cerebral cortex (Gai *et al.,* 2000). LBs are spherical cytoplasmic protein aggregates that are mainly composed of α-synuclein and its interacting partner synphilin-1 but also parkin, ubiquitin, neurofilaments (Forno *et al.* 1996; Spillantini *et al.* 1998; Gai *et al.,* 2000) and also proteasomal subunits and other components (Alves-Rodrigues *et al.,* 1998; McNaught and Jenner, 2001; Mc Naught and Olanow, 2006). LBs are located within the

perikarya or within neurites (Wakabayashi *et al,* 1992). Morphologically, LBs are concentric inclusions that typically possess a dense eosinophilic core and peripheral halo of radially arranged filaments comprised of fibillar α-synuclein and neurofilaments (Forno *et al.,* 1986; Wakabayashi *et al,* 1992 Spillantini *et al.,* 1998).

1.1.3.1 Protein aggregation and LB formation in PD

Defects in protein handling could be a crucial factor in the pathogenesis of PD (Alves-Rodrigues *et al.,* 1998; Chung *et al.,* 2001a; Shoesmith-Berke and Paulson, 2003). Indeed, abnormal proteins which are constantly produced due to normal cell metabolism, are normally cleared by the UPS in association with molecular chaperones, thus avoiding their accumulation (Betarbet *et al.,* 2005; McNaught and Olanow, 2006). However, ageing and impaired proteasomal function (McNaught and Jenner, 2001, Carrard *et al.,* 2002) accompanied by high levels of oxidative stress (Keller *et al.,* 2004), may lead to the accumulation of these proteins which eventually may be damaging to the cell (McNaught *et al.,* 2002; Chung *et al.,* 2001a,b). Moreover, intrinsic features of certain proteins such as α-synuclein might make them more prone to aggregation and more resistant to proteasomal degradation (Alves-Rodrigues et al., 1998). Indeed, several authors report that proteasomal inhibition results in accumulation of α-synuclein, the main component of LBs and a substrate for the UPS (Tofaris *et al.,* 2003; Mc Naught *et al.,* 2002a; Demasi and Davies, 2003; Sawada *et al.,* 2004). In addition to this, the high occurrence of ubiquitinated protein species within the LBs and the observation that parkin functions as an E3 ubiquitin-ligase make it plausible that proteins within the LBs are objects of parkin mediated ubiquitination so, the UPS appears to be an intersection of whether a toxic protein is degraded or it is packaged into an inclusion (Chung *et al.,* 2001b). At the same time protein aggregates have been reported to impair proteasomal function since heavily oxidised and cross-linked proteins present in these aggregates are poor substrates for the proteasome that prefers unfolded proteins as substrates (Wojcik and DeMartino, 2003; Grune *et al.,* 2004).

The current consensus points to LB formation as an aggresome-related process (Olanow *et al.,* 2004). Indeed, oxidised and damaged proteins are relatively resistant to degradation by normal proteolytic mechanisms and therefore are transported to centrosomes (i.e. perinuclear microtubule-organising centres) where they become associated with components of the UPS and are encapsulated by intermediate filaments

(IF's) to form large structures called aggresomes. Aggresomes are sites of enhanced proteolysis whose formation may serve to protect the nucleus and other organelles from exposure to the cytotoxic effects of abnormal proteins. By this means, LBs could be specialised aggresome-related structures that are formed in DA neurons as a way of sequestering and compartmentalisating those proorly degraded proteins and, ultimately, as a means of controlling excessive levels of abnormal proteins. Therefore, LBs may play a cytoprotective role that delays the onset of neuronal degeneration (reviewed by the following authors: Kopito, 2000; Olanow *et al.*, 2004; McNaught and Olanow, 2006). This idea is supported by the relatively severe neurodegeneration and early onset of symptoms in patients suffering from mutant-parkin-linked-PD, which lack LBs (Chung *et al.*, 2001b; McNaught *et al.*, 2001). Although the current theories point to a protective role of LBs some authors suggest that LBs are cytotoxic, at least in the later stages of the disease (reviewed by Ardley *et al.*, 2005; Harrower *et al.*, 2005; Mc Naught and Olanow, 2006).

Interestingly, Meredith *et al.* (2004) suggest that lysosomes might also be involved in LB formation based on α-synuclein accumulation in a MPTP animal model. These authors hypothesise that since ATP levels and UPS activity are decreased in damaged neurons, accumulated proteins are translocated to the lysosomes, a process assisted by molecular chaperones, for degradation. In the lysosome lipofucsin granules, lipids and neuromelanin accumulate until the lysosome collapses resulting in release of its contents into the cytoplasm where lipofucsin granules might provide nucleation centres for LB formation (Meredith *et al.*, 2004).

1.2 THE UBIQUITIN PROTEASOMAL SYSTEM (UPS)

The UPS plays an essential role in the degradation and clearance of short-lived, mutant, misfolded or damaged proteins in eukaryotes and ultimately in the regulation of crucial processes such as the cell cycle, transcription, antigen processing or signal transduction (Goldberg et al 1995; Ding and Keller, 2001). In neurons, the UPS plays an essential role in the control of normal neuronal function and homeostasis through the selective degradation of neuronal proteins (Ehlers, 2003). This system is soluble, has a slightly alkaline optimum pH and is ATP dependent, thus requiring magnesium as a cofactor (Ding and Keller, 2001b). Speese *et al.*, (2003) show that the UPS tightly controls levels

of presynaptic proteins and that the rate of UPS-dependent protein degradation is a primary determinant of neurotransmission strength.

Figure 1.3 illustrates the successive enzymatic steps involved in the UPS; firstly, ubiquitin, a heat stable 76-residue polypeptide, is activated by ubiquitin-activating enzyme (E1) in an ATP-dependent manner (Hersko and Ciechanover, 1998; Pickart, 2001). E1 forms a thiol ester linkage between a cysteine residue and carboxyl-terminal glycine in ubiquitin. Activated ubiquitin is then transferred to an ubiquitin-conjugating enzyme (E2; via another thiol linkage), which in conjunction with E3, an ubiquitin-protein ligase, identifies and mediates the attachment of polyubiquitin chains to the substrate. This process is referred to as ubiquitination and is the signal for degradation of the tagged protein by the 26S proteasome. The polyubiquitin chain is recognised by the proteasome, and the proteasome complex then rapidly degrades the labelled protein in an ATP dependent manner. This process produces short peptide fragments that are further degraded by peptidases to single aminoacids that can be recycled for new protein synthesis (Saric *et al.,* 2004).

Polyubiquitinated chains are attached via an isopeptide bond between the conserved C-terminal glycine residue of ubiquitin and the ε-amino group of the lysine (Lys or K) residue of the substrate (Hersko and Ciechanover, 1998; Verma and Deshaies 2000). However, there are also reports of proteins that are ubiquitinated without the requirement of Lys residues (Bloom *et al.,* 2003). Polyubiquitinated chains are formed by the sequential addition of mono-ubiquitin to a Lys residue of ubiquitin (isopeptide bonds between Gly76 and Lys48) which is already bound to the substrate (Chau *et al.,* 1989). A chain elongation factor (E4) may be required for the polyubiquitination of some proteasome substrates (Hartmann-Petersen *et al.* 2003).

Ubiquitin has seven lysine residues each of which can potentially bind the C-terminal glycine of the next ubiquitin moiety in the multi-ubiquitin chain, however, not all these linkages occur naturally (Chung. *et al.,* 2001a,b; Hartmann-Petersen *et al.* 2003). The most common ubiquitin linkage for targeting proteins for degradation by the proteasome is through Lys48 (Chau *et al.,* 1989), although Lys29-linked chains may also serve as degradation signals (Johson *et al.,* 1995). Other alternative linkages, for example at Lys63 are not recognised as a signal for proteasomal degradation, instead regulating

processes such as DNA repair, endocytosis or translational regulation (Hicke, 2001; Welchman *et al.*, 2005).

There is a range of different E2 enzymes that can associate with a variety of E3 enzymes, incorporating substrate specificity to the process (Ferrel et al., 2000; Chung *et al.*, 2001a; Hartmann-Petersen *et al.*, 2003). Indeed, since the number of E3 ligases exceeds the number of E2s, substrate specificity is mostly mediated by ligases (Ross and Pickart, 2004; Pines and Lindon, 2005). Multi-polyubiquitination is a reversible process. Several deubiquitinating enzymes appear to take part in the regulation and selectivity of the substrate targeted for degradation (Chung. *et al.*, 2001b; McNaught and Jenner, 2001; Hartmann-Petersen *et al.*, 2003).

Although the proteasome was initially characterised for its role in ATP and ubiquitin dependent proteolysis there is a significant amount of evidence that an ATP and/ or ubiquitin independent version of the proteasome also exists (Alves-Rodrigues *et al.*, 1998). Indeed, the first protein shown to be degraded by the 26S proteasome in an ubiquitin independent manner was ornithine descarboxylase (Murakami *et al.*, 1992). Other examples of proteins degraded in an ubiquitin-independent manner by the proteasome are c-Jun, calmodulin, troponin and p53 (reviewed by De Vrij *et al.*, 2004).

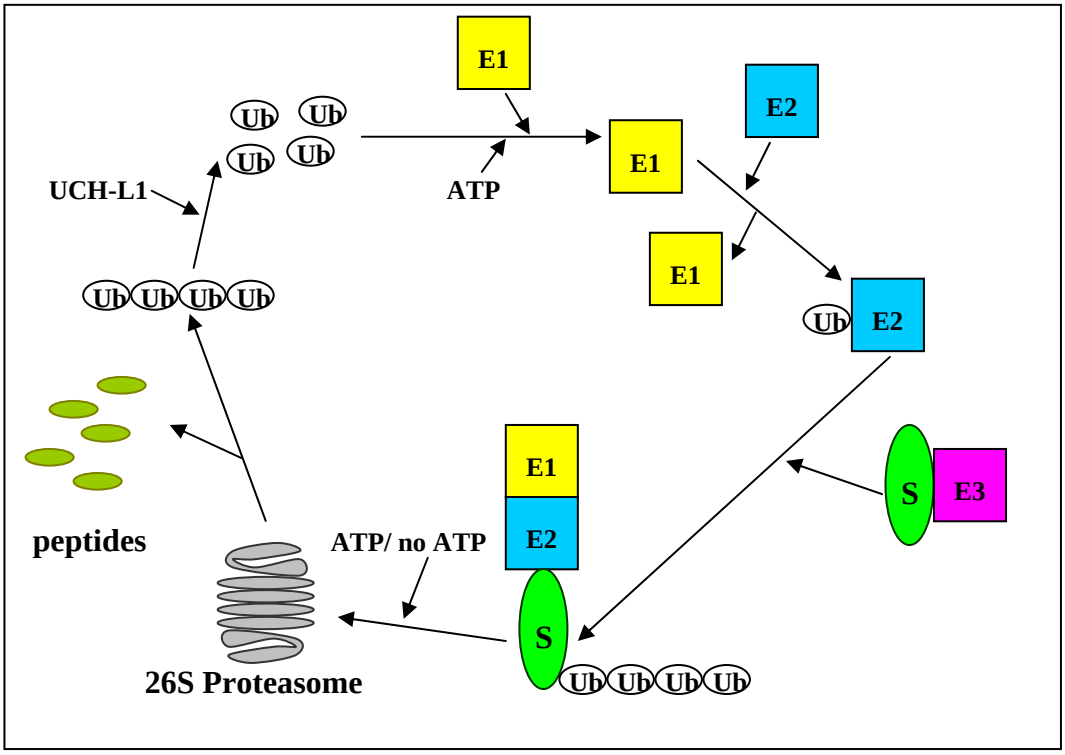


Figure 1.4. The Ubiquitin-Proteasome System (Taken from Betarbet et al. (2005) with modifications). *Ubiquitin is activated by E1 and covalently linked to the substrate with the help of E2 and E3 which is then degraded by the 26S proteasome in an ATP-dependent manner to generate small peptides around 4-10 aminoacids in length. Once the protein undergoes degradation the ubiquitin chain is released. Small oxidised proteins can undergo ATP-independent degradation by the 20S proteasome.*

1.2.1 Proteasome structure

The 26S proteasome is a ~2.5 MDa multicatalytic protease that is present in the cytoplasm and nuclei of all eukaryotic cells, which is responsible for the majority of intracellular proteolysis. As Figure 1.5 shows, two subcomplexes, the 20S catalytic core and the 19S regulatory particle (also known as PA700), make up the whole complex (Ferrell *et al.*, 2000; Ding and Keller, 2001). The 26S is involved in the ATP-dependent degradation of ubiquitinated and deubiquitinated proteins (Ciechanover, 1998). The proteasome is a very highly selective and specific proteolytic complex, involving both the 19S, that selectively recognises the substrates, and the 20S core, which can only degrade proteins that are unfolded. The barrel-shaped structure of the 20S core sequesters the active sites into the inner chambers (Wolf and Hilt, 2004).

The 20S proteasome can also be associated with one or two 11S (or PA28) particles, composed α– and β–subunits, that can be induced by interferon-γ to form the "immunoproteasome" (Chu-Ping *et al.*, 1992).

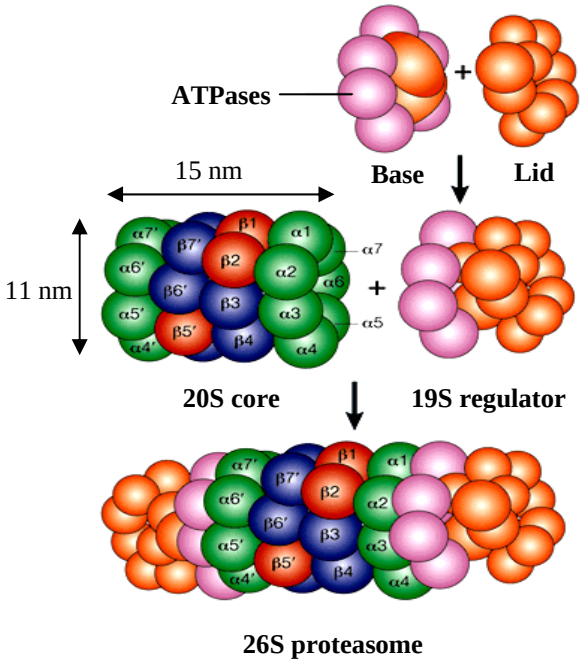


Figure 1.5. 26S proteasome complex (from Kloetzel; 2001 and Groll et al., 1997 with modifications). *The 26S proteasome is composed of 14 α- and 14 β-subunits arranged in 4 separated rings consisting of either 7 α- or 7β-subunits. The core particle has multiple catalytic centres located in the β-subunits that comprise the two inner rings of the 20S proteasome. Either end of the 20S core particle can cooperatively bind 2 regulatory cap subunits referred to as 19S. The regulatory subunits are involved in substrate recognition, unfolding and translocation to the catalytic site.*

1.2.1.1 20S catalytic core

The 20S catalytic core is composed of 14 α– and 14 β–subunits arranged in 4 separated rings consisting of either 7 α– or β–subunits which form a hollow cylindrical structure where proteolysis occurs (DeMartino and Slaughter, 1999; Jäger *et al.*, 1999). The core particle has multiple catalytic centres located in the β–subunits that comprise the two inner rings of the 20S proteasome. The best characterised proteolytic activities of the 20S proteasome are known as chymotrypsin-like (CLA), trypsin-like (TLA) or

postacidic-like activity (PLA; also known as peptidyl glutamyl peptide hydrolase or caspase-like activity) which preferentially recognise and cleave tyrosine or phenylalanine (hydrophobic residues), arginine or lysine (basic residues) and glutamate, respectively. Some biochemical data demonstrate that each catalytic activity is linked with a specific β–subunit. Thus three of the different β–subunits (present as duplicates) are presumably catalysts whereas the remaining subunits are of unknown function (De Martino and Slaughter, 1999; Brooks *et al.* 2000; Ferrell *et al.* 2000). In higher eukaryotes each of these three catalytic subunits are termed as β1 (PLA), β2 (TLA) and β5 (CLA). However, mutational studies on the different β-subunits suggested that each of the three active sites is formed by two subunits, the active subunit and a complementary non-catalytic subunit, whose inactivation results in reductions of the proteolytic activities. Therefore β4 and β5 form the CLA site, β2 together with β6 form the TLA site, and β1 and β7 form the PLA site (further information in table 4.3; Dick *et al.,* 1992; Hilt *et al.,* 1993; Arendt and Hochstrasser, 1997; Heinemeyer *et al.,* 1997). On the other hand, the two outer rings of the catalytic barrel, composed exclusively of α–subunits, which form two axial pores, ensure that only unfolded substrates can entry the catalytic chamber (Groll *et al.,* 2000) and play important roles in maintaining 20S stability and provide scaffolding for 20S binding proteins (De Martino and Slaughter, 1999; Brooks *et al.,* 2000; Ferrell *et al.,* 2000).

Structural and genetic studies identified the proteasome as an N-terminal nucleophile-hydrolase (Ntn) with a threonine (Thr) residue acting as the catalytic nucleophile (Fenteany *et al.,* 1995). Activation of the hydroxyl group of the Thr requires a proton acceptor at the active site. The surrounding area of the Thr contains a conserved lysine group, which at the neutral pH conditions in the active site environment of proteasomes is likely to be in a charged state, thus making it an unsuitable candidate for accepting a proton. Instead Lys is thought to lower the pK_a of the N-terminal amino group of Thr1 by its electrostatic potential, so that this group can act as the proton acceptor in proteolysis (Groll *et al.,* 1997; Groll and Huber, 2003).

Proteolysis of substrates by the proteasome occurs in a sequential manner and substrates are cut at many sites to yield small oligopeptides of between four and fourteen amino acids (Wolf and Hilt, 2004). This progressive mode of degradation might help to ensure that proteins are rapidly eliminated without retaining any biological function that could

be deleterious for the cell (Kisselev *et al.,* 1999). Even though the precise mechanism involved in such degradation is unclear it may involve conformational changes in the proteasome resulting in the opening of its central cavity and/or allosteric modifications of the catalytic sites (DeMartino and Slaughter, 1999, Ding and Keller, 2001b). Indeed, allosteric interactions between CLA and PLA have been described since substrates for the PLA appear to non-competitively inhibit CLA via binding non-catalytic sites (Kisselev *et al.,* 1999; Myung *et al.,* 2001). Proteasome allosterism is not fully understood but might be a very precise and diverse form of proteasomal regulation (reviewed by Gaczynska *et al.,* 2006). The proteasome subunit composition and the spacing between the individual proteolytic sites dictate the length and type of peptides generated by the proteasome (DeMartino and Slaughter, 1999, Ding and Keller, 2001b). Interestingly, genetic studies revealed that the chymotrypsin-like activity (harboured by β5) is the most important proteolytic activity for proteasomal function and also for cell growth, followed by trypsin-like (β2) and postacidic-like (β1) activities, since β5β1 and β5β2 double mutants of yeast are viable, whilst β2β1 mutants are not (Jäger *et al.,* 1999).

Initial studies suggested that the 20S proteasome rarely functioned as an isolated enzyme, the 26S proteasome being the major form in vivo. However, subcellular localization and stoichiometry of the different complexes showed that free 20S particles exceed the 19S or 11S (free or bound to 20S) over 3 to 4-fold (Rivett, 1998; Brooks *et al.,* 2000). Moreover, the 20S proteasome itself can degrade proteins without ubiquitin or ATP (Davies K.J.A., 2001; Grune *et al.,* 2003). Indeed, the 20S proteasome plays a major role in the degradation of mildly oxidised soluble proteins in the cytoplasm, nucleus and endoplasmic reticulum, a process which is ATP and ubiquitin independent (Davies, 2001; Grune *et al.,* 2003). The mechanism by which oxidised proteins are recognised by the 20S protesome involves the partial denaturation and unfolding of the protein which will lead to the exposure of hydrophobic patches of aminoacids at the surface of the oxidised proteins. These hydrophobic patches are able to bind to the α-subunits at the entrance of the core particle which in turn will result in the opening of the pores in the 20S particle helping it access into the catalytic chamber (Davies, 2001; Grune *et al.,* 2003). This is supported by Ding *et al.* (2003) who found that low levels of oxidative stress increased the amount of protein oxidation without affecting proteasome

activity. Of interest is the fact that the 20S complex has been reported to be 4-fold more resistant to oxidative stress than the 26S (Reinheckel *et al.,* 1998).

1.2.1.2 The 19S regulatory particle

Either end of the 20S proteasome can bind, in a cooperative and ATP dependent manner, an additional cap-like structured regulatory protein, known as 19S or P700, to form the 26S. The binding of the regulatory subunit to the catalytic 20S core enhances the ability of the proteasome to degrade both ubiquitinated proteins and non-ubiquitinated peptides (DeMartino and Slaughter, 1999; Ferrell *et al.,* 2000).

The 19S complex is universally composed of two different subcomplexes referred to as "lid", of unknown structure, and "base". The latter consists of a hexameric ring of 6 different non-redundant ATPases and three additional non-ATPase subunits whilst the lid is built up from eight different non-ATPase subunits (see figure 1.4; Chu-Ping *et al.,* 1994; Ferrell *et al.,* 2000; Glickman and Raveh, 2005). The 19S particle is involved in several ATP-dependent functions including (a) substrate recognition (recognition of the ubiquitin chain of the substrates) and unfolding, (b) disassembly of polyubiquitin chains from the substrate, (c) opening of the gates formed by the 20S α-subunits and (c) translocation of the unfolded sustrate to the catalytic chamber via the reverse-chaperone activity present in the base of the particle (reviewed by Hartmann-Petersen *et al.,* 2003). Molecular chaperons also cooperate with the UPS, facilitating and enhancing the correct folding and placement of the protein, as well as preventing proteins to aggregate or missfold (Imai *et al.,* 2003; Muchowski and Wacker, 2005).

These ATP dependent functions are not required for the hydrolysis of short peptides and the regulatory complex may activate that process by allosteric modification of the active centres (DeMartino and Slaughter, 1999; Ferrell *et al.,* 2000; Ding and Keller, 2001).

1.3 TOXINS USED IN PD MODELS

Certain pharmacological agents and environmental toxins cause lesions in specific cell populations and thereby mimic the pathological and symptomatic features of some neurodegenerative disorders. Such experimental models are useful for understanding the pathophysiology of PD and thus for assessing and developing new therapeutic strategies (Beal, 2001; Sanchez-Pernaute *et al.,* 2005; Shimohama *et al.,* 2003). Some of the most common toxins used in PD models include MPTP/ MPP^+, rotenone, 6-OHDA and DA. The structure of these compounds is illustrated in figure 1.5.

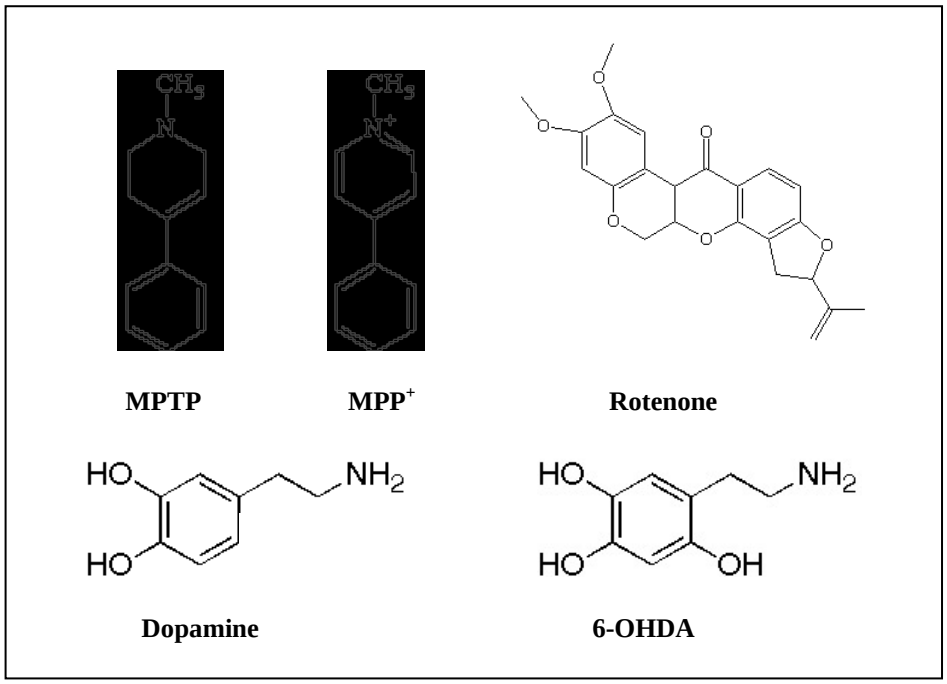


Figure 1.6. Chemical structures of MPTP, MPP^+, rotenone, 6-OHDA and dopamine.

1.3.1 MPTP model

One of the best-studied models of PD, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model, was first described to induce a human parkinsonism state after being injected as a narcotic analgesic (Davis *et al.,* 1979). Its use was later reported in a group of north-Californian drug addicts who developed strong parkinsonism symptoms after intravenously injecting a MPTP-contaminated "synthetic heroin" (Langston *et al.,* 1983). To date MPTP is one of the best experimental models of PD (Przedborski and

Vila, 2001). Indeed, many models exist for investigation of MPTP toxicity which range from whole animals to cell culture systems (Forno *et al.*, 1988).

1.3.1.1 Bioactivation and toxicity of MPTP

MPTP toxicity is believed to be mediated by at least two metabolic steps (see figure 1.6). Firstly, MPTP, which is highly lipophilic, readily crosses the brain blood barrier and can be taken up non-specifically by glial cells where it is oxidised, via monoamine oxidase B (MAO-B), to an intermediate, 1-methyl-4-phenyl-2, 3-dihidropyridium ion ($MPDP^+$), which is then auto-oxidised (non-enzymatically) to 1-methyl-4-phenylpyridinium ion (MPP^+), the active neurotoxin (Langston *et al.*, 1983; D'Amato *et al.*, 1986; Vidaluc, 1996; Speciale, 2002; Beal, 2001). Once produced, MPP^+ is taken up selectively by the DA transporter into DA neurons and can either (a) enter the mithochondria and inhibit the mitochondrial electron transport chain (Ramsay and Singer, 1986), (b) be sequestered into cytoplasmic vesicles by actions of the vesicular monoamine transporters (VMAT; Del Zompo *et al.*, 1993) or (c) remain in the cytosol where it can interact with different cytosolic enzymes (Klaidman *et al.*, 1993). MPP^+ toxicity is inversely proportional to the capacity of the cell to sequester the toxin via the VMAT (Takahashi *et al.*, 1997).

Mitochondrial dysfunction is mainly caused by direct inhibition of the electron transport enzyme NADH: ubiquinone oxidoreductase, also known as complex I; however MPP^+ has also been reported to inhibit complex III and IV of the electron transport chain. Blockage of mitochondrial function leads to decreased ATP levels, loss of mitochondrial membrane potential and possibly the formation of ROS, all of which may be contributing to the selective degeneration of the DA containing neurons (Langston *et al.*, 1983; Song *et al.*, 1998; Vidaluc, 1996; Bernardi *et al.*, 1999; Cassarino and Bennett, 1999; Conn *et al.*, 2001; Sherer *et al.*, 2001).

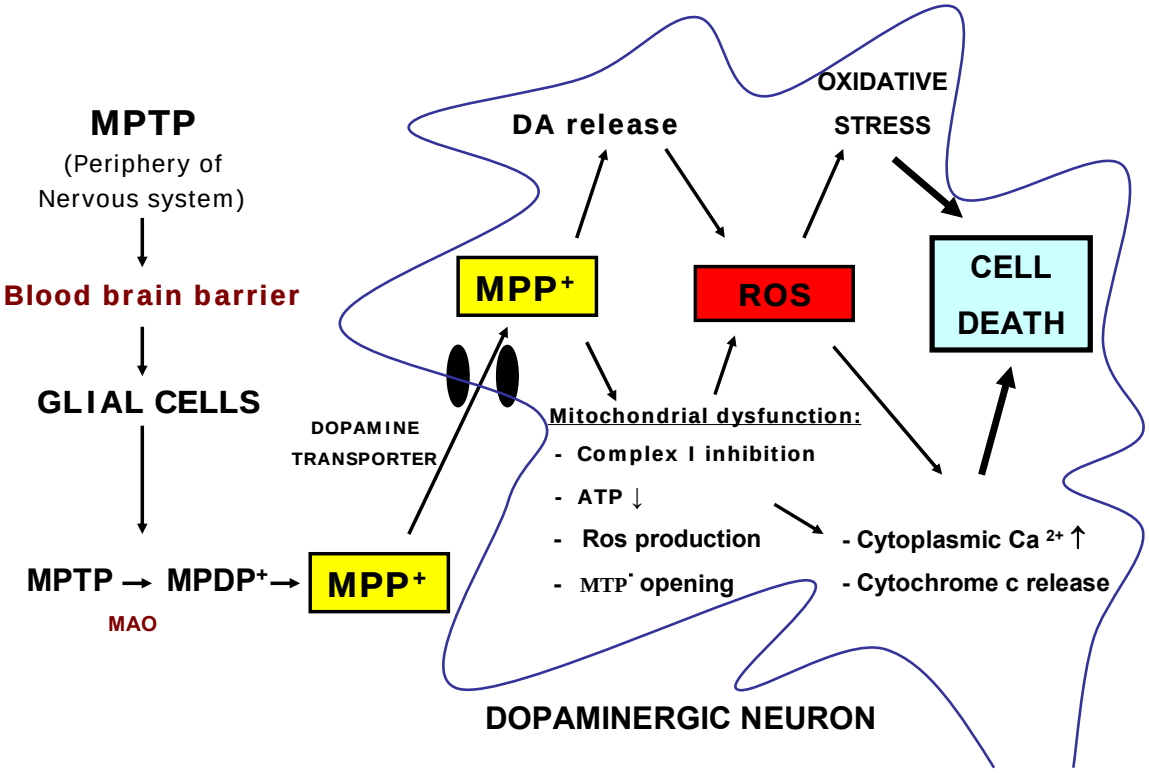


Figure 1.7. Summary of the mechanisms involved in MPTP toxicity (taken from Blum et al., 2001 with modifications). *MPTP can cross the blood brain barrier and be taken up by glial cells were it is bioactivated via MAO to MPP^+, where upon it is transported inside the DA neuron via the DA transporter. Once inside the cell, MPP^+ is accumulated inside the mitochondria where it inhibits complex I activity and thus ATP synthesis. MPP^+ also releases DA from the endogenous stores. Both events result in increased ROS which together with complex I inhibition increases cytoplasmic calcium levels and provokes the release of cytochrome c. All these events ultimately result in cell death.*

1.3.1.2 Role of oxidative stress in MPTP toxicity

Johannessen *et al.*, (1985) were the first authors to suggest that oxidative stress may contribute to MPP^+ toxicity. This hypothesis is supported by several authors. Indeed, it has been reported that MPP^+ induces the release of DA from endogenous stores, which, as detailed in previous sections, can be subsequently oxidised generating hydrogen peroxide, superoxide ($O_2^{-\bullet}$), quinone species and hydroxyl radicals ($OH^\bullet$) which are able to modify cellular macromolecules (Graham, 1978; Chiueh *et al.*, 1992; Vidaluc, 1996). The deleterious effects of DA release might actually precede mithochondrial dysfunction (Chan *et al.*, 1991). Indeed, Lotharius and O'Malley (2000) propose that the formation of ROS following MPP^+ treatment of mesencephalic cultures is not

initiated in the mitochondria but results from DA vesicular displacement within the cytoplasm that results in its oxidation. Another source of $O_2^{-\bullet}$ comes from the autooxidation of $MPDP^+$ which can be further catalysed to $OH^\bullet$ (Zang and Misra, 1992) and also from the leakage of reducing equivalents onto molecular oxygen due to mitochondrial dysfunction. Involvement of oxidative stress in MPTP neurotoxicity is further supported by the protection of several antioxidative agents (Lai *et al.*, 1993; Gonzalez-Polo *et al.*, 2004). For instance, Cassarino *et al.*, (1997) showed that MPP^+ induced ROS formation and increased the activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase in SH-SY5Y cells and in an *in vivo* model.

However other researchers place in doubt this hypothesis. Indeed, Lee *et al.* (2000a) suggested that MPP^+ toxicity is not primarily driven by oxygen free radicals in human neuroblastoma SH-SY5Y cells although it may exacerbate the vulnerability of the cells to oxidative damage. This was concluded after finding that toxic concentrations of MPP^+ did not increase lipid peroxidation and co-treatment with antioxidants did not attenuate MPP^+ toxicity. Consistently, MPP^+ toxicity was also found to be primarily due to the impairment in energy metabolism and not due to oxidative stress in a rat cell model (PC12 cells). As in the human cell line, MPTP/MPP^+ did not increase lipid peroxidation and again antioxidant administration did not protect the cells from toxic insult (Fonck and Baudry, 2001).

MPP^+ has also been shown to affect calcium homeostasis. Inhibition of complex I and subsequent ATP depletion can impair the plama membrane Ca^{2+}-ATPase which is involved in removing Ca^{2+} from the cell; this results in increased levels of free cytosolic calcium (Chen *et al.*, 1995). However, calcium can also accumulate in the cytosol via an impairment of the mitochondrial transition pore (MTP). MTP is a non-selective, high conductance pore which facilitates the passage of solutes between the mitochondrial matrix and cytoplasm (Cassarino and Bennet, 1999). The involvement of the MTP in MPP^+ toxicity was confirmed by Kass *et al.* (1988) who reported increased calcium levels after MPP^+ treatment of isolated hepatocytes with no involvement of the plama membrane Ca^{2+}-ATPase suggesting that the MTP was involved in the process. Indeed, MPP^+ via complex I inhibition is reported to induce the opening of brain MTPs, an event that results in the release of calcium but also cytochrome c (Cassarino *et al.*, 1999). Release of calcium may lead to the activation of kinases, proteases and nitric acid synthase which contribute to cell death (reviewed by Blum *et al.*, 2001).

Interestingly, MPP^+ binds with high affinity to neuromelanin, which is present in high concentrations in the SNpc of primates; this can provide partial explanation to selectivity of the toxicant towards DA neurons (D'Amato *et al.,* 1986).

1.3.1.3 Species differences and formation of insoluble aggregates

The MPTP model is performed in a wide range of species including vertebrates and invertebrates (eg. frogs, leeches or planaria) but it has also been used in cell cultures (reviewed by Shimohama *et al.,* 2003). Differences in species susceptibility to the toxin have been observed. For instance, rodents show variable sensitivity to MPTP; rats are resistant to the toxin whilst mice show different susceptibility and behavioural effects dependent on the strain utilised (Brownell *et al.,* 1998; Hamre *et al.,* 1999). In contrast, in humans and other primates MPTP causes a severe irreversible PD-like syndrome due to the degeneration of nigral DA neurons (Kopin and Markey, 1988; Langston and Irwin, 1986). Although there is only limited evidence for the presence of insoluble inclusions in MPTP models, this may be because chronic treatment has not been intensely studied (Beal, 2001). Indeed, chronic exposure of the toxin performed in macaques reproduced all the symptoms of PD but without the appearance of LB (Brownell *et al.,* 1998; 2003). Recently, Shimoji *et al.* (2005) also report the absence of inclusion body formation in adult wild-type C57BL6 mice following acute, semi-chronic and chronic exposure to MPTP. On the other hand, some proteinaceous inclusion bodies have been reported in MPTP-treated aged-monkeys although these inclusions were structurally different to the typical LB (Forno *et al.,* 1988). In addition to this, Meredith *et al.* (2002) report that chronic treatment of mice with MPTP and probenceid (a compound that retards MPTP and its metabolites clearance) results in the formation of LB-like inclusions which are immunoreactive for α-synuclein. This is consistent with Fornai *et al.* (2005) who report the formation of nigral inclusions following continous administration of MPTP in a mouse model, a process which was α-synuclein-dependent.

1.3.2 Rotenone

Epidemiologic studies give evidence of the involvement of pesticide exposure in PD pathogenesis. Moreover some pesticides, like rotenone or paraquat are currently used in *in vivo* and *in vitro* PD models and have been useful tools in giving a better understanding of the causes of the disease (Betarbet *et al.,* 2006; Brown *et al.,* 2006).

Rotenone is a naturally occurring compound derived from the roots of certain plants, which is commonly used as an insecticide. Its hydrophobic nature allows it to readily cross biological membranes (Perier *et al.,* 2003). Chronic exposure to rotenone in a rat model has been shown to specifically inhibit complex I of the electron transport chain, to produce selective nigrostriatal degeneration with the appearance of cytoplasmatic inclusions similar to Lewy bodies, which contained α-synuclein and ubiquitin. Moreover, exposure to this insecticide led to motor symptoms characteristic of PD (Betarbet *et al.,* 2000).

1.3.3 6-hydroxydopamine (6-OHDA)

6-OHDA, the hydroxylated analogue of DA, was the first agent used in an animal model of PD (Ungerstedt, 1971). This toxin selectively accumulates in DA neurons, leading to their death (Lotharius and O'Malley, 2000; Beal, 2001). 6-OHDA can not cross the blood brain barrier therefore, DA cell loss can only occur after direct intracerebral administration; in PD models injections are preferably given into the striatum or SN (Blum *et al.,* 2001). Since 6-OHDA can be generated by non-enzymatic reactions between DA, hydrogen peroxide and free iron, all present in the brain, this DA derivate can be considered as an endogenous neurotoxin (Jellinger *et al.,* 1995).

As with DA, free radical generation is involved in 6-OHDA toxicity (Blum *et al.,* 2001, Betarbet *et al.,* 2002). This is supported by the protection that antioxidants conferred against the toxin (Blum *et al.,* 2000). 6-OHDA lesions do not result in Lewy body formation and can produce non-specific damage to other neurons (Beal, *2001*). However, this toxin can reproduce the motor deficits seen in PD and has in the past been considered useful as a PD model for screening therapeutic strategies (Ungerstedt, 1971; Beal, 2001). A recent study suggests that 6-OHDA abolished both aerobic and anaerobic cell functions. Moreover, its autooxidation produces H_2O_2 which may

contribute to loss of anaerobic glycolysis likely via inhibition of lactic acid dehydrogenase (Mazzio *et al.*, 2004).

1.3.4 DA

DA is a natural neurotransmitter of the brain whose concentration in DA neurons oscillates from 0.1 to 1 mM. However, within dopaminergic neurons DA is normally sequestered into vesicles, which regulates its concentration in the cytoplasm and also in the synaptic cleft. Increased DA levels can be deleterious to the cell and have been reported in conditions like hypoxia or ischemia (reviewed by Blum *et al.*, 2001). As indicated earlier, loss of DA due to a loss of the DA neurons within the SNpc occurs in PD and is responsible for most of its motor symptoms (Barzilai and Melamed, 2003; Beal, 2001; Blum *et al.*, 2001).

DA itself is believed to be directly involved in the initiation of DA cell neurodegeneration (Zilkha-Falb *et al.*, 1997). As introduced earlier, DA is a source of ROS generation unique to DA neurons through its oxidation (reviewed by Blum *et al.*, 2001).

DA neurotoxicity has indeed been reported *in vivo*, in primary cultures and in several cell lines (Michel and Hefti, 1990; Filloux and Townsend 1993; Junn and Mouradian, 2001; Gomez-Santos *et al.*, 2003) and can cause cell death via both apoptotic and non-apoptotic mechanisms (Blum *et al.*, 2001). DA has also been reported to inhibit mitochondrial complex I (Ben-Shachar *et al.*, 2004). Interestingly extracellular concentrations of DA ranging from 100-500 μM have been reported to induce death of SH-SY5Y cells and to increase α-synuclein expression, a major component of LB (Gomez-Santos *et al.*, 2003).

1.4 THE NEURONAL CYTOSKELETON

The cytoskeleton is a dynamic and complex network of filamentous proteins that extends throughout the cytoplasm. In higher eukaryotes the cytoskeleton is composed of three distinct interacting filamentous systems known as microtubules (MTs), microfilaments (MFs) and intermediate filaments (IFs) formed from tubulin, neurofilament (NF; in the case of neuronal cells) and actin proteins respectively (Alberts *et al.*, 1994; Siegel 1999, Janmey, 1998). MTs and MFs, are composed of

phylogenetically very conserved proteins, whilst IFs are differentially expressed in different tissues (Nixon and Lewis, 1986). The cytoskeletal network is involved in many cellular processes, it provides mechanical strength to the cell, and is also involved in movement, adhesion, polarity and intracellular trafficking. Several proteins also associate with the cytoskeleton helping to organise and accomplish specialised roles of the individual filament systems (Yang *et al.*, 1999).

1.4.1 Microfilaments

MFs are composed of filamentous actin (F-actin) and a complex set of actin binding proteins (ABPs), which regulate the intrinsic polymerisation capacity of actin (Alberts *et al.*, 1994; Sayas *et al.*, 2002). F-actin results from the polymerisation of monomeric subunits of globular actin (G-actin), a process, which requires ATP as well as both monovalent and divalent cations (usually K^+ or Mg^{2+}; Alberts *et al.*, 1994; Carlier *et al.*, 2001). Polymerisation requires ATP binding but not ATP hydrolysis; in fact, ATP hydrolysis on F-actin results in weakening the bonds in the polymer and promotes depolymerisation (Carlier, 1991). MFs are polar structures composed of two structurally different ends, a slow-growing ("minus") end and a fast growing ("plus") end. MFs are also dynamic structures, indeed, actin molecules are continuously added and removed to and from the "plus" and "minus" end, respectively. Thus, no net change in the filament length occurs. This process is known as "Treadmilling" (reviewed by Alberts *et al.*, 1994).

1.4.2 Microtubules

MTs are composed of α- and β-tubulin heterodimers which align end to end to form protofilaments. α- and β-tubulin are ~ 450 amino acids highly homologous proteins. *In vivo*, usually, 13 protofilaments join laterally to form a hollow cylinder with an outer diameter of 25 nm. As with MFs, binding of GTP but not its hydrolysis, is necessary for MT polymerisation (reviewed by Siegel 1999; Carvalho *et al.*, 2003). A variety of MT-associated proteins (MAPs) can bind the microtubule wall (Alberts *et al.*, 1996; Siegel 1999; Downing and Nogales 1998).

Microtubules are constantly undergoing polymerisation/depolymerisation (a process known as dynamic instability; Mitchison and Kirschner, 1984) and are intrinsically polar structures. This means that the two ends of the MT exhibit different properties.

Indeed, this polarity is important to the cellular functions of MTs, which include cell movement, vesicle transport and chromosome segregation during mitosis (Downing and Nogales 1998; Dammerman *et al.,* 2003). In neuronal cells, MTs, in combination with MAPs are involved in neurite outgrowth and axon stabilisation (Nixon, 1998), and in intracellular transport (Shea and Flanagan, 2001). Also motor proteins can bind MTs, these proteins belong to two families: kinesins and dyneins. Each type of MT-depedent–motor protein carries a distinct cargo with it as it moves (Alberts *et al.,* 1996). MT polarity is also central to the ability of motor proteins to move unidirectionally on the polymer lattice and accomplish their diverse functions (Dammerman *et al.,* 2003).

MTs, but not free tubulin molecules can be covalently modified (e.g. acetylation or detyrosination). MTs modification gives an idea of the time that has elapsed since a particular MT polymerised, thus post-translational modifications mark MTs as mature. Although, the function of these modifications remains unresolved, it is thought that they provide sites for the binding of specific MAPs that further stabilise mature MTs (Laurent and Fleury, 1993; reviewed by Alberts *et al.,* 1996; Liao and Gundersen, 1998).

1.4.3 Neurofilaments

1.4.3.1 Neurofilament structure and assembly

There are six different classes of IFs classified according to amino acid sequence similarity and the intron structure of their genomic sequence (Xiao *et al.,* 2006). Neurofilaments (NFs), which are specific for neurons, belong to the fourth group of IFs and are composed of three subunit proteins: NF-L, NF-M and NF-H (Hirokawa and Takeda, 1998; Nixon R.A. 1998; Yabe *et al.,* 2001). The NF-L molecular weight (MW) corresponds to 61 kDa; however due to postranslational modifications (ie. phosphorylation and glycosylation) NF-L is detected at 68 kDa, when separated by SDS electrophoresis. Similarly, although the MWs of NF-M and NF-H are 102.5 and 111 KDa, respectively, phosphorylation of the carboxyl terminal tail domains, make NF-M and NF-H run at 150 and 190-210 kDa in polyacrylamide SDS gels (reviewed by Petzold, 2005).

Neurofilaments are composed of a short amino-terminal head domain rich in arginine and serine, a coiled-coil rod domain of approximately 310 amino acids, and a carboxyl-

terminus of varying length (see Figure 1.8). NFs in nerve axons are composed of a parallel array of 10-nm filaments with frequent crossbridges between NFs or between NFs and MTs or membranous organelles (Xu *et al.*, 1996; Hirokawa and Takeda, 1998; Nixon, 1998; Yabe *et al.*, 2001). The formation of the 10 nm IF results from the correct assembly of the NF-L, NF-M and NF-H subunits. The coiled-coil rod domain is mainly relevant for NF assembly, whilst variable head and tail domains are mainly responsible for protein-protein interactions, with the head domain also contributing to assembly (reviewed by Petzold, 2005). NF-H and NF-M have long hypervariable COOH-terminal tails containing from 40 to 60 KSP (lysine-serine-proline) repeats that are targets of phosphorylation (Hirokawa and Takeda, 1998; Yabe *et al.*, 2001).

The central rod domain is a very conserved region that consists of an extended α-helical region containing long tandem repeats of seven distinctive amino acids (called the heptad repeat) which promotes the formation of coiled-coiled dimers between two parallel α-helices (Alberts *et al.*, 1994; Xu *et al.*, 1996; Xiao *et al.*, 2006). Two dimers can then associate in an antiparallel manner to form a tetrameric subunit. Soluble tetramers are found in cells suggesting that they are the fundamental subunit from which IFs assemble. The antiparallel arrangement of dimers implies that the tetramer, and hence the IF that it forms is a non-polar symmetrical structure. This distinguishes IFs from actin filaments and MTs. The final step of NF assembly is less well-known but it appears that tetramers add to an elongating NF in a simple binding reaction in which they align along the axis of the filament and pack together in a helical pattern (Alberts *et al.*, 1994; Goldman *et al.*, 1999). Cross-bridging between NFs and other axoplasmic components is required for their association in parallel arrays (Nakagawa *et al.*, 1995; Leterrier *et al.*, 1996). This is accomplished by the carboxyl-terminal domains of both NF-M and NF-H which form side-arms which project from the core of the filament (Brownlees *et al.*, 2000). These filaments assemble *in vivo* as obligate heteropolymers of NF-L, which is indispensable for assembly, with sub-stoichiometric amounts of NF-M and/ or NF-H (Xu *et al.*, 1996; Goldman *et al.*, 1999). The molar ratios of these subunits are approximately 5:2:1 for NF-L, NF-M and NF-H, respectively (Xiao *et al.*, 2006).

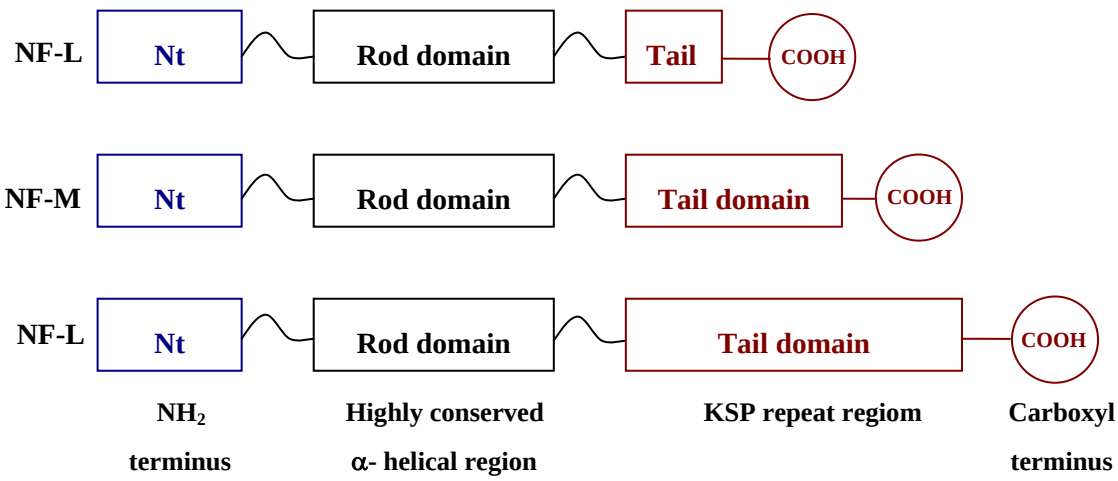


Figure 1.8 Structure of NF proteins. *NF-L, NF-M and NF-H share a tripartite structure composed of an amino terminal head domain, a highly conserved coiled-coil rod domain and a carboxyl terminus tail of varying length. NFs undergo post-translational modifications. Indeed, the carboxyl- tail domain of NF-M and mainly NF-H contain several KSP repeats that are target to phosphorylation (taken from Schmidt et al. 1991 with modifications).*

1.4.3.2 Post-translational modifications of NFs

It was first suggested by Gard and Lazarides (1982) that neurofilament function might be regulated by phosphorylation. The steady-state phosphate content of each NF subunit is regulated by a dynamic balance between the processes of phosphorylation and selective dephosphorylation. This balance is regulated differently in various sites within the neuron (Hirokawa and Takeda, 1998; Nixon, 1998; Yabe et al. 2001).

Indeed, NF-M and NF-H are some of the most highly phosphorylated proteins in the nervous system (Xiao *et al.*, 2006). As introduced earlier, the extensively phosphorylated carboxyl-tail domains project outward from the filament core in a way that phosphates are exposed on the surface of the NFs, thereby, suggesting that phosphorylation may regulate not only NF functions and assembly but the different interactions between the different cytoskeletal components during neurogenesis and axon radial growth (Nixon and Sihag, 1991; Hirokawa and Takeda, 1998; Grant *et al.*, 2001; Yabe et al. 2001).

NFs are phosphorylated at the Ser and Thr residues in both the amino-terminal domain and the KSP repeats in the carboxyl-tail domain. Phosphorylation and dephosphorylation are regulated by several protein kinases and phosphatases which are tightly regulated and interconnected (Petzold, 2005). N-terminal head phosphorylation is performed by secondary messenger-dependent kinases (PKA and PKC) whilst secondary–messenger independent kinases (proline-directed kinases eg. cyclin-dependent kinase-5, glycogen synthase kinase-α3; extracellular signal regulated kinases ERK 1 and ERK 2, stress activated protein kinases p38α, c-jun N-terminal kinase 1 (JNK 1) and 3 (JNK3)) are responsible for phosphorylation of the carboxyl-tails (reviewed by Xiao *et al.,* 2006). Phosphorylation of the carboxyl-tail domain of NFs is associated with axonal development and maturation (Shea *et al.,* 2003). Studies seem to agree in the fact that phosphorylation of NFs can occur in the cell perikaryon but also as they travel along the axon, consequently promoting incorporation into the cytoskeleton (Shea *et al.,* 1990, 1998). Although the serine residues of the KSP repeats within the carboxyl-terminal domain are heavily phosphorylated in axons, they are largely non-phosphorylated in perikarya and more proximal regions of axons (Sternberger and Sternberger 1983). Of importance is the fact that NF subunits express different levels of phosphorylation, even in the same axon but also between different axonal bundles and nerve tracks.

NF-L and NF-M head domain and NF-H KSP repeats can also suffer glycosylation (linked to serine and threonine residues). The role of this post-translational modification is not fully understood, however it might be implicated in NF trafficking and function (reviewed by Petzold, 2005).

1.4.3.3 The role of NFs in axons

During maturation axons elongate to establish a physical contact with their target followed by radial growth (up to 10-fold in diameter; Xu *et al.,* 1996). Axons are enriched with NFs, which extend along their length and form the primary cytoskeletal component, especially in mature nerve cells. The major function of NFs is to provide mechanical stability to neurons (reviewed by Alberts *et al.,* 1994; Nixon, 1998). In fact, NFs in conjunction with MTs, MAPs, actin and associated motor proteins constitute the dynamic axonal cytoskeleton (Grant *et al.,* 2001). The relative proportion of NF subunits varies during neuronal development. During embryonic neurogenesis NF-L

and NF-M are co-expressed whilst NF-H expression occurs later in development (Nixon and Sihag, 1991; Shea and Beermann, 1994; Julien 1999).

NFs determine axonal calibre, which in turn is responsible for determining the conduction velocity at which nerve impulses are propagated along the axon (Xu *et al.,* 1996; Hirokawa and Takeda, 2003). This was proven with two animal models in which a lack of NFs resulted in severe inhibition of radial growth (Ohara *et al.,* 1993; Eyer and Peterson, 1994). Indeed, phosphorylation of NF-H and NF-M side arms (Xu *et al.,* 1996; Siegel, *et al.;* 1999) and cross bridges between NFs are thought to be crucial in determining and maintaining axonal calibre (Nakagawa *et al.,* 1995). However, it is now proposed that NF-L and NF-M stoichiometrics are more important for axonal growth than NFs phosphorylation. Indeed, NF-M is the "preferred" subunit for NF-L copolymerisation and main regulator of axonal calibre (Elder *et al.,* 1998; Rao *et al.,* 1998).

Of importance is the fact that NFs may also bind actin filaments, thus associating MFs, NFs and MTs with the cell membrane (Leterrier *et al.,* 1996). Indeed, Shea and Beermann (1994) suggested that NFs role in stabilising the axonal cytoskeleton results from interactions between NFs and MTs, which are mediated by NF-H and MAPs.

1.4.3.4 Transport of NF proteins

NFs, as all the cytoskeleton components, are synthesised within the neuronal perikaryon and then delivered to the axon by a process known as axonal transport, which can be divided into "fast axonal transport" and "slow axonal transport". The first one includes the transport of membranous organelles and the second, the transport of the cytoskeletal proteins (such as, NFs, MTs and associated proteins; Yabe *et al.,* 1999). In fact, the MT system in association with its complementary motor proteins is required to transport the cellular components (Terada, 2003). Roy *et al.,* (2000) reported that NFs spend a maximum of 20 % of the time moving and the rest of the time are paused. Thus, these findings suggest that slow and fast transport may involve a unique system in which proteins simply spend different lengths of time associated with their motors (Shea and Flanagan, 2001).

For a long time, the form in which NFs were transported along the axon (ie. monomers or polymers) remained unclear; however, it is now accepted that NFs are transported in

different formats, including insoluble hetero-oligomers, short filaments as well as subunits (Lasek *et al.,* 1993; Roy *et al.,* 2000; Terada, 2003; Yuan *et al.,* 2006). Transport is most rapid during neurogenesis and slows into the adults as axons undergo radial growth and myelination; this is when NF-H phosphorylation is maximal (Grant *et al.,* 2001).

It is been suggested that phosphorylation/dephosphorylation of the carboxyl-terminal domains of NF-H and NF-M determines axonal transport rate (Prahlad *et al.,* 2000; Roy *et al.,* 2000; Shea *et al.,* 2003) and also regulates the interactions between NFs and the anterograde and retrograde axonal motor proteins kinesin and dynein (Shea and Flanagan, 2001; Shea *et al.,* 2003; Jung *et al.,* 2005). However, two studies by the same research group challenges the "classical" hypothesis that carboxyl-terminal tail phosphorylation of NFs regulates axonal transport, since they show that axonal transport rate along the optic nerve of mice lacking the hyperphosphorylated tail domain NF-H was unaltered (Rao *et al.,* 2002; Yuan *et al.,* 2006).

It is of interest the fact that normal segregation of highly phosphorylated NFs in axons has been found to be disrupted in some neurons in pathological states associated with perikaryal accumulation of neurofilaments (Hirokawa and Takeda, 1998; Nixon R.A. 1998; Yabe et al. 2001). This is further discussed in section 5.1.1 and 5.1.2.

1.5 AIMS OF PROJECT

Mitochondrial impairment, glutathione depletion and oxidative stress have been implicated in the pathogenesis of Parkinson's disease, linked recently to proteasomal dysfunction (reviewed by Betarbet *et al.,* 2005). The initial aim of this study was to investigate how these factors influence the various activities of the proteasome in human SH-SY5Y neuroblastoma cells treated with the PD mimetics MPP^+ or DA. The project also investigated the effects of glutathione depletion on proteasome activity of human neuroblastoma cells either following or not toxin treatment. It is worth noting that most of previous work in that area was performed in rodent cells. Moreover, little information is available on PD mimetics on the three proteasomal activities.

Another aim was to determine whether treatment with neurotoxins caused a direct effect on proteasome activity of commercial, purified 20S proteasome or in the cell extracts. The effect of antioxidants on proteasomal activity of both toxin-treated cells and

purified 20S, was also studied. Moreover, modifications or losses of several proteasomal subunits have been reported with ageing (Bulteau *et al.*, 2001) and in PD (McNaught *et al.*, 2003), thus possible changes on the individual 20S proteasomal subunits following treatment with MPP^+ and DA were also investigated.

Finally, given that NFs, ubiquitinated proteins and proteasomal subunits are found in proteinaceous inclusions, such as LBs and, the UPS activity is impaired in SNpc of PD brains (McNaught and Jenner, 2001), this project also aimed to investigate whether NFs can undergo proteasomal degradation. Additionally, the post-translational modifications and distribution of the NF network after toxin treatment of SH-SY5Y cells were also studied.

CHAPTER II

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell culture

2.1.1.1 Reagents

Dulbecco´s Modified Eagle's medium (DMEM; 12-614F), foetal bovine serum (14-801-F), penicillin/ streptomycin (17-603E), L-glutamine (17-603E), trypsin/EDTA solution (02-007E), were all purchased from Cambrex, Berkshire, UK.

DMEM/HAM´s F12 medium (D6421), MEM non-essential amino acids solution (M7145), Trypan blue solution 0.4 % (v/v; T8154), from Sigma-Aldrich Chemical Company, Poole, UK

2.1.1.2 Plastic ware

All sterile plastic were supplied by Sarstedt, Leicester, UK.

- Cryotube vials (Nunc brand products), Merk Ltd., Leicester, UK.

- Microtitre plates, 96-well flat-bottomed non sterile, black (015-210190W), Fisher Scientific UK.

- Nunc Lab-Tech CC chamber slides (permanox, 177445), Scientific Laboratory Supplies, Ltd., Nottingham, UK.

2.1.2 Specialised laboratory reagents

- Acrylogel 3 solution Electran (containing 2.5% NN´-methylenebisacrylamide, final ratio 29:1:0.9; 443735T), VWR International Ltd., Poole, UK.

- Adenosine 5'-triphosphate (ATP; A3377), Sigma-Aldrich Chemical Company, Poole, UK.

- Ascorbic acid (vitamin C; A5960), Sigma-Aldrich Chemical Company, Poole, UK.

- Bio-Rad protein assay dye reagent concentrate (500-0006), Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.

- 5-Bromo-4-chloro-3-indolyl-phosphate (di-sodium salt; BCIP; MB1018), Melford Laboratories Ltd. Ipswich, UK.

- $_{L}$-buthionine-[S,R]-sulfoximine (BSO; B2515), Sigma-Aldrich Chemical Company, Poole, UK.

- Cycloheximide (C-7698), Sigma-Aldrich Chemical Company, Poole, UK.

- Dimethlsulfoxide (DMSO; D/4120/PB08), Fisher Scientific UK Ltd., Loughborough, UK.

- 5,5'- Dithio-bis(2-nitrobenzoic acid), (DTNB; D-8130), Sigma-Aldrich Chemical Company, Poole, UK.

- 2D gel starter kit- (163-2105), Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.

- ECL Western Blotting detection Reagents (RPN2109), GE Healthcare Bio-Sciences, Bucks, UK.

- Folin-Ciocalteu´s phenol Reagent (J/4100/08), Fisher Scientific UK, Leicester, UK.

- GBX developer/replenisher (P7042), Sigma-Aldrich Chemical Company, Poole, UK.

- GBX fixer/replenisher (P7167), Sigma-Aldrich Chemical Company, Poole, UK.

- Glutathione (G-4251), Sigma-Aldrich Chemical Company, Poole, UK.

- Glutathione reductase (EC 1.6.4.2; G3664), Sigma-Aldrich Chemical Company, Poole, UK.

- Igepal CA-630 (I3021), Sigma-Aldrich Chemical Company, Poole, UK.

- MPP^{+} Iodide (D048), Sigma-Aldrich Chemical Company, Poole, UK.

- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; M2128), Sigma-Aldrich Chemical Company, Poole, UK.

- N-acetyl cysteine (A7250), Sigma-Aldrich Chemical Company, Poole, UK.

- β- nicotinamide adenine dinucleotide phosphate, reduced form (β- NADPH; N1630), Sigma-Aldrich Chemical Company, Poole, UK.

- Nitro Blue Tetrazolium (NBT; MB1019), Melford Laboratories Ltd., Ipswich, UK.

- Nitrocellulose 0.22 μM pore size (WP2HY00010), Genetic Research Instrumentation, Essex, UK.

- Precision Plus protein dual colour standards (161-0374), Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.

- Pre-stained SDS molecular weight standard mixture (SDS-7B), Sigma-Aldrich Chemical Company, Poole, UK.

- Ready IPG strip, pH 3-10, non-linear, 7cm (163-2002), Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.

- Silver staining kit, Protein (17-1150-01), GE Healthcare Bio-Sciences, Bucks, UK.

- ± α- Tocopherol (vitamin E; T3251), Sigma-Aldrich Chemical Company, Poole, UK.

- XAR-5 Kodak Film (F5388), Sigma-Aldrich Chemical Company, Poole, UK.

- Vialight bioassay kit (HS Plus; RT07-221) , Cambrex, Berkshire, UK.

Enzymes

- 20S proteasome (mammalian; EC 3.4.25.1; PW8729), Biomol International, Exeter, UK.

- Trypsin (EC 3.4.21.4) Type II-S, porcine pancreas, Sigma-Aldrich Chemical Company, Poole, UK.

- Calpain-2 (EC 3.4.22.53), rat, recombinant, high purity, *E. coli* (208718), Calbiochem, Nottingham, UK. .

Fuorogenic substrates

- Boc-Leu-Arg- Arg-AMC·HCl (I1585), Bachem, Merseyside, UK.

- Suc-Leu-Leu-Val-Tyr-AMC, (I1395), Bachem, Merseyside, UK.

- Z-Leu-Leu-Glu-AMC (539141), CN Biosciences, Nottingham, UK.

Protease Inhibitors

- MDL 28,170 (208722), Calbiochem, Nottingham, UK.

- Lactacystin Proteasome inhibitor (sc-3575), Autogen Bioclear UK Ltd., Calne, UK.

- Protease Inhibitor cocktail (P8340), Sigma-Aldrich Chemical Company, Poole, UK.

2.1.3 Antibodies

2.1.3.1 Primary Antibodies

- Anti-ERK (K23) antibody (sc-94), Santa Cruz Biotech, Santa Cruz, California, USA.

- Neurofilament 200KDa monoclonal antibody (clone N52) (N0142), Sigma-Aldrich Chemical Company, Poole, UK.

- SMI 31 anti-phospho-neurofilaments, Sternberger Monoclonals Inc, Maryland, USA.

- Proteasomal subunit β4, Rabbit polyclonal antibody (PW8890), Biomol International, Exeter, UK.

- Proteasomal subunit β5, Rabbit polyclonal antibody (PW8895), Biomol International, Exeter, UK.

- Proteasomal subunit β6, Rabbit polyclonal antibody (PW900), Biomol International, Exeter, UK.

- Proteasomal subunit β2, mouse monoclonal antibody (clone MPC168; PW8145), Biomol International, Exeter, UK.

- Proteasomal "core subunits" (α5/α7/β1, β5,β5i,β7), Rabbit polyclonal antibody (PW8155), Biomol International, Exeter, UK.

- Proteasome subunits α 1, 2, 3, 5, 6 and 7 mouse monoclonal antibody (clone MPC231; PW8195), Biomol International, Exeter, UK.

2.1.3.2 Secondary Antibodies

- Goat anti-mouse immunoglobulins alkaline phosphatase conjugated (D0486).

- Goat anti-mouse immunoglobulins horseradish peroxidase conjugated (P0447).

- Goat anti-rabbit immunoglobulins alkaline phosphatase conjugated (D0487).

- Goat anti-rabbit immunoglobulins horseradish peroxidase conjugated (P0448).

- Rabbit anti-mouse immunoglobulins FITC conjugated (F0261).

- Rabbit anti-mouse immunoglobulins alkaline phosphatase conjugated (D0487).

All purchased from DAKO Ltd., Cambridgeshire, UK.

2.1.4 Specialised equipment

- Bio-Rad mode 680 microplate reader, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.

- Bio-Rad protean IEF Cell System, Bio-Rad Laboratories Ltd, Hempstead, UK.

- Bio-Rad power Pac 300, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.

- Bio-Rad Trans-Blot electrophoretic transfer system, Bio-Rad Laboratories Ltd, Hempstead, UK.

- Cell scraper (C2808), Sigma-Aldrich Chemical Company, Poole, UK.

- Leica CLMS confocal laser microscope, Leica, Germany.

- Fluostar optima plate reader, BMG Labtech Ltd., Bucks, UK.

- Fujifilm FLA-5100 gel scanner, Fujifilm Life Sciences Products, Sheffield, UK.

- Fujifilm intelligent dark box, Fujifilm Life Sciences Products, Sheffield, UK.

- MIKRO 22R microfuge, Hettich, Germany.

- Mini- PROTEAN III system, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.

- Nikon Eclipse TS 100 inverted microscope, Nikon, Japan.

- Neubauer double cell clear sight haemocytometer (AC1000), Weber Scientific International (Division of Hawksley Technology), West Sussex, UK.

- Sanyo CO_2 incubator MCO-17AIC, Sanyo Gallenkamp PCL, Leicestershire, UK.

- Sanyo Harrier 18/80 refrigerated centrifuge, Sanyo Gallenkamp PCL, Leicestershire, UK.

- Soniprep 150, MSE scientific instruments, UK.

- Tecan SPECTRA Fluor plate reader, Tecan, UK Ltd., Reading, UK.

- Walker class II microbiological safety cabinet, Walker safety cabinets Ltd., Derbyshire, UK.

2.1.4.1 General laboratory reagents

All general laboratory reagents were of the highest grade and purchased from Sigma-Aldrich Chemical Company, Poole, UK, unless otherwise specified in the text.

2.2 METHODS

2.2.1 Cell culture

A clone of human SH-SY5Y neuroblastoma cell line was obtained from European Collection of Animal and Cell Cultures (ECACC).

2.2.1.1 Maintenance of SH-SY5Y cells

Cell culture was carried out in a class II safety cabinet using aseptic technique. Cells were cultured in 25 cm^2 (T25), 75 cm^2 (T75) and 175 cm^2 (T175) flasks in Dulbecco´s Modified Eagles Medium (DMEM)/HAMS F12 (1:1) containing: 10% (v/v) heat inactivated foetal bovine serum (heat inactivated at 60 ºC for 30 min), 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 % (v/v) non essential aminoacids ("growth medium").

Cells were maintained as a monolayer and incubated at 37 ^oC in a humidified atmosphere of 95 % (v/v) air / 5 % (v/v) carbon dioxide until 70-90 % confluent.

2.2.1.2 Sub-culture

When cells required subculturing, growth medium was removed using a Pasteur pipette and the cell monolayer was washed twice with pre-warmed DMEM (37 ^oC). Cells were detached from the growing surface using trypsin (100 μg/ml) / Ethylenediamine (EDTA; 40 μg/ml) in DMEM at 37 ^oC (trypsinisation). Trypsin activity was quenched by the addition of growth medium (ten times the volume of trypsin solution). The suspension was transferred to a sterile centrifuge tube and centrifuged at 150 x g for 5 min. The supernatant was removed and the pellet re-suspended in 1ml of fresh growth medium. Following cell count, a volume of cell suspension (dependent on the number of cells required) was transferred to an appropriate sterile flask containing fresh growth medium. Cells were then incubated as described in section 2.2.1.

2.2.1.3 Viable cell counting and seeding

To seed cells at a given density a volume (typically 20 µl) of cell suspension was diluted 1:10 in Trypan blue solution (0.4% (v/v)). A viable cell count was performed in five fields of Neubauer haemocytometer using light microscopy. Cell density was calculated as follows:

Cell/ml = average cell number (from five fields) x 10^4 x dilution factor

This was then used to calculate the volume of cell suspension required to achieve a specific cell density in a known volume of fresh growth medium. Once seeded cells were incubated as described in section 2.2.1.

2.2.1.4 Cryo-preservation of cells

Long-term storage of cells was achieved in the gaseous phase of liquid nitrogen in freezing medium containing, 95 % (v/v) FBS and 5 % (v/v) sterile dimethyl sulphoxide (DMSO). Cells were grown as described in section 2.2.1, harvested by trypsinisation, a viable count performed (see section 2.2.1.3) and cells resuspended in ice-cold freezing medium at a density of 2 x 10^6cells/ml. The suspension was immediately transferred to cryovials on ice (1 ml aliquots) and stored at -80 ^oC overnight before transferring to liquid nitrogen for long term storage.

2.2.1.5 Resuscitation of cryo-preserved cells

Cryovials were removed from liquid nitrogen storage and rapidly thawed in a 37 ^oC water bath. Cell suspensions were immediately transferred to a sterile centrifuge tube containing 10 ml of fresh growth medium. Suspensions was centrifuged 150 x g for 5 min. The supernatant was removed and cells re-suspended in 1 ml fresh growth medium using a Pasteur pipette before transfer to a sterile T25 flask containing 10 ml of growth medium. Cells were incubated as described section 2.2.1 and sub- cultured as described in section 2.2.1.2.

2.2.2 Assessment of cell viability: MTT tetrazolium salt assay for anchorage dependent cells

3-[4-5-Dimethylthiazol-2-y1]-2,5-diphenyl tetrazolium bromide (MTT) is a substrate that is taken up by cells and reduced by mitochondrial and endoplasmic reticulum

dehydrogenase enzymes, to a purple formazan product that accumulates within viable cells (Cookson *et al.,* 1995). In general, cell viability was assessed in 96-well plates. Typically, 10 µl of 5mg/ml MTT in DMEM were added to the culture medium of growing cells and incubated at 37 ^{o}C in a humidified atmosphere of 95 % (v/v) air / 5 % (v/v) carbon dioxide for 1 hour. After this time medium was carefully removed from the cells and the formazan product solubilised in 100µl DMSO. The plate was agitated on an orbital shaker to aid dissolution of the formazan product prior to reading the absorbance at 570 nm. Absorbance results were expressed as mean percentage cell viability compared to controls ± standard error of the mean (SEM).

2.2.3 ATP assay

The ViaLight HS plus kit was used according to the manufacturer´s guidelines. The kit is based upon the bioluminescent measurement of ATP that is present in all metabolically active cells. The method utilises an enzyme, luciferase, which catalyses the formation of light from ATP and luciferin according to the following reaction:

$$\text{ATP} + \text{Luciferin} + \text{O2} \xrightarrow[\text{Mg++}]{\text{LUCIFERASE}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \textbf{LIGHT}$$

Cells were cultured in 96-well plates as described in section 2.2.1. To assay ATP, 50 µl of cell lysis reagent was added to each well (containing 100 µl of medium). After 10 min, 100 µl of extracts or ATP standards (containing 0, 12.5, 50, 250, 500, 5000, 5000, 25000 pmols) were transferred to a white walled microtitre plate where 100 µl of ATP monitoring PLUS reagent were added to each well. The emitted light intensity (directlyproportional to ATP concentration) was measured (365nm) and the ATP content calculated from the ATP calibration graph. The assay was conducted at ambient temperature (18-22 ºC). Results were calculated as pmols ATP/ µg protein and converted to mean % of ATP (in comparison to control samples) ± SEM.

2.2.4 Preparation of cell lysates

2.2.4.1 Total protein extraction from cells for detection of neurofilament proteins in response to protease inhibitors and toxins

Cells ($1.5x10^6$) were seeded in T25 flasks and left 24 h to allow attachment and recovery. Medium was carefully removed from each flask and replaced with 5 ml of fresh growth medium supplemented with or without different treatments depending on the specific experiment. After the required time (normally 24, 48 and 72 h) cells were detached by trypsinisation (see section 2.2.1.2) and the resultant pellet lysed in 250 µl extraction buffer (50 mM Tris, pH 6.8; 150 mM NaCl; 5 mM EDTA; 1 % sodium dodecyl sulphate (SDS); 0.2 % (v/v) protease inhibitor cocktail) and transferred to an eppendorf tube on ice. For experiments where a significant number of cells were floating, these were harvested and included in the extraction; medium and DMEM washes were collected and centrifuged at 150 x g for 5 min. The resultant pellet was added to the adherent cell extract. Total extracts were sonicated (6 x 3 seconds) on ice and heated at 100ºC for 5 min. Aliquots of samples were stored at -20 ºC prior to protein estimation by the Lowry method (see section 2.2.5.1)

2.2.4.2 Protein extraction from cells for measuring 26S/20S proteolytic activity

Cells were seeded at a density of 500,000 cells/well in 6-well plates and incubated for 24 h to allow attachment and recovery. Medium was carefully removed from each well and replaced with 2 ml of fresh growth media supplemented with the treatment. After the required time period, cells were washed twice with 1ml of DMEM and detached using a cell scraper in 250 µl ice-cold homogenisation buffer [20 mM Tris/HCl, pH 7.2; 0.1 mM EDTA; 1 mM 2-mercaptoethanol; 5 mM ATP; 20 % (v/v) glycerol; 0.04 % (v/v) Igepal CA-630]. Cell lysates were transferred to an eppendorf tube on ice. For experiments where a significant number of cells were floating, these were harvested and included in the extraction; medium and DMEM washes were collected and centrifuged at 150 x g for 5 min. The resultant pellet was added to the adherent cell extract. Finally, total extracts were vigorously vortex mixed and kept on ice for immediate analysis of

2.2.5 Estimation of protein in cell extracts

2.2.5.1 Mini-Lowry method

The protein content of samples was estimated using the Lowry method (Lowry *et al.*, 1951, with modifications). A calibration graph was constructed using bovine serum albumin (BSA) to represent 0-80 μg protein. Equal volumes of extraction buffer used in the samples were added to each standard (typically 10-30 μl) and standards made up to a final volume of 100 μl in distilled water.

The working Lowry reagent [1ml; 2 % (w/v) $NaCO_3$, 0.01 % (w/v) $CuSO_4$, 0.027 % (w/v) NaK tartrate in 0.1 M NaOH] was added to each standard and sample, vortex mixed and incubated at room temperature for 15 min. The reaction was developed over 30 min at room temperature by adding 100 μl Folin Ciocalteu´s phenol reagent diluted 1:1 in distilled water. All samples were vortex mixed and absorbance measured at 750 nm.

2.2.5.2 Bio-Rad protein assay

The Bio-Rad protein assay was used in accordance with the manufacturer´s guidelines. A calibration graph ranging from 0-50 μg was prepared from BSA as detailed in section 2.2.5.1) Samples and standards were diluted to 800 μl in distilled water. 200 μl of Bio-Rad dye reagent were added to each standard and sample, vortex mixed and after an incubation period of 5 min, absorbances were read at 595 nm.

2.2.6 Fluorogenic peptide assay for protease activity.

2.2.6.1 20S/26S proteasomes activity

Proteasome specific substrates are typically three to four amino acid residue peptides with a fluorogenic tag at the C terminus. The proteasome cleaves an amido bond between an amino acid and the fluorogenic tag, resulting in the realease of a highly fluorescent product. From the different fluorophores used in these substrates, 7-amino-4methylcoumarin (AMC) has the highest fluorescence and is the most commonly used

fluorogenic reporter group in proteasome substrates. Substrates chosen for the assay were Suc-LLVY-AMC (50 μM), Boc-LRR-AMC (100 μM) and Z-LLE-AMC (37.5 μM) for chymotrypsin-like (CLA), trypsin–like (TLA) and post acidic-like (PLA) proteasomal activities, respectively. (Canu *et al.* 2000; David *et al.* 2002, Kisselev and Goldberg 2005). 100μl of SH-SY5Y cell lysates (~ 20-30 μg protein; see section 2.2.4.2) or 0.05 μg of purified human 20S proteasome were incubated at 37^{o}C with a fluorogenic substrate in assay buffer (50 mM HEPES, 5mM EGTA pH 8.0) in a total volume of 200μl. Readings were taken every 5-10 min for 3h (360nm excitation; 465 nm emission). Results were calculated as ΔFU/s/ μg protein and then converted to % activity in comparison to control samples.

2.2.6.2 Fluorogenic peptide assay for calpain activity.

Calpain activity was measured with the method described by Sasaki *et al.* (1984) with modifications. Rat, recombinant calpain-2 was utilised in this fluorogenic assay. 0.36 μg/μl of calpain-2 were pre-incubated at 37^{o}C in assay buffer (60 mM imidizole pH 7.3, 5 mM L-cysteine, 2.5 μM 2-mercaptoethanol, 5 mM $CaCl_2$ and 4 % (v/v) DMSO) for 15 min before the addition of the fluorogenic substrate Suc-Leu-Tyr-AMC (1 mM) in a 100 μl total assay buffer volume. Fluorescence was measured (360 nm excitation; 465 nm emission) every min for 20 min. Results were calculated as ΔFU/s/μg protein and then converted to % activity in comparison to control samples.

2.2.7 Measurement of total glutathione levels

Total glutathione levels were determined using the DTNB-GSSG reductase-recycling assay, based on the method of Anderson (1985) with minor modifications. The assay works on the basis of the following reactions:

$$2\ GSH + DTNB \rightarrow GSSG + TNB$$

$$\uparrow \qquad\qquad \downarrow$$

$$2\ GSH + NADP+ \leftarrow GSSG + NADPH + H+$$

Cells were plated out in T25 flasks at 80 % cell density, incubated overnight for recovery, then medium was removed and treatments were added. Following treatment,

2.2.8 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

2.2.8.1 Preparation of polyacrylamide resolving gels

The Bio-Rad mini protean III apparatus was assembled according to the manufacturer´s guidelines. In brief two glass plates were held, typically 1.5mm apart, by vertical spacers.

For each gel, 10 ml gel mix were prepared as detailed in Table 2.1 and degassed under vacuum for 10 min. For each 10 ml gel mix, 100µl 10 % (w/v) ammonium persulphate (APS) and 10 µl N, N, N', N'-tetramethyl-ethylenediamine (TEMED) were used as polymerisation agents and added immediately before the gel was poured.

Gels were poured with a 2.5 cm space at the top of the glass plates to allow the addition of a stacking gel and covered with a layer of distilled water. The gel mix was allowed to polymerise at room temperature for 30-45 min.

Table 2.1 details the methods for preparation of polyacrylamide resolving gels. Separation of proteins within a sample can be varied depending on the percentage of

acrylamide within the resolving gel. For greater separation of high molecular weight proteins, a lower percentage acrylamide gel would be used and vice versa.

REAGENT	**7.5% (w/v) polyacrylamide gel**
40% Acrylamide stock	1.9 ml
1.5 M Tris buffer pH 8.8	2.5 ml
10 % SDS	100 µl
Millipore (distilled) water	5.50 ml

Table 2.1. Preparation of 7. 5 % acrylamide resolving gels for SDS-PAGE.

2.2.8.2 Preparation of stacking gel

Typically, 3 ml of 4 % (w/v) acrylamide stacking gel mixture were used per gel and degassed under vacuum over 10 min. The volumes of reagents required to prepare 100 ml of stacking gel mix are shown in Table 2.2. To polymerise 3 ml of the stacking gel stock, 12 µl TEMED and 30 µl of APS were added immediately before pouring the stacking gel on top of resolving gel to the top of the glass plates. Combs were positioned (10 wells) within the gel to form individual wells. The gel was allowed to polymerise for 30 min at room temperature before removal of the combs and transfer of the gel to an electrophoresis running chamber to be submerged in SDS-PAGE buffer (Tris 25 mM pH 8.3, 192 mM glycine, 01 % (w/v) SDS).

REAGENT	Volume (to make 100ml)
40% (w/v) Acrylamide stock	10 ml
0.5 M Tris buffer pH 6.8	25 ml
10 % (w/v) SDS	1ml
Millipore (distilled) water	64 ml

Table 2.2. Preparation of 100 ml stock 4 % polyacrylamide stacking gel.

2.2.8.3 Preparation of samples for SDS-PAGE

Samples (typically 20-50 μg protein) were diluted 1:1 in 2 x concentrated reducing electrophoresis sample buffer (4 % (w/v) SDS; 20 % glycerol; 0.1 M Tris- HCl pH 6.8; 100 mM DTT; 0.01% (w/v) Bromophenol blue). Samples were heated to 100 ^{o}C for 5 min then microfuged at high speed for 10 seconds to recover all the sample prior to loading and separation at a constant current of 200 V. Current was stopped as the dye front approached the bottom of the gel.

2.2.8.4 Acetone precipitation of protein

Acetone precipitation was used when samples required the concentration of protein. The required sample volumes were transferred to an eppendorf tube and vortex mixed with 5 times its volume of ice-cold acetone prior to incubation at -20 ^{o}C for a minimum of 2 h. Protein precipitate was then harvested at 14000 x g for 10 min. The supernatant was carefully removed and discarded and the pellet re-suspended in an appropriate volume of reducing electrophoresis sample buffer for loading.

2.2.9 2D SDS- PAGE

Two dimensional electrophoresis initially separates proteins based on their net charge using an electric field (isoelectric focusing), proteins with the highest pI, that is negatively charged, will migrate to the positive anode and proteins with low pI

(positively charged) will migrate to the negative cathode. Then SDS-PAGE electrophoresis separates these proteins based on their denatured molecular weight, higher molecular weight proteins will experience higher resistance in the gel so will migrate less distance than low molecular weight proteins.

For 2D SDS-PAGE a Bio-Rad readyPrep 2D-starter kit and the Bio-Rad protean IEF Cell System were used following the manufacturer's instructions.

2.2.9.1 IPG Strip Re-hydration

The required number of 7 cm ready IPG strips (pH 3-10) were removed from the -20 ºC freezer. 100 µl of isoelectric (IGF) buffer were pipetted into the required lanes of a disposable re-hydration tray and IPG strips (backing plastic removed) were gently placed, gel side down, onto the IGF buffer avoiding air bubbles to form. Then the tray was covered and left overnight at room temperature to allow strip re-hydration..

2.2.9.2 Sample preparation

2-5 µg of purified 20S proteasome (in 50 mM HEPES, 5mM EGTA pH 8.0) were incubated with 2mM MPP^+ or DA in phosphate buffer saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4 pH 7.4) for 2 h at 37º C prior to solubilisation in IGF buffer to cup loading samples onto the strips .

2.2.9.3 Isoelectric focusing (IEF)

IEF tray was cleaned with ethanol and then water prior to positioning damped electrode wicks over each electrode wire. Each IPG strip was transferred to the corresponding lanes of the focussing tray, gel side down and + end of the strips to the anode end of the tray, avoiding air bubbles underneath the strip. IPG strips were then covered with mineral oil and samples were cup-loaded under the cathode (-) end of each IPG strip. The tray was then placed in the protean IEF cell and focussing was run at 50 µA / strip at 20 ºC and linear ramping of 250 V/ 20 min (step 1) followed by linear ramping of 4000 V/ 2 h (step 2) and linear ramping of 4000V /10000V-h (step 3, total process time 5 h). After electrofocussing mineral oil was drained off, strips placed into a clean disposable tray and stored at -20 ºC until ready to run second dimension electrophoresis.

2.2.9.4 2D SDS-PAGE

2.2.9.4.1 Preparation of resolving gel

The Bio-Rad mini protean III apparatus was assembled according to the manufacturer´s guidelines (see section 2.2.8.1). For analysis of purified 20S proteasome subunits a 15 % gel was prepared as detailed in Table 2.3 and Table 2.4. For each 26 ml gel mix, 260 µl 10% (w/v) ammonium persulphate (APS) and 26 µl TEMED were used as polymerisation agents, added immediately before the gel was poured.

REAGENT	**BUFFER A**
1.5 M Tris Buffer pH 8.8	26.65 ml
0.1% (w/v) SDS	0.2 g
30% (w/v)Glycerol	30 ml
Millipore (distilled) water	140 ml

Table 2.3. Preparation of 200 ml of Buffer A for 2D SDS-PAGE. Buffer was pH to 8.8 with HCl and stored at 4 ºC.

REAGENT	**15 % GEL**
BUFFER A	10 ml
40 % (w/v) Acrylamide	9.8 ml
10 % (w/v) SDS	200 µl
Distilled water	6 ml

Table 2.4. Preparation of 26 ml of resolving gel for 2D SDS-PAGE

2.2.9.4.2 IPG strip equilibration

This step ensures that cysteines are reduced and alkylated to minimise vertical streaking in the second dimension.

To reduce cysteine residues, 1 ml of equilibrium buffer I [6 M urea, 2 % (w/v) SDS, 0.375 M Tris-HCl pH 8.8, 2 % (w/v) DTT and 20 % (v/v) glycerol] was added to each lane containing an IPG strip (gel side down) and samples placed on an orbital shaker for 10 min. This procedure was repeated 3 times using fresh equilibration buffer I. Strips were then washed in 1 ml of equilibration buffer II [6M urea, 2 % (w/v) SDS, 0.375 M Tris-HCl pH 8.8 and 20 % (v/v) glycerol] to alkylate free residues. This procedure was repeated 3 times using fresh equilibration buffer II.

Once resolving gels and strips were equilibrated, strips were removed from the disposable equilibration tray and dipped briefly into SDS-PAGE buffer [Tris 25 mM pH 8.3, 192 mM glycine, 0.1 % (w/v) SDS] and laid, gel side up, onto the back plate of the resolving SDS-PAGE gel. Strips were overlaid with melted agarose and the gels submerged in SDS-PAGE running buffer. 20S subunits were separated at a constant current of 200 V. Separation was stopped as the dye front approached the bottom of the gel.

2.2.9.5 Silver staining of the 2D SDS-PAGE (mass spectroscopy compatible)

For this purpose the GE healthcare silver staining kit was used following the manufacturer´s instructions. All glassware used was previously washed with detergent, rinsed with distilled water and immersed in 5 % (v/v) nitric acid for a minimum of one hour. In brief, 2D gels were first fixed in 40 % (v/v) ethanol and 10 % (w/v) glacial acetic acid for 30 min, and then washed three times in millipore distilled water for 5 min. Gels were sensitised in 30 %(v/v) ethanol, 0.2 % (w/v) sodium thiosulphate and 0.83 M sodium acetate for 30 min and then washed with millipore distilled water once for 15 min prior to a second wash left overnight. The following day, silver reaction was conducted with 0.25 % (w/v) silver nitrate; no formaldehyde was added at this step to allow mass spectroscopy compatibility. Gels were rinsed twice in millipore distilled water for 1 min before developing the gels in 0.236 M sodium carbonate and 0.0148 % (w/v) formaldehyde for the required time. Development of the gels was stopped with

43.2 mM EDTA-Na_2·2 H_2O for 10 min. Gels were stored at 4^oC in millipore distilled water. Pictures of the gels were taken on a Fujifilm FLA 5100 gel scanner.

2.2.10 Western blotting and immunoprobing of proteins

2.2.10.1 Western blotting

Proteins separated by SDS-PAGE were transferred electrophoretically onto a nitrocellulose membrane by Western blotting using the Wet blotting (Bio-Rad Trans-Blot electrophoretic) system method. Four pieces of filter paper and one piece of nitrocellulose were cut to the same size as the gel and pre-saturated with electroblotting buffer [48 mM Trizma base, 39 mM glycine, 20 % (v/v) methanol, 0.0375 % (w/v) SDS]. The gel was laid on the top of the sheet of nitrocellulose. This was sandwiched on each side with two pieces of filter paper and two fibre pads. Care was taken during this procedure to ensure no air pockets. The nitrocellulose sandwich was held in a plastic case submerged in electroblotting buffer in a tank. For overnight blotting 30 V were applied over 16 h at room temperature. The process could also be carried out in 1 h 30 min at 100 V according to the manufacturer's instructions.

2.2.10.2 Immunoprobing

2.2.10.2.1 Preparation of nitrocellulose for immunoprobing

Protein transferred to nitrocellulose during the blotting process was stained with copper pthalocyanine 3,4',4'',4''' tetrasulphonic acid tetrasodium salt (0.05 % (w/v) in 12 mM HCl) to ensure adequate transfer and to allow for imaging (Fujifilm intelligent dark box). The nitrocellulose was then cut as required for immunoprobing and de-stained in 12 mM NaOH and washed in distilled water.

2.2.10.2.2 Blocking of non-specific antibody binding and immunoprobing with primary and secondary antibodies

Non–specific antibody binding was prevented by blocking of nitrocellulose for 1 h in 3 % (w/v) marvel milk / Tris saline buffer (50 mM Trizma base, 200 mM NaCl, pH 7.4; TBS) with gentle shaking. Nitrocellulose sections were incubated with primary antibody diluted in blocking agent overnight at 4 ^oC with gentle shaking. Information regarding the epitope specificity and required dilutions of primary monoclonal antibodies used are

detailed in Table 2.5. Unbound primary antibody was removed by washing with TBS / 0.1 % (v/v) Tween 20 for 6 x 10 min washes with vigorous shaking. Nitrocellulose sections were incubated with an alkaline phosphatase or horseradish peroxidase (HRP) conjugated secondary antibody diluted 1:1000 in 3 % Marvel/TBS to allow development via colorimetric or enhanced chemiluminescence methods respectively (see sections 2.2.10.3 and 2.2.10.4). Secondary antibodies were incubated for 2 h at room temperature with gentle shaking. Unbound antibody was again washed with TBS / 0.1 % (v/v) Tween 20 for 6 x 10 min.

Antibody	Epitope specificity	Working dilution (Western Blotting)	Working dilution (Immunocytochemistry)
N52 (mAb)	Anti-NF-H /NF-M (phosphorylation independent)	1:500 - 1:1000	1:200
SMI 31 (mAb)	Anti-NF-H /NF-M (phosphorylation dependent)	1:1000	1:500
Total ERK (pAb)	Anti- total ERK 1/2	1:500 - 1:1000	N/A
PW8890 (pAb)	Proteasomal β4	1:1000	1:1000
PW8895 (pAb)	Proteasomal β5	1:1000	1:1000
PW8155 (pAb)	Proteasomal "core subunits" (α5/α7/β1, β5,β5i,β7)	1:1000	1:1000
PW900 (pAb)	Proteasomal β6	1:1000	1:1000
PW8145 (mAb)	Proteasomal β2	1:1000	1:1000
PW8195 (mAb)	Proteasomal α subunits (α1, 2, 3, 5, 6 and 7)	1:1000	1:1000

Table 2.5. Epitope specificity and working dilutions required for primary antibodies for Western blotting and immunocytochemistry techniques. *pAb= polyclonal antibody; mAb= monoclonal antibody.*

2.2.10.3 Alkaline phosphatase development system

Blots were washed for 5 min in distilled water then equilibrated for a further 5 min in substrate buffer (0.75 M Tris pH 9.5). Antibody reactivity was developed in the dark by addition of alkaline phosphatase substrate solution [20 ml substrate buffer; 44 µl NTB (75 mg/ ml in 70% (v/v) DMF); 33 µl BCIP (50 mg/ ml)] prepared immediately prior to use. The reaction was allowed to proceed until bands appeared. To stop the reaction the substrate was poured off and the nitrocellulose rinsed with distilled water. Nitrocellulose was dried between sheets of filter paper for further analyses and storage.

2.2.10.4 Enhanced Chemiluminescence (ECL) development system

ECL was performed using a kit from Amersham Pharmacia Biotech UK Ltd., according to manufacturer's instructions. In brief, equal volumes of solution A and solution B were mixed to a final volume of 1ml / 9 x 6 cm^2 nitrocellulose then immediately incubated with the nitrocellulose for 1 min.

ECL substrate was drained and the nitrocellulose was placed protein-side down onto SaranWrap and sealed. Care was taken to avoid trapping air pockets. The nitrocellulose was placed protein-side up into a film cassette and held in place with masking tape prior to overlaying with photographic film. Film was exposed to nitrocellulose in the dark for the required time, dependent on the primary antibody used and then removed from the cassette and placed immediately into developing solution (diluted 1:5 in water) for 1 min. Exposed film was then rinsed in water and transferred to fixing solution (diluted 1:5 in water) for a further minute. The film was washed again and then allowed to dry at room temperature.

When ECL was performed using a Fujifilm Intelligent dark box system, the process was similar except for the fact that no film was required. Instead, after one minute incubation of the nitrocellulose with the kit solution, the nitrocellulose was placed directly into the dark box and chemiluminescence revealed digitally following the manufacturer's instructions.

2.2.10.5 Stripping and re-probing membranes

In most cases, primary and secondary antibodies could be completely removed from membranes and re-probed several times. Nitrocellulose membranes were submerged in stripping buffer [SDS 100 mM, 2-mercaptoethanol, 2 % (w/v), 6.25 mM Tris-HCl pH 6.7] and incubated at 50 ^{o}C for 30 min with occasional agitation. The membrane was washed 3 x 10 min in TBS/0.1 % (v /v) Tween-20, then blocked and re-probed as detailed in section 2.2.10.2.

2.2.10.6 Quantification of Western blots

To allow for quantitative comparison of protein band intensity following Western blotting and immunoprobing, a process of band quantification was performed using the Aida Image Analyser v.4.03, according to the manufacturer's guidelines. In brief pixel intensity of equal sized areas around bands were obtained and quantified based on the number of pixels in each area multiplied by the grey shade value of each pixel.

2.2.11 Detection of reactive oxygen species by confocal microscopy

Cells were seeded in Nunc Lab-Tech CC chamber slides at 10,000 cells per well and allow to attach and grow. On addition of treatments cells were left incubating at 37 ^{o}C for the required time. Medium was then carefully removed and 100 μM 2,7-dichlorodihydrofluorescein diacetate (DCDHF) in DMEM loaded onto cells for 50 min. The dye was removed and cells washed with Hanks buffered salt solution (HBSS; 140 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 20 mM HEPES pH 7.4, 5 mM $NaHCO_3$, 5.5 mM glucose) prior to immunocytochemical analyses.

2.2.12 Immunocytochemical analysis of proteins

SH-SY5Y cells were seeded in 300 μl growth media into an eight well permanox chamber slide at a density of 10,000 cells/well. After overnight recovery, treatments were added and cells incubated for the required time.

With care, medium was removed, cells washed three times in DMEM to remove serum and fixed in 200 μl ice-cold 90 % methanol / TBS at -20 ºC for 10 min. Methanol was removed and cells were washed three times with TBS. Cells were further permeabilised

in 200 µl Triton X- 100 / TBS for 10 min at room temperature and washed in TBS as before prior to immunoprobing them.

Non-specific antibody binding was prevented by blocking with 3 % (w/v) bovine serum albumin in TBS (BSA/TBS) for 1h at room temperature with gentle shaking. Cells were incubated with primary antibody diluted in BSA/TBS overnight at 4 ^{o}C. Cells were washed with TBS for 3 x 5 min then incubated with Fluorescein Isothiocyanate (FITC) conjugated secondary antibody diluted 1:50 for 2h at room temperature in the dark. Excess secondary antibody was removed by 3 x 5 min washed in TBS in the dark. Slides were carefully air-dried. Vectasheld preservative solution ± propidinium iodide was applied to the slide before a cover slip was placed over the cells and sealed into place. The slide was then stored at -20 ^{o}C in the dark to prevent bleaching of fluorescence signal prior to viewing by confocal laser microscopy.

2.2.13 Statistical analysis

Data were presented as ± the standard error of the mean (SEM) at a 95 % confidence limit. Statistical analysis was performed using a two-tailed, homoscedastic, Student's t-test. Statistical significance was accepted at $p < 0.05$ (*) or 0.01 (**).

CHAPTER III

EFFECT OF PD MIMETICS ON PROTEASOMAL ACTIVITY IN A SH-SY5Y HUMAN NEUROBLASTOMA CELL MODEL

3. EFFECT OF PD MIMETICS ON PROTEASOMAL ACTIVITY IN SH-SY5Y CELLS

3.1 INTRODUCTION

3.1.1 UPS involvement in PD pathogenesis

As further detailed in the general introduction, mammalian cells appear to possess two major pathways for general protein degradation, lysosomal proteases and the proteasome complex. The latter is a component of the UPS, which plays an essential role in the degradation and clearance of short-lived, mutant, misfolded or damaged proteins in eukaryotes and ultimately in the regulation of crucial processes such as the cell cycle, transcription, antigen processing and signal transduction (Goldberg *et al* 1995; Ding and Keller, 2001).

The presence of missfolded and aggregated proteins and ubiquitin-positive inclusions within the specific brain region affected in many neurodegenerative diseases, including PD, suggest that the UPS might be crucial in their pathogenesis (reviewed by McNaught and Olanow, 2006). So far, of all neurodegenerative disorders, PD is most directly associated with UPS dysfunction (discussed by Ardley *et al,* 2005). As introduced in section 1.1.2.5 this is supported by the finding that proteasomal activity is decreased in SNpc from PD patients event which may contribute to neurodegeneration of dopaminergic neurons and LB formation in sporadic PD (Mc Naught *et al.,* 2001, 2002; Mc Naught and Jenner 2001), and the discovery of some rare mutations in genes coding for components and substrates of the UPS which give rise to familial forms of PD (Huang *et al.,* 2004; Betarbet *et al.,* 2005; further detailed in section 1.1.2.4).

Accumulation of aberrant proteins can occur when the cell proteolytic systems of the cell (e.g. UPS) are impaired or when the amount of these proteins exceeds the cell's capacity (reviewed by Betarbet *et al.,* 2005). Indeed, oxidatively damaged proteins (mostly ubiquitin-conjugated) have been reported to accumulate with age and age related diseases like PD. Moreover, proteasome activity has been shown to be decreased with age (Carrad *et al.,* 2002). On the other hand, aggregated proteins have been found to bind to the 20S proteasome leading to its irreversible inhibition (Bence *et al.,* 2001; Davies, 2001). Interestingly, certain proteins are more prone to aggregation; this is the

case for α-synuclein (reviewed by Maries *et al.,* 2003). In fact, oxidative damage can enhance α-synuclein's ability to misfold and aggregate (Giasson *et al*., 2000). Although controvertial, α-synuclein is thought to be a substrate for the proteasome. Indeed, proteasome inhibition can lead to accumulation of α-synuclein in several *in vitro* models (reviewed by Betarbet *et al.,* 2005). Miwa *et al.* (2005) showed that proteasome inhibition in the nerve terminals of nigrostriatal DA neurons in the SNpc led to neuronal degeneration and the formation intracytoplasmic inclusions rich in α-synuclein. Interestingly, synphilin-1 one of α-synuclein's binding partners has been reported to undergo proteasomal degradation and cells over-expressing synphilin-1 are more susceptible to proteasomal dysfunction (Lee *et al.,* 2002). Moreover, futher supporting a key role of the UPS in PD pathogenesis, proteasome inhibition has been shown to cause selective loss of dopaminergic neurons in both an *in vivo and in vitro* rat model (Fornai *et al.,* 2003).

Besides UPS impairment, mitochondrial complex I inhibition is also repeatedly implicated in the pathogenesis of the disease (reviewed by Abou-Sleiman *et al.,* 2006). Indeed, there is increasing evidence that these two events interact with each other in the complex multifactorial cascade of deleterious processes underlying PD pathology (Duke *et al.,* 2006).

3.1.2 Proteasomal impairment in PD cellular models

Several studies link mitochondrial impairment with the UPS in PD since toxins which can inhibit mitochondrial complex I activity can lead to proteasome impairment. For instance, exposure of rat primary mesencephalic cultures to rotenone and MPP^+ for 6 h, has been reported to reduce proteasome activity via ATP depletion and not via ROS production (Höglinger *et al.,* 2003). Shamoto-Nagai *et al.* (2003) reported that rotenone impaired proteasomal activity, in SH-SY5Y human cells after 72 h, however through oxidative modification of the proteasome itself. Very recently, a number of related reports have been published. Rotenone-infused rats were shown to exhibit selective loss of the nigrastriatal pathway with proteasomal activities reduced in ventral midbrain region of rats with lesions, whilst proteasomal activities were increased in rats without lesions (Betarbet *et al.* 2006). The same authors also report rotenone treated SK-N-MC neuroblastoma cells exhibit increased proteasomal activity prior to a reduction in activities over a 4 week period. Using the same cell line, Wang *et al.,* (2006) studied the

effect of 6 pesticides, including rotenone, ziram or dieldrin on the 26S proteasome activity using a 26S proteasome reporter system. They showed that some of these pesticides inhibited proteasomal activity at low concentrations with rotenone also inducing oxidative stress.

3.1.3 SH-SY5Y human neuroblastoma cell line

Animal models are useful tools for revealing links between symptoms and pathological defects that result following exposure to neurotoxins such as MPTP/ MPP^+ or rotenone. However, their application is limited for a number of reasons, including ethical concerns, species differences and complex cellular interactions occurring at organ and tissue level. On the other hand, *in vitro* cell models allow the investigation of sub-cellular biochemical processes with a tighter control of the environmental conditions of a specific cell type. Employment of a neuroblastoma human cell line may allow development of a model with analogy to dopaminergic neurons *in vivo*. Thus a study of the effects of neurotoxins on cultures of human neuroblastoma cells may allow a more detailed analysis of changes in the UPS.

The SH-SY5Y cell line is the third cloned sub-line from a parent neuroblastoma cell line SK-N-SH. SK-N-SH was established in 1970 from the mestastatic bone tumour of a young female (Biedler *et al.,* 1973). SH-SY5Y human neuroblastoma cells are a useful *in vitro* model for the study of MPTP neurotoxicity since this cell line contains the necessary components for the synthesis, metabolism and transport of DA. Furthermore, SH-SY5Y cells predominantly express MAO-A and only low levels of MAO-B (Fitzgerald *et al.,* unpublished) and contain the DA uptake system (Song and Ehrich, 1998; Storch et al. 2000). The uptake of DA and MPP^+ by this cell line is a dose and time dependent process (Song and Ehrich, 1997; Song and Ehrich, 1998).

3.1.4 Aims of chapter

At the time this study started, little information was available about the mechanisms by which decreased proteasomal function occurred in SNpc of PD patients, or whether toxins linked to mitochondrial dysfunction such as MPTP and its active metabolite MPP^+ could impair the UPS.

The aim of this chapter is to determine whether treatment of SH-SY5Y human neuroblastoma cells with the neurotoxins MPP^+ and DA leads to an alteration in

extracted proteasome activity. Although it is fully recognised that DA is primarily a neurotransmitter, since treatment with DA leads to cell damage, for convenience I will refer to both MPP^+ and DA as (neuro)toxins. The toxins chosen for this study are of relevance for PD since MPP^+ inhibits mitochondrial complex I and is the active metabolite of MPTP, a neurotoxin commonly used in PD models; DA relevance resides in the fact that DA-containing neurons are selectively lost in PD and DA metabolism generates a vast amount of oxidative stress which may make neurons within SNpc more prone to damage. All three proteasomal activities (CLA, the focus of most previous work, TLA and PLA) were studied.

Moreover, the concomitant effects of the neurotoxins on ATP, glutathione levels, and cell viability are also monitored because these parameters are known to influence the UPS and are relevant for PD. In order to mimic the situation in vivo (see introduction section 1.1.2), glutathione levels were also reduced using $_L$- buthionine-[S,R]-sulfoximine (BSO), an specific inhibitor of γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH biosynthesis (Stokes *et al.*, 2000). Thus, analysis of the possible implications of oxidative stress in the mechanisms underlying toxin-induced proteasomal impairment in our cell system were performed by (a) the use of a precursor for the synthesis of the antioxidant glutathione, N-acetyl-cysteine (NAC; also a mild antioxidant on its own right), and (b) further stressing the toxin-treated cells by depleting glutathione levels with BSO.

Finally, since PD is a chronic disorder, the effects of 3 and 7 weeks treatments with low levels of toxins on proteasomal activity were also monitored.

3.2 RESULTS

3.2.1 Characterisation of a proteasome activity assay for SH-SY5Y cells

3.2.1.1 Effect of protein content

The fluorogenic peptide assay for proteasomal activity (described in the methods section 2.2.6.1) had not been used before for SH-SY5Y cell extracts in my laboratory. Therefore, a preliminary study was performed to determine the amount of protein (and thus cell number) to give a representative activity rates.

SH-SY5Y cells were plated out in 6-well plates at different densities (i.e 250,000, 300,000, 400,000 and 500,000 cells per well), left incubating overnight and extracted into 250 μl of homogenisation buffer as described in section 2.2.4.2. A fluorogenic peptide assay for CLA was performed as described in section 2.2.6.1.

With 300,000 - 500,000 cells/well activity rates/μg protein were similar, irrespective of whether 25 μl or 100 μl sample were used (Table 3.1); using 250,000 cells/ well, rates were lower. Based on these results a cell density of 400,000-500,000 cells/ well and 100 μl of assay volume (corresponding to 24-33 μg protein/well) were chosen as suitable for use in the assay.

	25µl			100µl		
CELL DENSITY	**ΔFU/s**	**ΔFU/s/ µg**	**SEM**	**ΔFU/s**	**ΔFU/s/ µg**	**SEM**
250,000	0.0272	0.0063	0.0098	0.1282	0.0116	0.0039
300,000	0.0818	0.0193	0.0275	0.2694	0.0238	0.0173
400,000	0.1358	0.0227	0.0089	0.5669	0.0236	0.0304
500,000	0.1814	0.0217	0.0112	0.7156	0.0213	0.0212

Table 3.1. ***Effect of cell density and sample volume on fluorogenic peptide assay for chymotrypsin-like activity (CLA).*** *Cells were plated out in 6 well plates at different densities (ie. 250,000, 300,000, 400,000 and 500,000) and CLA of cell extracts monitored as detailed on section 2.2.6.1. Two different volumes of the samples, 25 and 100 µl were tested in order to determine the optimal parameters of the assay. Results are presented as ΔFU/s and ΔFU/s/µg ± SEM. Data are from a representative experiment with assays replicated three times.*

3.2.1.2 Relative proteasomal activities in SH-SY5Y cells

The fluorogenic substrates used for measuring the three proteasomal activities chosen in our laboratory were Suc-LLVY-AMC, Boc-LLR-AMC and ZLLE-AMC for CLA, TLA and PLA respectively.

Table 3.2 illustrates apparent fluorescence rate values for CLA, TLA and PLA; results indicate that TLA is greatest, followed by CLA and finally PLA in our cell line.

	ΔFU/s/µg	**SEM**
CLA	0.0289	0.0068
TLA	0.2446	0.0204
PLA	0.0165	0.0012

Table 3.2. ***Relative apparent proteasomal activities from SH-SY5Y cells.*** *Cells were grown in 6-well plates and after overnight recovery and extracted in 250 µl homogenisation buffer (section 2.2.4.2). Then cell extracts were incubated with Suc-LLVY-AMC, Boc-LLR-AMC and ZLLE-AMC to monitor CLA, TLA and PLA respectively in a fluorogenic peptide assay (section 2.2.6.1). Results are presented as ΔFU/s ± SEM (n=3).*

3.2.1.3 Specificity of the different fluorogenic substrates for proteasomal activity

To determine the specificity of the three substrates towards proteasomal activity, an irreversible proteasome inhibitor (lactacystin) and a calpain inhibitor (MDL 28,170) were used in the fluorogenic peptide assay as described before.

Final concentrations of 1 μM and 10 μM lactacystin and 0.1 μM and 1 μM of MDL 28,170 were added to control cell extracts and proteasomal activities were measured. Both inhibitors were also tested on purified 20S proteasome and calpain enzyme; calpain activity was measured as detailed in section 2.2.6.2.

Table 3.3 shows the inhibitory effects of lactacystin and MDL 28,170. Lactacystin inhibited CLA of purified 20S proteasome in a dose dependent manner; with 0.1, 1 and 10 μM lactacystin, inhibiting the enzyme by 40 %, 78 % and 95 % respectively. With 10 μM lactacystin, TLA of 20S samples was partally inhibited (70% inhibition cf. controls) but PLA was unaffected. In cell extracts, lactacystin inhibited CLA in a dose dependent manner; however, none of the other proteasomal activities were inhibited by lactacystin. Finally, 1 and 10 μM lactacystin also inhibited calpain, altought to a lesser extent than MDL 28,170 (25 % and 40 %, respectively). The assay was performed with 0.36 μg/μl of calpain, concentration which was found to give consistent activity rates in the fluorogenic assay.

On the other hand, calpain was inhibited by 51 % with 0.1 μM MDL 28,170, whilst higher doses of the inhibitor (1 and 10 μM) virtually abolished the activity. MDL 28,170 also inhibited CLA and TLA from purified 20S proteasome by 25 % and 58 % respectively when used at 10 μM, but had no effect on PLA. In cell extracts, MDL 28,170 did not inhibit CLA or PLA; however TLA was inhibited by 67 % with 1 μM MDL 28,170.

			% Inhibition		
SAMPLE	**Inhibitor**	**Concentration (μM)**	**CLA**	**TLA**	**PLA**
20S	**Lactacystin**	**0.1**	40 *	0	0
		1.0	78 **	0	0
		10.0	95**	72 *	0
	MDL 28,170	**0.1**	0	0	0
		1.0	0	0	0
		10.0	25 **	58**	0
Extracts	**Lactacystin**	**1.0**	70 **	0	0
		10.0	94 **	0	0
	MDL 28,170	**1.0**	0	0	0
		10.0	0	67 **	0
Calpain		**Concentration (μM)**	**Calpain**		
	Lactacystin	**0.1**	0		
		1.0	25 **		
		10.0	39 **		
	MDL 28,170	**0.1**	51 **		
		1.0	99 **		
		10.0	98 **		

Table 3.3. ***Inhibitory effects of lactacystin and MDL 28,170 on 20S proteasome, SH-SY5Y cell extracts and calpain enzyme***. *Cell extracts (20-25 μg), 0.05 μg of 20S protease or 0.036 μg of calpain were utilised in a fluorogenic petide assay as described in sections 2.2.6.1 and 2.2.6.2. Lactacystin or MDL 28,170 (0.1, 1 and 10 mM) were directly added to the assay to assess specificity of the substrates and inhibitors. Results were calculated as ΔFU/s/μg and converted to % activity cf. controls. Statistical analysis was carried out using a two-tailed t-test. Data are from a representative experiment with assays replicated three times. The experiment was repeated 3 times. Statistical significance was accepted when *= p<0.05 or ** = p< 0.01 in comparison to untreated control.*

Although lactacystin is a widely used and fairly specific proteasome inhibitor, it did not inhibit PLA activity in cell extracts or purified 20S whilst TLA was only inhibited at the highest concentration used (ie. 10 μM). Thus, a different proteasome inhibitor, epoxomicin, was utilised to investigate the specificity of the TLA substrate in particular for measuring (TLA) proteasomal activity. The specificity of epoxomicin was also determined by using commercial calpain.

Figure 3.1 shows that 1, 10 and 20 μM epoxomicin all inhibited TLA in purified 20S proteasomes by 65 % (panel A; similar to lactacystin) and TLA in cell extracts by 25 % (panel B). Since the effect of epoxomicin was not dose dependent, a 10 μM dose was used in subsequent experiments with cell extracts.

Finally, the effect of 10 μM epoxomicin on CLA and PLA (in addition to TLA) from SH-SY5Y cell extracts was investigated in order to correlate TLA data shown in figure 3.1 with the other two proteasomal activities. Figure 3.2 shows CLA was particularly sensitive to epoxomicin. As before, TLA was only partly inhibited by epoxomicin (~ 30 %) but PLA was inhibited by 74 % in comparison to controls.

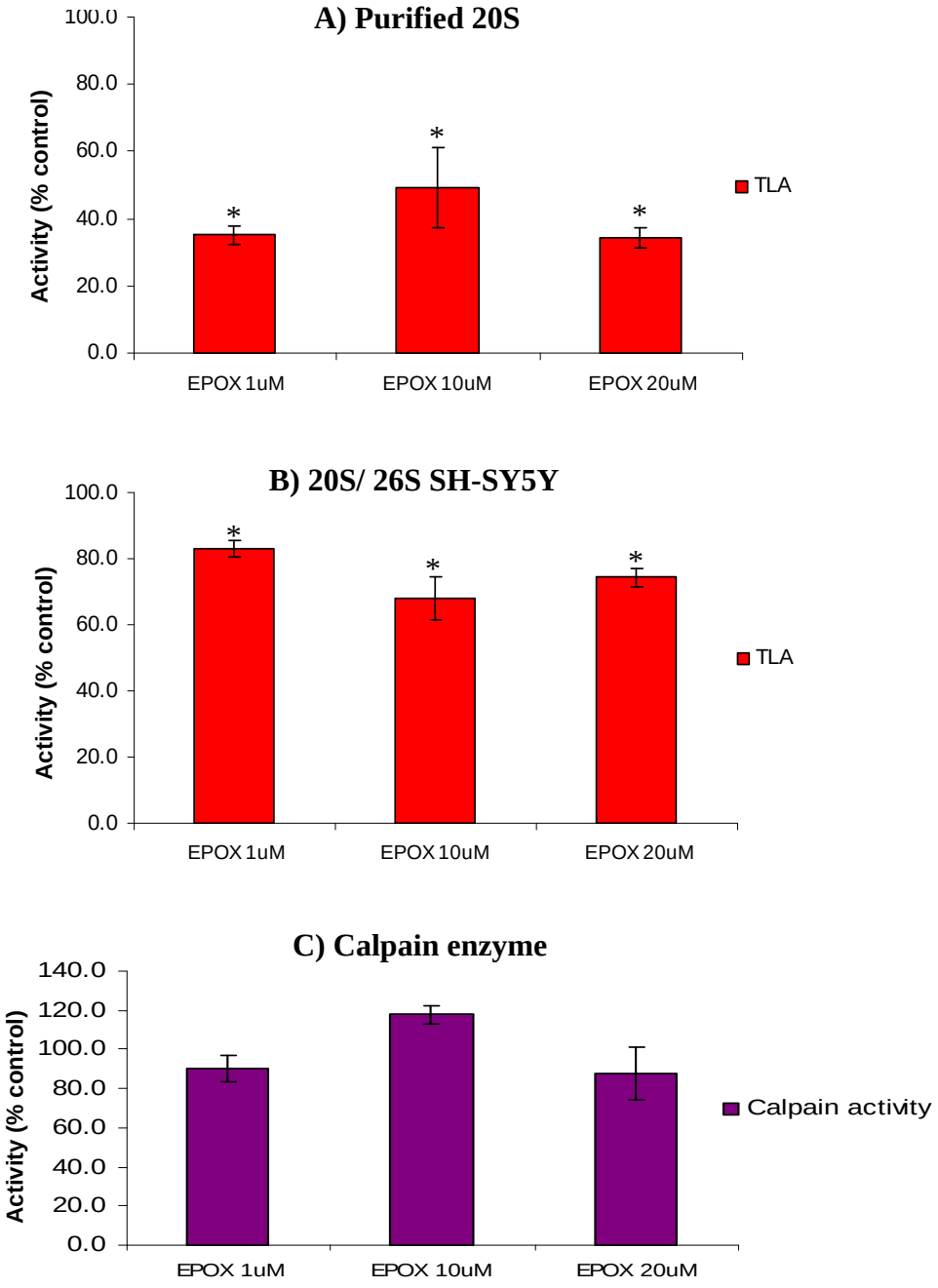


Figure 3.1. Inhibitory effect of epoxomicin on TLA from SH-SY5Y cells, 20S proteasome or calpain activity. *Cells extracts (20-25 μg), purified 20S proteasome (0.05 μg) or calpain-2 (0.036 μg) were utilised in a fluorogenic peptide assay as detailed in sections 2.2.6.1 and 2.2.6.2 respectively. 1, 10 and 20 μM epoxomicin were directly added to control cell extracts, purified 20S proteasome or calpain-2 to assess the specificity of the substrates utilised in the assay towards TLA. Specificity of epoxomicin towards proteasome was also assessed by testing the effect of this inhibitor on calpain enzyme. Results were calculated as ΔFU/s/μg ± SEM and then converted in mean % in comparison to control samples. The data are from a representative experiment with assays replicated three times. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when *= p<0.05 cf. control.*

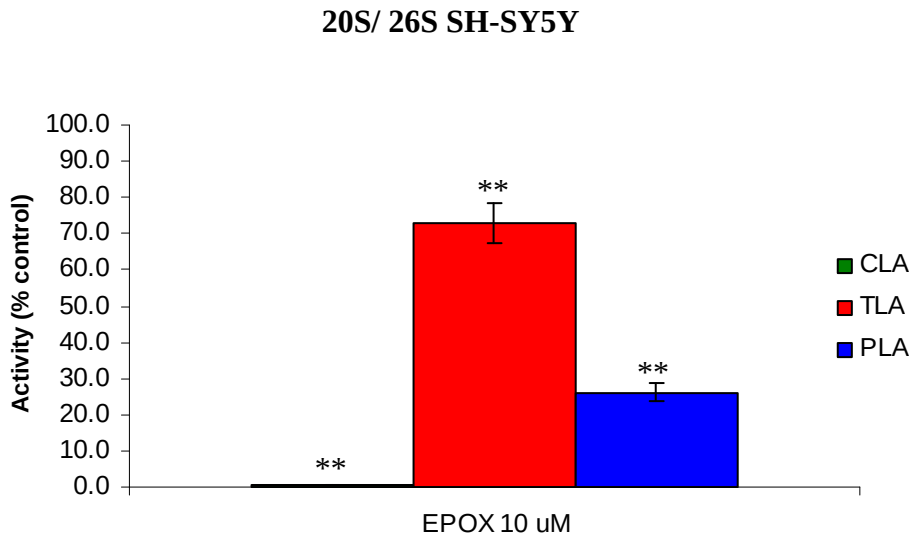


Figure 3.2. ***Inhibitory effect of 10 μM epoxomicin on CLA, TLA and PLA from SH-SY5Y cells.*** *Cells extracts were utilised in a fluorogenic peptide assay as detailed in section 2.2.6.1. Epoxomicin (10 μM) was directly added to control cell extracts to assess the specificity of the substrates utilised in the assay and epoxomicin towards proteasome activity. Results were calculated as ΔFU/s/μg ± SEM and then converted in mean % in comparison to control samples. The data are from a representative experiment with assays replicated three times. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when * = p<0.05 or ** = p<0.01 of control.*

3.2.2 Effect of MPP^+ and DA on proteasomal activity

3.2.2.1 Assessment of cell viability after exposure to toxins

Before studying the effect of toxins on proteasomal activity, concentrations of the toxins that were highly and mildly cytotoxic to mitotic SH-SY5Y cells over a 72-h period were established using a MTT reduction assay (see section 2.2.2). This assay is dependent on cell integrity and viability and is, more specifically, a measure of metabolic activity. Figure 3.3 shows that MPP^+ and DA reduced cell viability in a dose dependent manner. MPP^+ (100 μM) did not reduce cell viability until 48 h (29 % reduction ca. control samples) and, similarly, cell viability was reduced by 30 % at 72 h. Increasing MPP^+ to 2 mM reduced cell viability by 51 %, 53 % and 63 % after 24, 48 and 72 h, respectively.

DA at 100 μM did not affect cell viability until 72 h, whilst 500 μM concentration of the latter toxin led to a 64 %, 70 % and 73 % decrease in cell viability after 24, 48 and 72 h respectively (ca. control samples), similar to 2 mM MPP^+. 2 mM DA reduced cell viability by 35 %, 30 % and 15 % in comparison to controls after 24, 48 and 72 h respectively. Therefore, high doses of DA appears more damaging to the cells than MPP^+.

Finally, BSO toxicity was also assessed. Although, 2mM BSO lead to a 90% reduction in glutathione levels as early as 24 h (see Figure 3.3), cell viability was not affected over 72 h.

In subsequent experiments, 100 μM MPP^+ and DA were selected for use in SH-SY5Y cells as a mildly toxic dose (~20-30 % reduction in cell viability after 72 h) and 2 mM MPP^+ and 500 μM DA as a highly toxic dose (~60- 70 % cell viability reduction ca. controls).

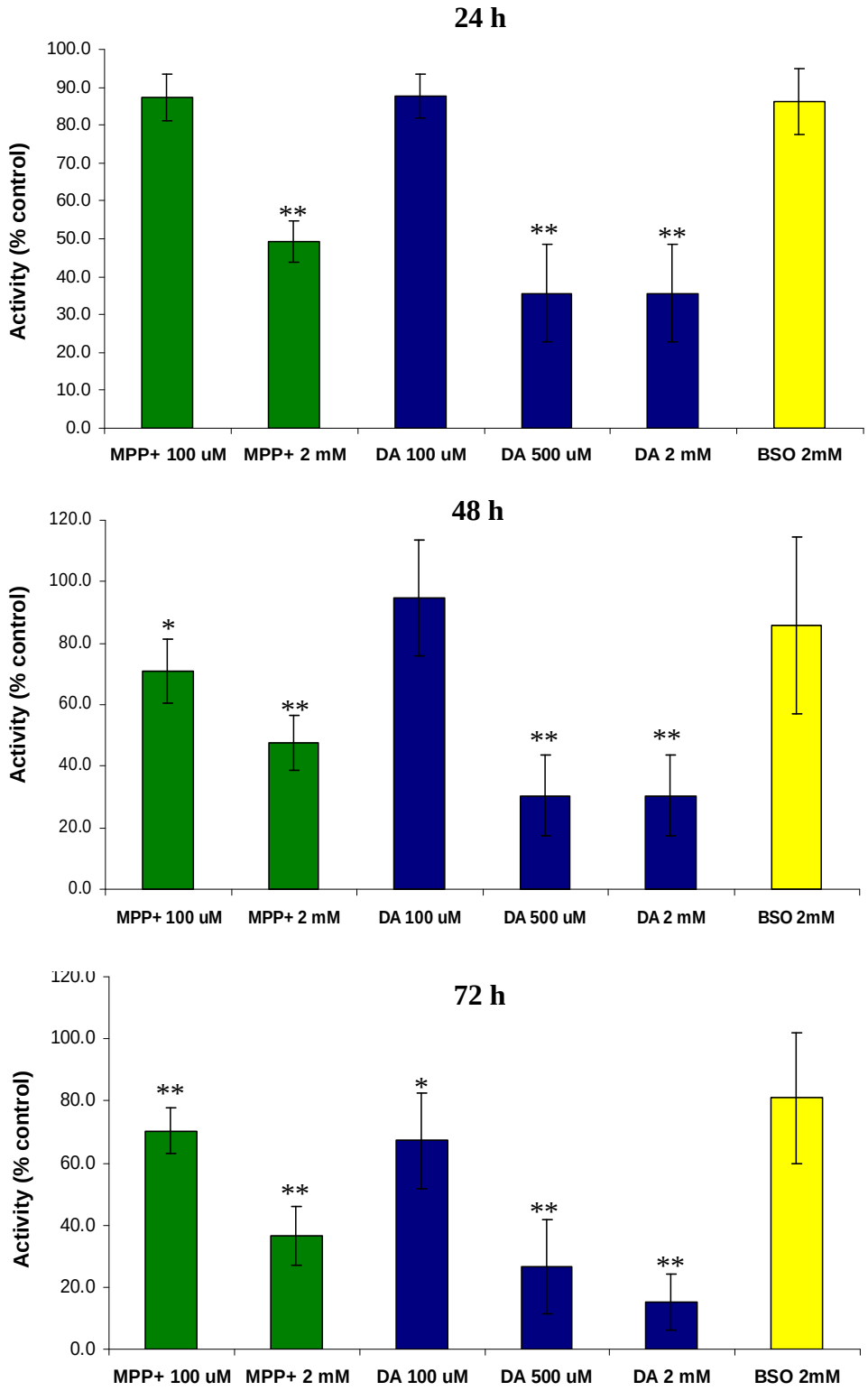


Figure 3.3. ***Assessment of cell viability in SH-SY5Y cells after MPP$^+$, DA and BSO exposure over time.*** *Cells were seeded in 96 well plates at a density of 10000 cells/ well. After 24h medium was exchanged for fresh growth medium supplemented with/ without 100 μM and 2 mM MPP$^+$, DA 100 μM and 500 μM and BSO 2 mM and incubated for 24, 48 and 72 h prior to MTT reduction assay (section 2.2.2). Data are from three independent experiments. Statistical significance was assessed vs. control mitotic cells using a two-tailed t-test where *= p< 0.05 and **= p< 0.01.*

3.2.2.2 Effect of MPP^+ and DA on proteasomal activities

The effects of a 72 h exposure to mildly and highly toxic doses of MPP^+ and DA on the three proteasomal activities were initially studied. In order to assess the true contribution of proteasomal activity to each value, the effects of 10 μM lactacystin and 10 μM epoxomicin were also monitored (concentrations chosen in section 3.2.1.3).

Table 3.6 shows that 100 μM of MPP^+ or DA did not affect CLA or PLA after 72h. Higher doses of MPP^+ (2 mM) reduced CLA and PLA to 20.2 % and 10.2 % of controls, respectively. On the other hand 500 μM DA also reduced CLA and PLA, but to a lesser extent, than MPP^+ (by 44.4 % and 51 %, respectively). Interestingly, neither toxins reduced TLA at any concentration tested; indeed, 100 μM MPP^+significantly increased this activity by 62.7 % in comparison to control. However, this increase in activity or the lack of effect of the toxins on TLA could be due to the unspecific nature of the TLA substrate towards proteasomal activity (see section 3.2.1.3).

This was confirmed by using the proteasome inhibitors lactacystin and epoxomicin on control and toxin-treated cells. Results show that whilst lactacystin did not inhibit TLA in treated cells, epoxomicin similarly reduced TLA in control and toxin-treated cells (by 25-30 % of controls); thus proteasomal TLA in the cell extracts accounts only 25-30 % of the total activity rates obtained. On the other hand, for CLA and PLA the assay is truly measuring proteasomal activity since epoxomicin virtually abolish CLA (also lactacystin) and inhibited PLA by around 90 % in most cases (except with 2 mM MPP^+ where inhibition was 68 %). Thus, toxins are reducing CLA and PLA.

Activity	Treatment	ΔFU/s/μg	SEM	% Activity ca. control	% Activity with lactacystin 10μM	% Activity with epoxomicin 10μM
CLA	Control	0.0289	0.0041	100.0	3.3 *	1.1 *
	MPP^+ 100 μM	0.0238	0.0024	82.5	6.0 *	0.0 **
	MPP^+ 2 mM	0.0058	0.0034	20.2 *	0.0 *	0.0 **
	DA 100 μM	0.0302	0.0072	104.5	27.3 **	0.0 *
	DA 500 μM	0.0160	0.0060	55.6 *	8.2 **	0.0 **
TLA	Control	0.245	0.0077	100.0	78.9	74.6 *
	MPP^+ 100 μM	0.398	0.0116	162.7 **	90.0	68.7 *
	MPP^+ 2 mM	0.212	0.0155	86.9	70.5	72.6 *
	DA 100 μM	0.274	0.0118	112.2	100.0	77.1 *
	DA 500 μM	0.283	0.0168	115.8	93.1	67.8 **
PLA	Control	0.0165	0.0008	100.0	76.2	12.8 *
	MPP^+ 100 μM	0.0162	0.0009	98.6	83.0	10.7 *
	MPP^+ 2 mM	0.0017	0.0018	10.2 **	100.0	36.6 *
	DA 100 μM	0.0165	0.0017	100.3	80.0	8.9 *
	DA 500 μM	0.0079	0.0009	48.2 **	100.0	0.0 **

Table 3.4. ***Effect of MPP^+ and DA on proteasomal activities from SH-SY5Y cells after 72h exposure.*** *Cells were grown in 6-well plates and after overnight recovery; cells were treated with MPP^+ and DA. After 72h incubation cells were extracted in 250 μl homogenisation buffer and cell extracts were incubated with Suc-LLVY-AMC, Boc-LLR-AMC and ZLLE-AMC to monitor CLA, TLA and PLA respectively in a fluorogenic peptide assay (section 2.2.6.1). 10 μM lactacystin or epoxomicin (final concentrations) were directly added to the extracts in the assay in order to assess substrate specificity for proteasomal activity. Results are presented as ΔFU/s ± SEM and then converted in mean % in comparison to control samples. The data are from a representative experiment with assays replicated three times. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when *= $p<0.05$ or ** = $p< 0.01$ cf. control.*

Since only 25-30 % of the total activity measured by the TLA substrate in SH-SY5Y cell lysates is proteasomal TLA in all samples tested, the effects of the toxins on proteasomal TLA were calculated by correcting each activity rate using the % inhibition of activity obtained with epoxomicin (Table 3.4).

Table 3.5 shows that neither toxin reduced TLA activity; indeed a significant activation of the latter activity occured with 100 μM MPP^+ and 500 μM DA.

Treatment	**"Uncorrected TLA" (AFU/s/μg ±SEM)**	**% Inhibition with epoxomicin**	**"Corrected TLA" (AFU/s/μg ± SEM)**	**% control**
Control	0.245 ± 0.008	25.4 *	0.062 ± 0.002	100
MPP^+ 100 μM	0.398 ± 0.012	31.3 *	0.120 ± 0.003	194 **
MPP^+ 2 mM	0.212 ± 0.015	27.4 *	0.058 ± 0.004	93.5
DA 100 μM	0.274 ± 0.012	22.9 *	0.061 ± 0.003	99.3
DA 500 μM	0.283 ± 0.017	32.2 **	0.088 ± 0.005	142.5 *

Table 3.5. Effect of MPP^+ and DA cells on proteasomal TLA from SH-SY5Y cells after 72 h exposure. *Cells were grown in 6-well plates and after overnight recovery; cells were treated with MPP^+ and DA. After 72h incubation cells were extracted in 250 μl homogenisation buffer and cell extracts were incubated with Boc-LLR-AMC to monitor TLA in a fluorogenic peptide assay (section 2.2.6.1). Epoxomicin (10 μM final concentration) was directly added to the extracts in the assay in order to assess substrate specificity for proteasomal activity. Total activity rates were corrected against the % inhibition of TLA with epoxomicin and then converted into mean % in comparison to control samples. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when *= $p<0.05$ or ** = $p< 0.01$ cf. control (n=3).*

3.2.3 Direct effect of MPP^+ and DA on SH-SY5Y cell extracts

To determine whether toxins can directly impair proteasome function, control cell extracts were incubated with a range of MPP^+ and DA concentrations (0.1-20 mM) during the assay.

Figure 3.5 shows that toxins at 100 μM did not reduce CLA in cell extracts; however, 2 mM MPP^+ and DA significantly reduced CLA to 87.0 % of controls. Whilst 20 mM DA had a similar effect to 2 mM DA, 20 mM MPP^+ reduced CLA by a much greater extent (to 16 % of controls) than 2 mM MPP^+.

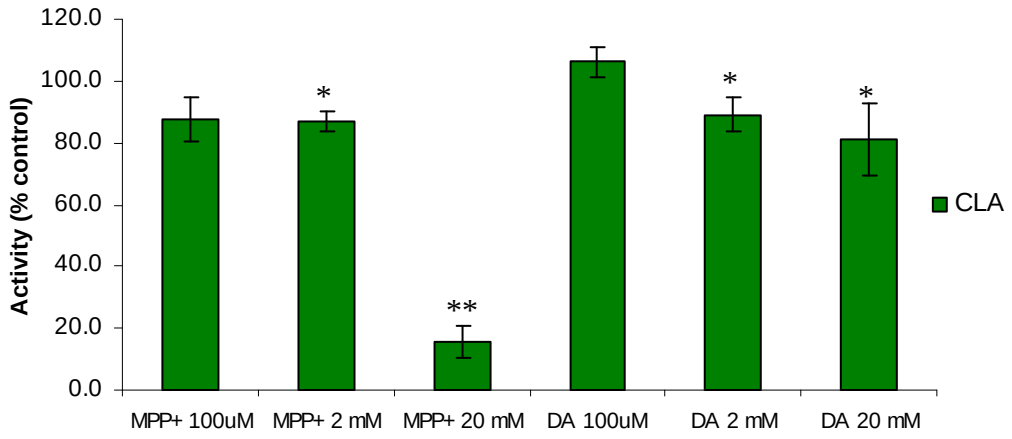


Figure 3.4. ***Direct effect of MPP^+ and DA on 20 S proteasomal activity of SH-SY5Y cell extracts.*** *Cells were grown in 6-well plates and after overnight recovery, extracted in 250 μl homogenisation buffer. Cell extracts were incubated with Suc-LLVY-AMC to monitor CLA in a fluorogenic peptide assay (section 2.2.6.1). 100 μM, 2 and 20 mM MPP^+ and DA (final concentrations) were directly added to the extracts in the assay in order to assess direct effect of the toxins on CLA activity. Results were calculated as ΔFU/s/μg ± SEM and then converted in mean % in comparison to control samples. The data are from a representative experiment with assays replicated three times. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when *= p<0.05 or ** = p< 0.01 cf. control.*

3.2.4 Effect of MPP^+ and DA on CLA, ATP and glutathione levels of SH-SY5Y cells.

CLA is the most well characterised proteasomal activity and its substrate is very specific (see section 3.2.1.3) since 10 μM lactacystin and epoxomicin virtually abolished its activity in both cell extracts and pure 20S proteasome. Therefore, future studies will focus on CLA. The next aim was to assess whether the reduction in CLA in response to MPP^+ and DA treatment was associated with changes in ATP and glutathione levels.

ATP and glutathione levels from SH-SY5Y cells were determined by enzymatic methods detailed in sections 2.2.3 and 2.2.7, respectively. Control SH-SY5Y cells typically contain 5.504 nmols of ATP and 20.798 pmols of glutathione per μg of protein (Table 3.6).

	Value ±SEM
pmols ATP / μg protein	5.504 ± 1.173
pmols glutathione / μg protein	20.798 ± 3.702

Table 3.6. ***ATP and glutathione levels of SH-SY5Y cells.*** *Cells were seeded in either, 96-well plates or T25 flasks and after overnight recovery ATP and glutathione levels were monitored as described sections 2.2.3 and 2.2.7, respectively. Data are from three independent experiments with assay replicated three times.*

Figure 3.5 and Figure 3.6 represent a time course of CLA, ATP and glutathione levels after treatment with MPP^+ and DA, respectively, in comparison to controls.

Figure 3.5 shows that MPP^+ affects CLA, ATP and glutathione levels in a dose and time-dependent manner. 100 μM MPP^+ had no significant effect on CLA activity at 24 h and 48 h; however it reduced CLA by 30 % at 72h. This reduction in CLA was preceded by a 30% decrease in glutathione levels after 24 h, which further decreased after 72h. Finally, 100 μM MPP^+ did not reduce ATP levels after a 24-h exposure, but, after 48 h and 72 h, ATP levels were reduced by approximately 20 %.

With 2 mM MPP^+, CLA was not affected at 24 h but was significantly reduced by 46 % and 78% at 48 and 72 h, respectively. Similarly, ATP levels were not affected until 48 h treatment with 2 mM MPP^+ (48 % reduction ca. controls), which further decreased at 72h (69 % reduction ca. controls). Glutathione levels, on the other hand were reduced earlier (31 %, 67% and 82 % after 24 h, 48 and 72 h, respectively in comparison to control samples).

Figure 3.6 shows that 100 μM DA did not reduce CLA nor glutathione levels till 72 h treatment (30 % and 23 % reduction ca. controls, respectively). However, 100 μM DA resulted in a significant increase (76 % ca. controls) in glutathione levels at 24h. ATP levels were not affected by this dose of DA at any time tested. With 500 μM DA, again, CLA was only reduced after 72 h. As before glutathione levels increased in comparison to controls after 24 h, but were significantly decreased by 17 % and 77 % after 48 and 72h, respectively. Finally, this high dose of DA virtually abolished ATP levels as early as 24 h.

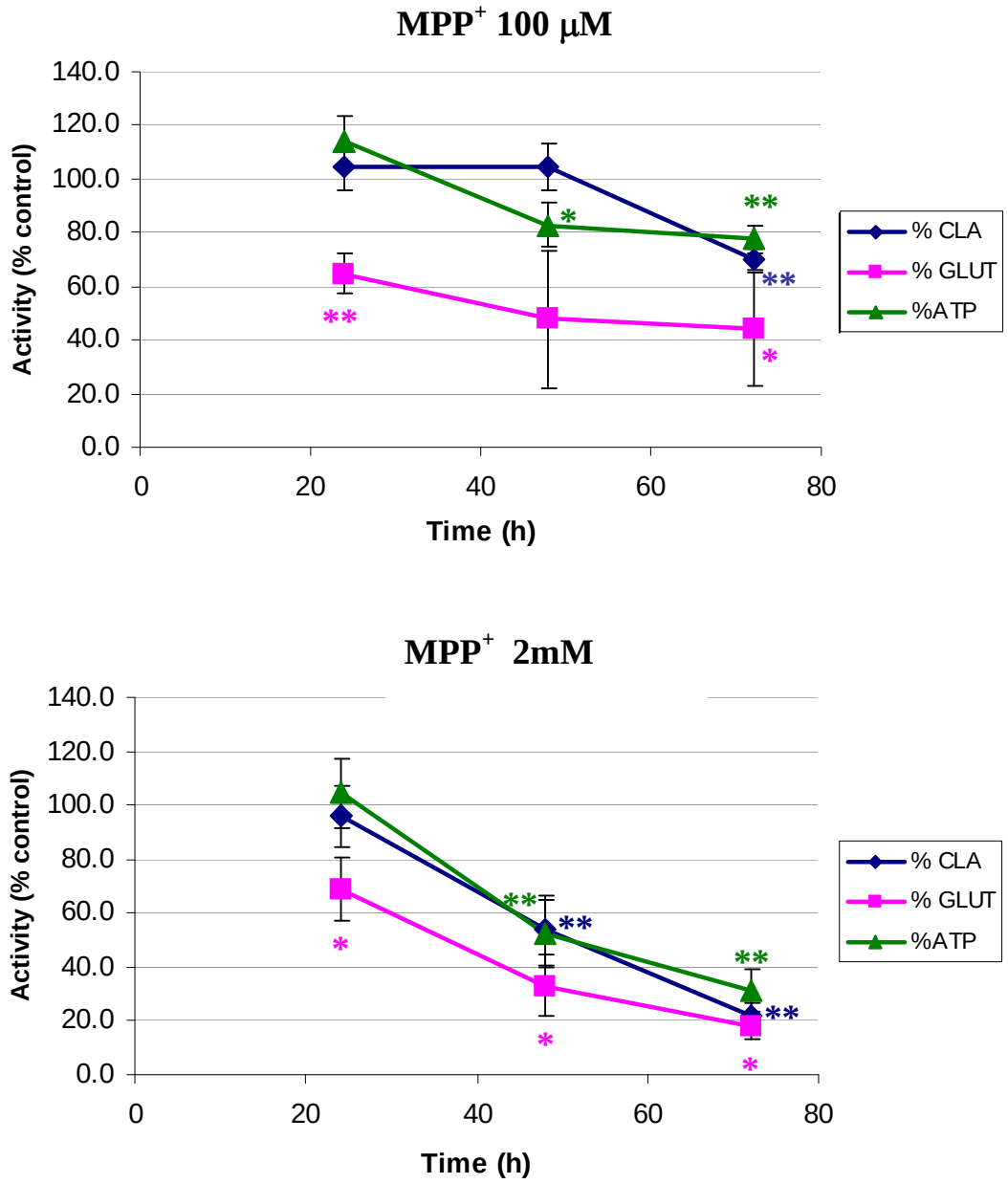


Figure 3.5. ***Effect of MPP+ on CLA, ATP and glutathione levels of SH-SY5Y over time.*** *Cells were seeded in either, 6-well plates, T25 flasks or 96-well plates and treated, after overnight recovery, with 100 µM and 2mM MPP+ for 24, 48 and 72h. After required time CLA, ATP and glutathione levels were monitored as described sections 2.2.6.1, 2.2.3 and 2.2.7 respectively. Results are presented as mean % in comparison to controls. The data are from five independent experiments. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when *= p<0.05 or ** = p< 0.01 cf. control.*

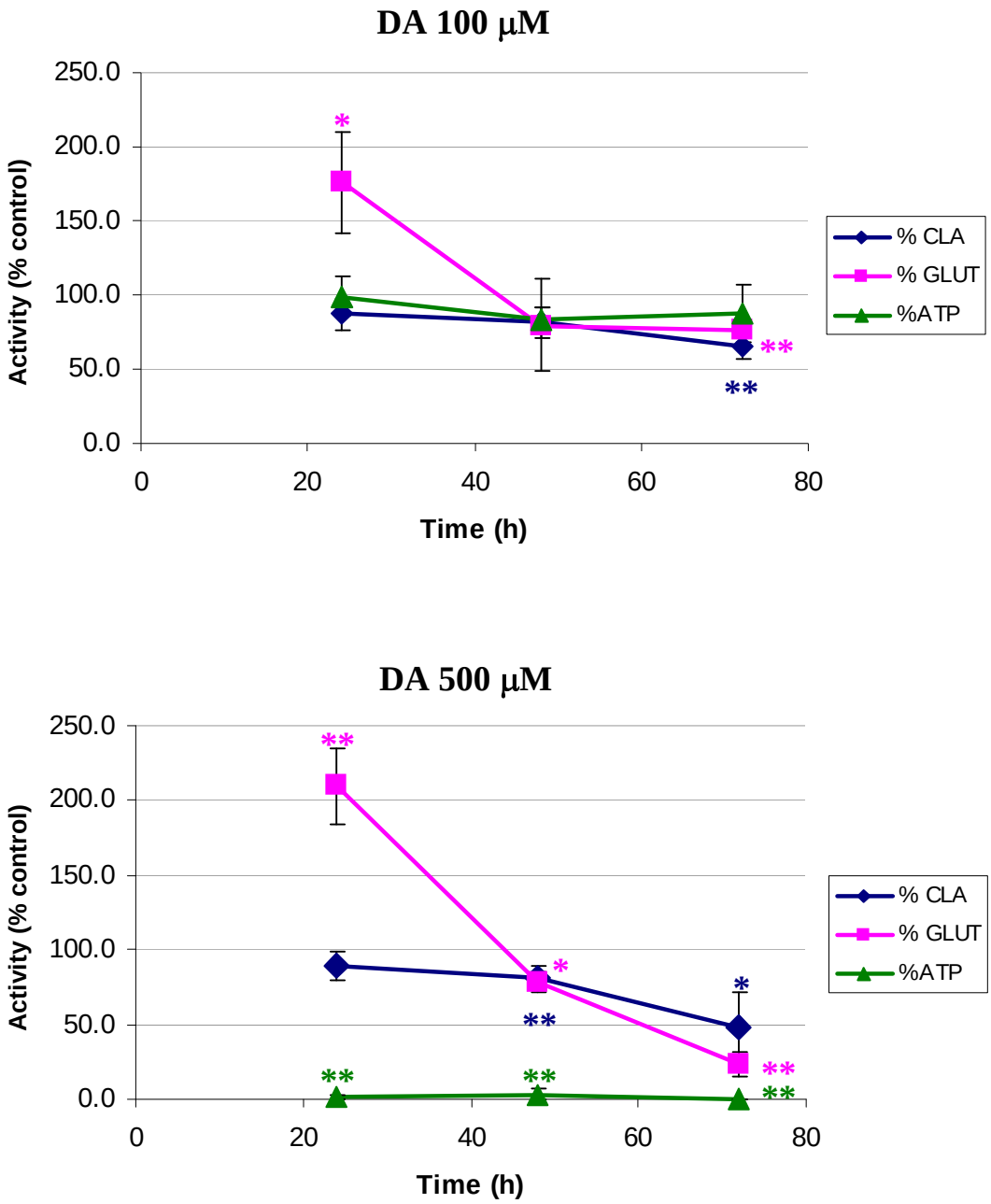


Figure 3.6. ***Effect of DA on CLA, ATP and glutathione levels of SH-SY5Y cell extracts.*** *Cells were seeded in either, 6-well plates, T25 flasks or 96-well plates and treated, after overnight recovery, with 100 μM and 500 μM DA for 24, 48 and 72h. After required time CLA, ATP and glutathione levels were monitored as described sections 2.2.6.1, 2.2.3 and 2.2.7, respectively. Results are presented as mean % in comparison to controls. The data are from five independent experiments. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when *= p<0.05 or ** = p< 0.01 cf. control.*

3.2.5 Effect of toxins on the proteasome is partly due to oxidative stress.

3.2.5.1 Effect of N-acetyl cysteine on proteasomal activity after toxic insult

To determine whether the decrease in proteasomal activity caused by the toxins could be partly due to oxidative stress, the effect of N-acetyl cysteine (NAC; at 1 mM) on proteasomal activity of SH-SY5Y cells after 72-h exposure to 2mM MPP^+ and DA was monitored.

Figure 3.7 shows 1 mM NAC itself did not affect CLA after 72h but that MPP^+ and DA decreased CLA to 13.7 % and 0.5 %, respectively after the same time period. In the case of DA, the decrease in proteasomal activity was partly reversed by 1 mM NAC (activity increasing from 0.5 % to 52.3 % of control). However, 1 mM NAC did not significantly protect CLA from MPP^+ toxicity.

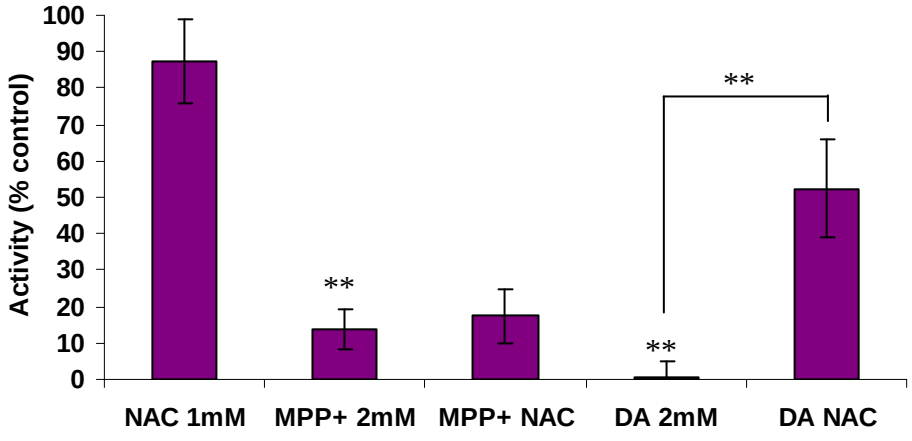
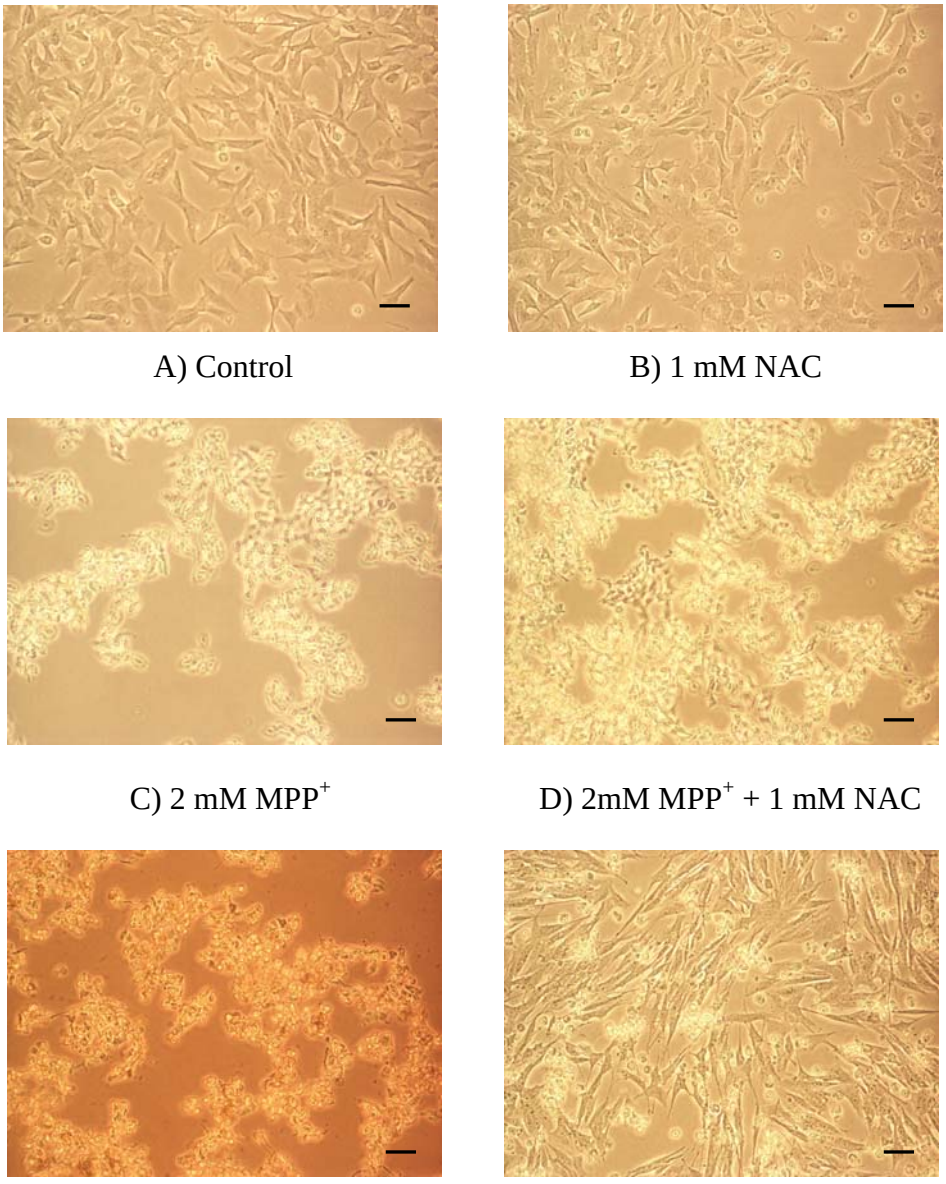


Figure 3.7. ***Effect of NAC on CLA from SH-SY5Y after 72h toxic insult with MPP^+ and DA.*** *Cells were grown in 6-well plates and after overnight recovery, were treated with 2 mM MPP^+ or DA+/ - 1 mM NAC. After 72h incubation cells were extracted in 250 μl homogenisation buffer and cell extracts were incubated with Suc-LLVY-AMC to monitor CLA in a fluorogenic peptide assay (section 2.2.6.1). Results were calculated as ΔFU/s/μg ± SEM and then converted in mean % in comparison to control samples. The data are from a representative experiment with assays replicated three times. Experiment performed 3 times. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when ** = p< 0.01 cf. untreated and toxin-treated control.*

The protection afforded by NAC against DA was also observed at the morphology level. Figure 3.8 shows that control cells (panel A) and cells treated with NAC (panel B) are flat and elongated and have some axon-like structures. On the other hand, when treated with 2mM MPP^+ or DA cells were rounded and many were floating (panel C and E, respectively). NAC had no effect on the morphology of cells treated with MPP^+, but blocked the effect of DA on cell morphology (panel D and F, respectively).



A) Control

B) 1 mM NAC

C) 2 mM MPP^+

D) 2mM MPP^+ + 1 mM NAC

E) 2 mM DA

F) 2mM DA + 1 mM NAC

Figure 3.8. Morphological changes in *SH-SY5Y cells after 72h treatment with 2 mM MPP^+, 2 mM DA and 1 mM NAC.* *Images were taken with a Nikon eclipse TS100 inverted microscope (x 400 magnification). Scale bar= 25 μm.*

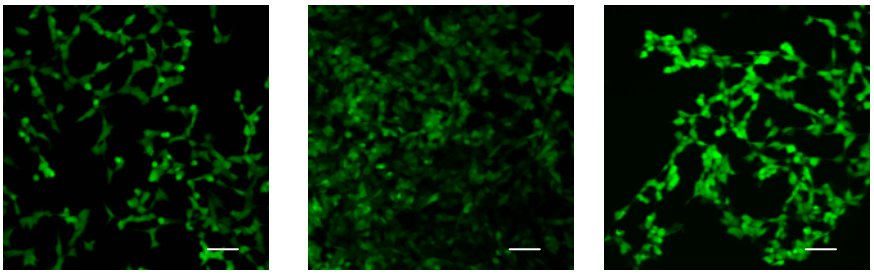
3.2.5.2 Assessment of reactive oxygen species (ROS) formation in SH-SY5Y cells by confocal microscopy

To confirm the possible implication of ROS in MPP^+ and DA toxicity in our model, DCHDF was used as a general indicator of ROS formation (Crossthwaite *et al.,* 2002). Following enzymatic or base-catalysed cleavage of the diacetate group, DCFDH is readily oxidised to the highly fluorescent product dichlorofluorescein (DHF). Formation of DHF can be monitored by confocal laser microscopy (see section 2.2.11).

Results show that 2 mM MPP^+ increased ROS in SH-SY5Y cells after 48 and 72 h (Figure 3.9). Despite using a lower dose of DA (100 μM) than MPP^+, ROS levels were much higher in the DA samples, even after 24 h.

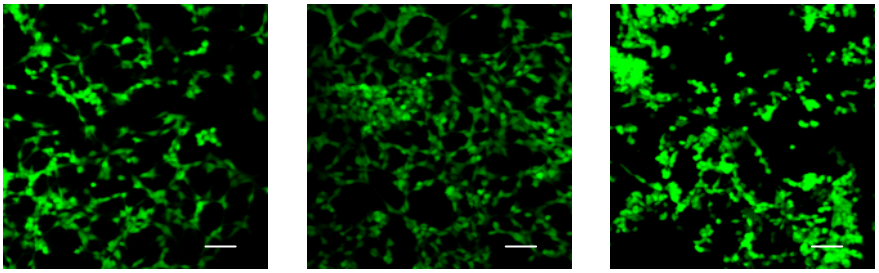
The effect of NAC on MPP^+ and DA 72 h treatment was also monitored. Results show that NAC reduced ROS formation in control and also in toxin-treated cells. Interestingly, with DA samples NAC appeared to virtually abolish ROS formation.

24h



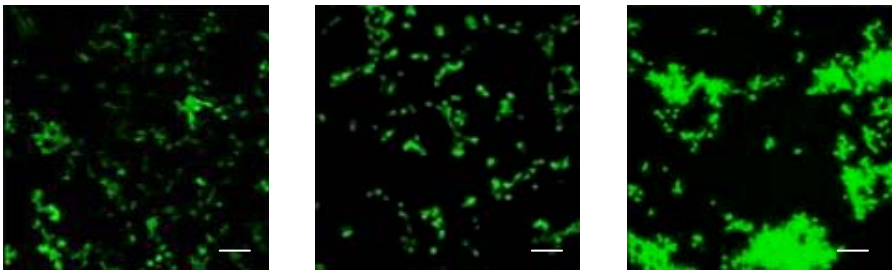
Control MPP$^+$ 2mM DA 100 μM

48h

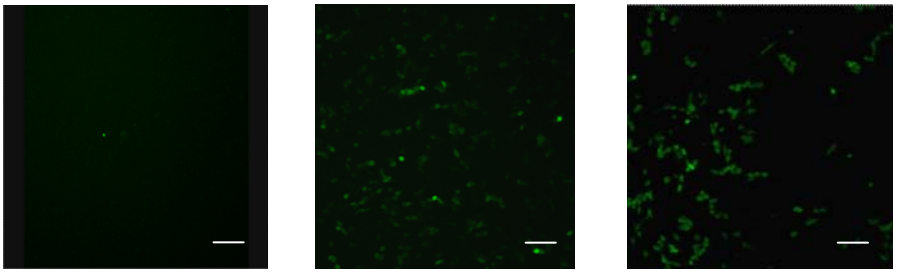


Control MPP$^+$ 2mM DA 100 μM

72h



Control MPP$^+$ 2mM DA 100 μM



NAC 1 mM MPP$^+$ 2mM + NAC DA 100 μM + NAC

Figure 3.9. ***Assessment of ROS formation by confocal microscopy.*** *SH-SY5Y cells were plated out in permanox 8-well chamber slides at a density of 10,000 cells per well. After overnight recovery cells were treated with 100 μM DA and 2 mM MPP^+ for 24, 48 and 72 h prior to analysis of ROS formation by confocal microscopy as described in section 2.2.11. The effects of NAC were also monitored on 72h control and toxin-treated cells. Note: 100 μM DA was used because higher levels resulted in complete loss of cells from the chamber slides. Scale bar = 100 μm.*

3.2.5.3 Effect of glutathione depletion in CLA and ATP levels on SH-SY5Y

In section 3.2.2.2 it was shown that glutathione levels are depleted in SH-SY5Y cells after treatment with MPP^+ and DA. In fact, with MPP^+, glutathione depletion preceded reduction in CLA. Therefore, to investigate whether the reduction in CLA was mainly driven by glutathione, glutathione levels were artificially depleted by 2 mM BSO.

As expected, 2 mM BSO virtually depleted glutathione levels as early as 24h (Figure 3.10). ATP levels were also reduced by 20-30 % after 48 and 72 h and by 65 % after 96 h. Although CLA was reduced by approximately 30 % at 72 h, the reduction in CLA was only significant (> 50 %) after 96 h treatment with 2 mM BSO.

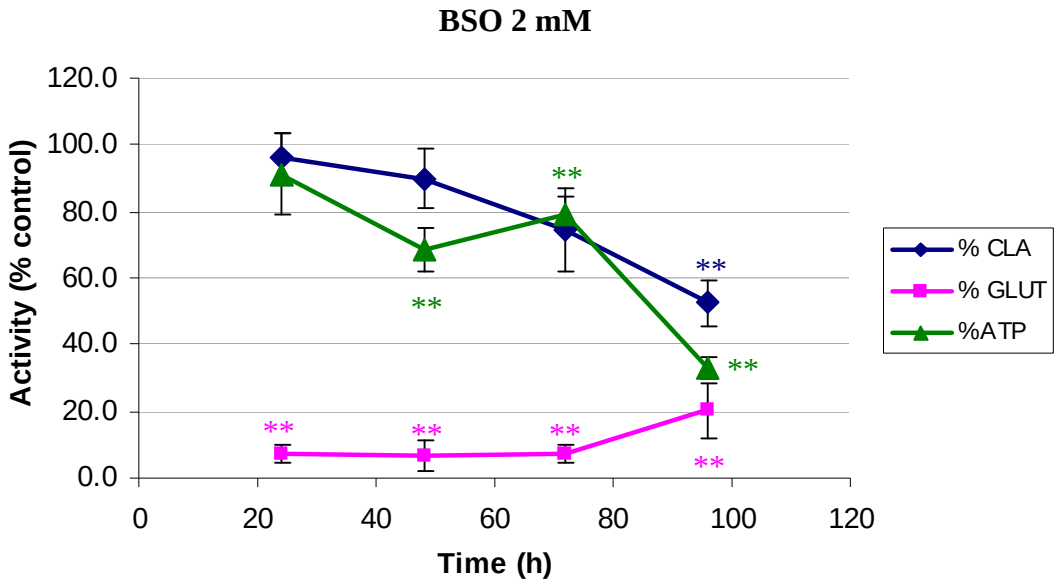


Figure 3.10. ***Effects of 2 mM BSO on CLA, ATP and glutathione levels of SH-SY5Y over time.*** *Cells were seeded in either, 6-well plates, T25 flasks or 96-well plates and treated, after overnight recovery, with 2mM BSO for 24, 48, 72 and 96 h. After required time CLA, ATP and glutathione levels were monitored as described sections 2.2.6.1, 2.2.3 and 2.2.7, respectively. Results are presented as % in comparison to controls. The data are from three independent experiments. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when ** = p< 0.01 cf. control.*

3.2.5.3.1 Assessment of reactive oxygen species (ROS) formation after glutathione depetion

Figure 3.11 shows that 2 mM BSO increased ROS in comparison to controls by 24 h. ROS were not further increased after this time point.

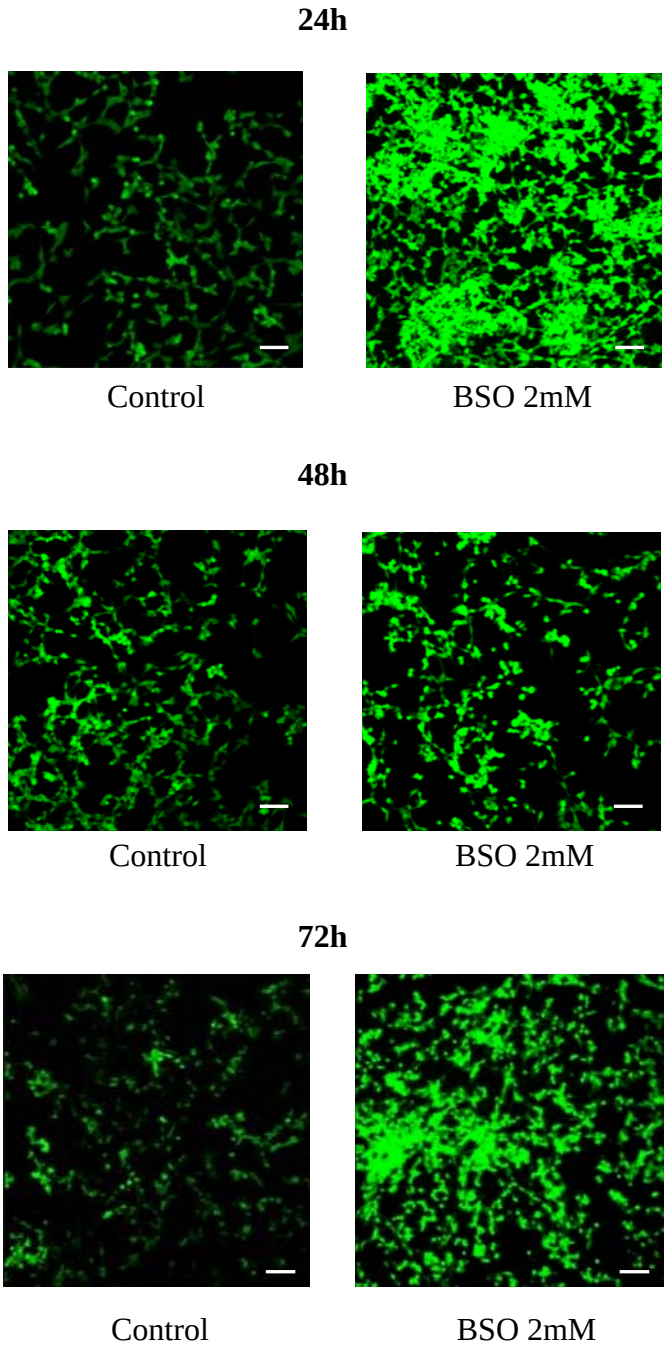


Figure 3.11. Assessment of ROS formation by confocal microscopy. *SH-SY5Y cells were plated out in permanox 8-well chamber slides at a density of 10,000 cells per well. After overnight recovery cells were treated with 2 mM BSO for 24, 48 and 72 h prior to analysis of ROS formation by confocal microscopy as described in section2.2.11. Scale bar = 100 μm.*

3.2.5.3.2 Effect of glutathione depletion on CLA after 72 h toxic insult

To investigate whether glutathione reduction can further exacerbate proteasome impairment caused by MPP^+/ DA, 2 mM BSO was used to deplete glutathione in cells treated with 100 μM MPP^+ or DA for 72 h. Cells were extracted and a fluorogenic peptide assay was performed as explained in sections 2.2.4.2 and 2.2.6.1, respectively.

Figure 3.12 shows that, although BSO further reduced CLA in the MPP^+ treated cells, the reduction was not significant. In the presence of DA, on the other hand, BSO resulted in a significant increase in CLA.

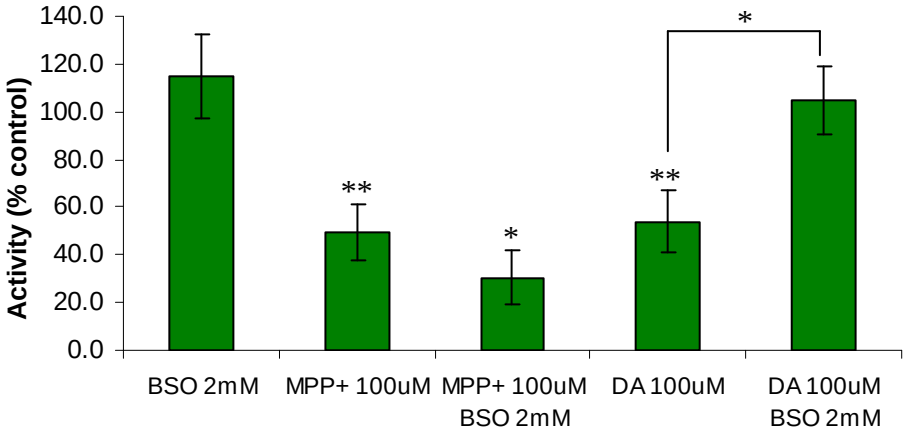


Figure 3.12. ***Effect of glutathione depletion on CLA, of SH-SY5Y after 72h toxic insult with 100 mM MPP^+ and DA.*** *Cells were seeded in 6-well plates and after overnight recovery, treated with 100 μM MPP^+ and DA supplemented with or without 2 mM BSO for 72h. Then, cells were extracted as detailed in section 2.2.4.2 prior to CLA measurement as described section 2.2.6.1. Results were calculated as ΔFU/s/mg and transformed into % in comparison to the relevant control (untreated control or BSO control). The data are from six independent experiments. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when *= $p<0.05$ or ** = $p< 0.01$ cf. untreated or toxin-treated control.*

3.2.6 Effect of chronic exposure to low doses of MPP^+ and DA on proteasomal activity

Finally, proteasomal activity was monitored after chronic exposures (3 and 7 weeks) to low doses of MPP^+ and DA. Figure 3.13 shows that 10 μM MPP^+ significantly reduced CLA by 9 % and 30 % in comparison to controls after 3 and 7 weeks, respectively. TLA and PLA were not reduced until 7 weeks treatment with MPP^+ (14 % and 47 % reduction respectively ca. controls). 10 μM DA reduced CLA by 49 % and 17 % after 3 and 7 weeks, respectively. Interestingly, TLA was considerably increased with 10 μM DA at both times tested. Finally, 10 μM DA reduced PLA by 74 % and 19 % in comparison to controls after 3 and 7 weeks, respectively.

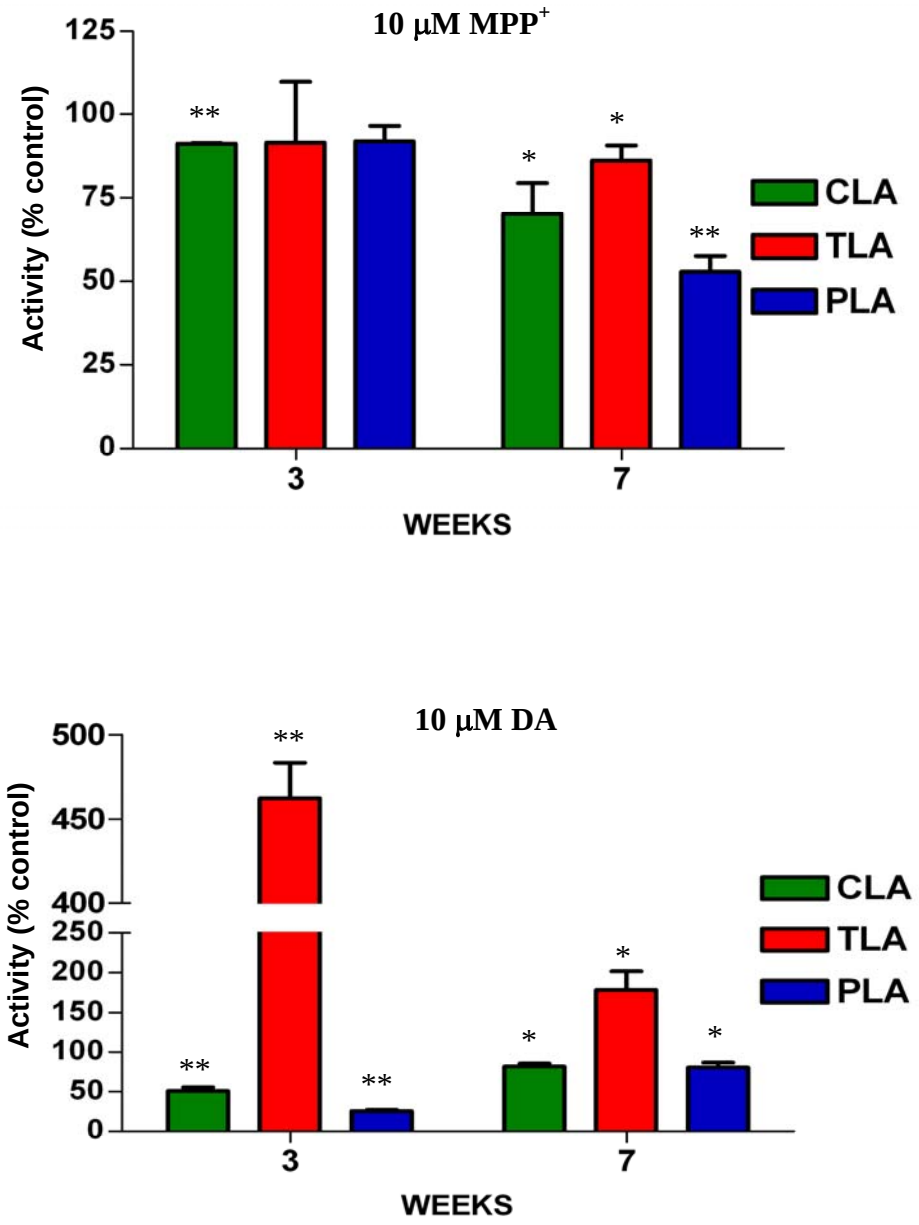


Figure 3.13. ***Effect of chronic treatment with 10 mM MPP+ and DA on proteasomal activities.*** *Cells were seeded in 6-well plates and after overnight recovery, treated with 10 μM MPP+ and DA for 3 or 7 weeks. Proteasomal activities were monitored as described section 2.2.6.1. Results were calculated as ΔFU/s/μg + SEM and transformed into % in comparison to controls. The data are from three independent experiments. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when * = p<0.05 cf. toxin control or ** = p< 0.01 cf. control.*

3.3 DISCUSSION

3.3.1 Specificity of fluorogenic substrates for measuring proteasomal activities and protease inhibitors

As detailed in the main introduction, the eukaryotic proteasome possesses three proteolytic activities, known as chymotrypsin-like, trypsin-like and post-acidic-like (also termed PGPH) activities with distinct specificities against peptide substrates (Rodgers and Dean, 2003 and Kisselev and Goldberg, 2005). A convenient and sensitive way to monitor the activity of proteasomes in cells or tissues is the incubation of cell lysates or tissue homogenates with fluorogenic peptide substrates, which are three or four amino acid residue peptides with a fluorogenic reporter group at the C terminus. The proteasome cleaves an amido bond between an aminoacid and the reporter group, resulting in the release of a highly fluorescent product which can be measured. There are different fluorophores but substrates containing 7-amino-4-methylcoumarin (AMC) are the most commonly used. Despite being used widely, relatively little information is available on how specific these substrates are for proteasomal activity when used in biological samples which may contain many other proteases (Kisselev and Goldberg, 2005). Furthermore, the specificity of proteasome inhibitors and substrates towards the proteasome may vary between different cell lysates and tissue homogenates (Rodgers and Dean, 2003; Kisselev and Goldberg, 2005).

To attempt to assess the specificity of the assays in SH-SY5Y cell lysates, the ability of the proteasome inhibitors lactacystin and epoxomicin, and also the calpain inhibitor MDL 28,170 to inhibit the production of a fluorescent product was compared in SH-SY5Y cells lysates and purified 20S proteasome samples. In this study fluorogenic substrates chosen for measuring proteasomal activity were Suc-LLVY-AMC, Boc-LRR-AMC and Z-LLE-AMC for CLA, TLA and PLA, respectively.

Epoxomicin is claimed to inhibit exclusively proteasomal activity (Kisselev and Goldberg, 2001) whilst lactacystin is known to be more active against CLA than TLA and PLA (Rodgers and Dean, 2003; Kisselev and Goldberg, 2006). Indeed, lactacystin covalently binds to the terminal Thr of the chymotrypsin–like subunit β5 (Groll and Huber, 2004). On the other hand, MDL 28,170 is a short hydrophobic N-blocked dipeptidyl aldehyde lacking charged residues which is able to penetrate cell membranes

by passive diffusion. This calpain inhibitor is reported to also inhibit cathepsin B, and very weakly α-chymotrypsin (Mehdi, 1991).

Lactacystin inhibited CLA in a dose dependent manner in pure 20S; inhibition was virtually complete at the highest concentration (10 μM). On the other hand, 20S-TLA was only inhibited by high doses of lactacystin (10 μM) and PLA was not inhibited at any concentration tested. Epoxomicin reduced PLA to a great extent whilst TLA was only partly inhibited even at the highest concentration of the inhibitor tested (i.e 20 μM). Thus, results suggest that epoxomicin is specific for CLA and to a lesser extent PLA followed by TLA, whilst lactacystin is most specific for CLA followed by TLA. Lactacystin does not inhibit PLA. This is consistent with Kisselev and Goldberg (2005) who report that epoxomicin is the most specific inhibitor for the three proteasomal activities and with Rodgers and Dean, (2003) who showed that lactacystin is most active for CLA. Limited data is available about the specificity of inhibitors against PLA but it is known that PLA is less well inhibited by lactacystin or epoxomicin that CLA (Rodgers and Dean, 2003). On the other hand, MDL 28,170 virtually depleted calpain activity when used at 1 μM or over. Since 10 μM MDL 28,170 also partly inhibited TLA and to a lesser extent CLA our results suggest this inhibitor losses specificity for calpain when used at high concentrations (Rodgers and Dean, 2003).

Specificity of the fluorogenic substrates was tested using the inhibitors with cell extracts. Again, lactacystin inhibited CLA from SH-SY5Y cell lysates in a dose-dependent manner with 10 μM abolishing this activity. Similarly 10 μM epoxomicin, also depleted CLA in cell lysates; these data and the fact that CLA in the 20S and cell extracts was only weakly/ not inhibited by MDL 28,170 would suggest that Suc-LLVY-AMC was fairly specific for CLA. This is consistent with the studies of Rodgers and Dean (2003) who found Suc-LLVY-AMC the most specific substrate to measure CLA. Indeed, this activity was inhibited by more than 75 % with lactacystin in THP1 and J774 cell lysates and in liver cytosol homogenate (Rodgers and Dean, 2003). On the other hand, since lactacystin did not inhibit TLA in cell extracts and epoxomicin only partially inhibited TLA (~ 25 % ca. controls), results would suggest that the TLA substrate is less specific than the CLA substrate for proteasomal activity. Our results are supported by Kisselev and Goldberg (2001), who found that the contribution of non-proteasomal proteolysis to the cleavage of substrates of TLA is always higher than to

the cleavage of PLA and CLA sites. They showed that substrates utilised to measure TLA present a high Km (>0.5 mM) and the specific activity at low concentrations of the substrates is low (Kisselev and Goldberg, 2001). Rodgers and Dean (2003) also found TLA was weakly inhibited by the proteasome inhibitors and that TLA substrate measured other proteases activities. Finally, since PLA was markedly inhibited by epoxomicin (87.2 % inhibition ca. controls) but not by MDL 28,170 our results suggest that Z-LLE-AMC is a fairly specific substrate towards proteasomal PLA.

It would have been of interest to measure calpain activity in our cell system to further confirm a possible activation of these proteases with MPP^+ and DA. However, the protocol used (from Sasaki *et al.*, 1984 with modifications) did not give reproducible activity rates. In fact, although calpains are calcium activated proteases, the assay gave decreasing activity rates with increasing concentrations of calcium, suggesting that it was not reliable.

3.3.2 Effects of MPP^+ and DA on proteasome activity

Our data indicate that MPP^+ and DA reduced CLA and PLA from SH-SY5Y cells in a dose dependent manner after 72 h. The use of proteasomal inhibitors in control and toxin-treated cell lysates confirmed that the activities affected by the toxins were proteasomal. CLA and PLA were similarly reduced by the toxicants, whilst proteasomal TLA (obtained from inhibition assays with epoxomicin) was not reduced by MPP^+ or DA; in fact a significant increase in TLA was observed with 100 μM MPP^+ and 500 μM DA.

The decrease in CLA and PLA after toxic insult with MPP^+ and DA in our cell system is consistent with data reported by several authors. For instance, Keller *et al.* (2000) found that DA toxicity induced a time and dose-dependent decrease in proteasome activity in the PC12 cell line. Elkon *et al.* (2004) also found a decrease in the three proteasomal activities in PC12 cell line, this time caused by 6-hydroxy-DA (6-OHDA), a derivate of DA widely used in animal models of PD. Interestingly, they also report an increase of the proteasomal activities with low doses of the toxin. This is in accordance with the increase in TLA activity in our cell system with 100 μM MPP^+ and 500 μM DA which might be a cellular compensatory effect to mild oxidative stress. Moreover, some studies also showed that toxins that inhibit mitochondrial complex I, like rotenone and other pesticides also lead to proteasome inhibition (Shamoto-Nagai *et al.* 2003; Höglinger *et al.* 2003; Wang *et al.* 2006). Finally, MPTP has also been reported to impair

the three different proteasomal activities in the SN of common marmosets (Zeng *et al.,* 2006). Controversially, in our cell system TLA was not reduced by MPP^+ or DA despite the use of the same fluorogenic substrate (Z-LLE-AMC) as used by several authors who show a decrease in this activity after rotenone (Betarbet *et al.,* 2006) or MPTP (Zeng *et al.,* 2006) treatment. This suggests that in our human cell system, TLA is less sensitive to MPP^+ and DA treatment than CLA or PLA.

3.3.3 Effects of PD mimetics on proteasome activity, glutathione and ATP levels

As indicated earlier, mitochondrial dysfunction linked to energetic failure and oxidative stress (ROS formation), accompanied by a depletion of both reduced and oxidised gluthatione content, are well documented contributors to the disease state (Cassarino and Bennet, 1999; Beal M.F. 2001; Buhmann *et al.* 2004, Chinta *et al.* 2006). It is therefore important to understand the contribution of these factors on proteasome activity in the cells following treatement with the PD mimetics MPP^+ and DA.

3.3.3.1 Effects of MPP^+ and DA on CLA

MPP^+ and DA reduced proteasomal CLA in a dose and time dependent manner. Proteasomal activities were corrected by protein content in order to eliminate any possible decrease in activity due to cell death. The results, as further detailed in the last section, are consistent with several studies showing that some pesticides, including rotenone (Betarbet *et al.,* 2001; Betarbet *et al.,* 2006 and Wang *et al*., 2006), MPTP/ MPP^+ and DA or derivates can lead to proteasome reduction (Keller *et al.,* 2000; Elkon *et al.* 2004; Shamoto-Nagai *et al.* 2003; Höglinger *et al.* 2003 and Zeng *et al.,* 2006). The link between these toxins, which inhibit mitochondrial function, and proteasome impairment further strengthens the potential role of the proteasome in the pathogenesis of PD. Indeed, proteasomal function has been reported to be decreased in SNpc from PD patients (McNaught and Jenner 2001; McNaught *et al.,* 2003).

Contrary to previous reports, this thesis reports that CLA activity could be also directly impaired by addition of the toxins to the cell extracts. Concentrations of 2 mM of MPP^+ and DA led to a reduction in CLA, MPP^+ being more damaging than DA over a 2 h incubation period. This disagrees with Höglinger *et al*. (2003) who exclude a direct reduction of CLA after incubating primary mesencephalic cell lysates with 30 μM

MPP^+ (and also 30 nM rotenone) for ten min. However, this approach is limited since time exposure might be too short to affect proteasome activity with low concentrations of the toxins. Also Wang *et al.* (2006) report that no direct effect on 20S proteasome activity in SK-N-MC cells lysates treated with 6 different pesticides, including the complex I inhibitor rotenone. Therefore, to my knowledge, this study is the only one showing direct effect of MPP^+ and DA on proteasome activity.

3.3.3.1.1 Mechanisms of MPP^+-mediated CLA impairment

MPP^+ decreased glutathione levels prior to reducing CLA or ATP levels; indeed, CLA was only reduced if ATP was also reduced. These findings, suggest that MPP^+-induced proteasomal impairment in my system may be mainly driven by ATP levels. This is supported by the fact that the antioxidant, NAC, failed to protect the cells from MPP^+ effects on the proteasome. This is similar to Höglinger *et al.* (2003) who found that in rat primary mesencephalic cultures, ATP levels needed to be reduced to a threshold low level by MPP^+ or rotenone prior to induce cell death linked with a 30-60 % reduction in UPS activity. Furthermore, these authors found that restoring ATP levels up to 40 % by glucose supplementation, restored proteasomal activities. However, in my cell system only a 20 % reduction in ATP levels in comparison to controls was required for MPP^+ to induce proteasome dysfunction. This suggests that human cells are more sensitive than rat cells. On the other hand, Betarbet *et al.* (2006) found that chronic exposure of human SK-N-MC cells to rotenone impaired the UPS without decreasing ATP levels whilst α-tocopherol protected the cells from rotenone insult indicating that oxidative stress is involved in the toxicity of this complex I inhibitor.

It is important to note that, glutathione levels may also play a role in MPP^+-mediated proteasomal impairment in SH-SY5Y cells, since a sustained reduction in glutathione levels precede the decrease in CLA. Some evidence that depletion in glutathione levels by BSO may exacerbate the effect of MPP^+ on CLA is presented in this thesis. Moreover, confocal microscopy analysis showed that addition of NAC to MPP^+-treated cells (2 mM) could counteract the increase of ROS caused by the toxin, however as stated before without rescuing CLA or cell morphology. Indeed, oxidative stress (ie. free radical formation) has been proposed by several authors to contribute to MPTP/ MPP^+ toxicity (Conn *et al.*, 2001; Song *et al.*, 2004) and, contrary to the results obtained in this thesis, is supported in some systems by the protection afforded by anti-oxidant

agents (Lai *et al.,* 1993; Gonzalez-Polo *et al.* 2004; Chinta *et al.* 2006). Interestingly, Cassarino *et al.* (1997) found that MPTP treatment of mice and MPP^+ exposure of SH-SY5Y human neuroblastoma cells increased oxygen free radical production and also antioxidant enzyme activities. However, involvement of ROS in MPP^+ toxicity is controversial. Lee *et al*. (2000) found that MPP^+ did not increase levels of lipid peroxidation at toxic concentrations in SH-SY5Y cells. Moreover, they also found that pre-treatment of the cells with antioxidants or antioxidant enzymes did not reduce MPP^+ cytotoxicity, concluding that that MPP^+ increased the vulnerability of cells to oxidative stress rather than inducing cell death directly from oxygen free radicals generation. In addition, Lotharius and O'Malley (2000) reported that MPP^+-induced ROS formation is not mitochondrial in origin but results from vesicular DA displacement and intracellular DA oxidation.

In conclusion, it appears that ROS might not be the main factor controlling MPP^+-induced proteasome impairment; glutathione depletion combined with either ATP depletion and/or oxidative stress are needed before proteasomal activity is reduced.

3.3.3.1.2 Mechanism of DA-mediated CLA impairment

With regards to DA, low levels of this toxin (100 μM) did not reduce ATP levels at any time tested, whilst CLA and glutathione levels were reduced after 72 h. With higher doses of DA, ATP levels were virtually abolished as early as 24 h, presumably via complex I inhibition (Gluck and Zeevalk 2004; Ben-Shachar *et al.* 2004; Gimenez-Xavier *et al.,* 2006), but CLA was not reduced until 72 h treatment; this was after glutathione levels were decreased (by 48h). Thus, it is hypothesised that DA toxicity may be primarily driven by oxidative stress within the cells and not via ATP depletion.

This was supported by the fact that the antioxidant NAC, could significantly reduce the effect of dopamine on CLA and also on cell morphology. Indeed, confocal microscopy studies revealed that low doses of DA (100 μM) induced vast amounts of ROS in SH-SY5Y cells, which could be markedly alleviated by the NAC. Moreover, consistent with my findings, Elkon *et al.* (2004) found that a reduction in proteasome activity caused by 6-OHDA in PC12 cells was alleviated by the addition of the antioxidant NAC. This glutathione precursor also rescued cells from protein oxidation and abolished the activation of caspase-3 (ie. apoptosis; Elkon *et al.,* 2004). Additionally, data supporting a key role for glutathione on DA toxicity and UPS impairment was shown by Jha *et al.*

(2002), since a reduction of total glutathione levels after treatment of PC12 cells with the DA metabolite, 6-OHDA, led to reduced ubiquitin conjugated levels suggesting that ubiquitination of proteins is inhibited in a glutathione-dependent fashion.

Surprisingly, BSO appeared to protect proteasome from DA insult; CLA was significantly increased to virtually control levels when BSO was added to DA. This could be explained by a similar transient compensatory response to that observed by others with 6-OHDA (Elkon *et al.,* 2004). On the other hand, Stokes *et al.* (2000) found that in SK-N-SH neuroblastoma cells, DA toxicity was enhanced when GSH was depleted; but only when glutathione levels were virtually depleted (98 % reduction with 2mM BSO), suggesting that GSH stores are in excess in neuroblastoma cells.

Finally it is important to note that although DA concentrations used in this study (100-500 μM) would be considered as being above normal physiological values, since extracellular concentrations are reported to be of the order of nM in the SN and striatum, the intracellular striatum concentration is estimated to be around 70 μM, whilst the concentration in neuronal endings can be in the mM range (Blum *et al.,* 2001; Gimenez-Xavier *et al.,* 2006; Morikawa *et al.,* 1996). Thus it would seem likely that extracellular DA concentrations in the present study would lead to intracellular concentrations that are found in vivo in dopaminergic neurons with disrupted vesicles or vesicles not storing DA efficiently.

In conclusion, reduction in glutathione levels and the subsequent increase in oxidative stress might be a critical parameter on DA-induced proteasomal impairment.

3.3.3.2 Effect of BSO on CLA

It has been suggested that low levels of oxidative stress increases proteasome activity but higher levels can inhibit proteasomal fuction (Reinheckel *et al.*, 1998). Indeed, depletion of glutathione levels (with BSO) in my cell system led to impaired CLA. However, although ROS levels were greatly increased after 24 h treatment with BSO, proteasomal function only decreased after 96 h. BSO also reduced ATP levels in the cells (by 48 h), suggesting that other factors, like ATP, might also be contributing in the process. Therefore, ROS by itself might not be sufficient to cause proteasomal impairment.

3.3.4 Chronic exposure to low levels of toxins impairs proteasomal function

Chronic exposure to low levels of MPP^+ reduced CLA levels after 3 weeks treatment, and TLA and PLA after 7 weeks treatment with the toxin. In contrast, DA was more damaging to the proteasome, reducing CLA and PLA after 3 weeks treatment. These data are consistent with Betarbet *et al.* (2006) who found that chronic exposure to rotenone also decreased the three proteasomal activities in SK-N-MC cells after 4 weeks. As reported before in this thesis, TLA appears to be the least sensitive of the three activities. Interestingly, TLA was increased after 3 and 7 week exposure of cells to DA. It has been suggested that activation of the proteasome can occur under mild oxidative stress conditions (Reinheckel *et al.*, 1998). This might be a compensatory mechanism in order to help the cells to cope with oxidatively damaged proteins. Indeed, treatment of PC12 cells with 6-OHDA, a naturally occurring DA analogue (Blum *et al.*, 2001), increases proteasome activity at low doses (10-100 μM) with a reduction in activity with higher doses discussed (Elkon *et al.*, 2004). This is in agreement with Höglinger *et al.* (2003) who showed that 6-OHDA also transiently increased all three proteasomal activities in rat mesencephalic cell cultures. Recent data from Betarbet *et al.* (2006) also showed compensatory increases in proteasomal activities in neuroblastoma cells prior to a reduction in activities over a 4 week period. However, as discussed in section 3.3.1, the TLA substrate is unspecific for measuring proteasomal TLA. My previous data suggest that calpains may contribute to the activity measured in the TLA assay since MDL 28,170 inhibited measured TLA by 67 % of control cells. Nevertheless, further studies with different types of proteases inhibitors are needed to elucidate true proteasomal TLA in these experiments.

CHAPTER IV

MPP^+, MPTP AND DA DIRECT EFFECT ON PURIFIED 20S PROTEASOME

4. MPP$^+$, MPTP AND DA DIRECT EFFECT ON PURIFIED 20S PROTEASOME

4.1 INTRODUCTION

4.1.1 The 20S proteasome

As explained in the general introduction, the 20S proteasomal catalytic core is a 28 subunit multicatalytic particle consisting of four heptameric rings. The two outer rings consist of seven α-subunits each and the two inner rings consist of seven β-subunits. The multiple catalytic centres are located in three β–subunits (β5, β 2 and β1; Koop *et al.*, 1997; De Martino and Slaughter, 1999; Ferrell *et al.*, 2000; Kisselev and Goldberg, 2001; De Vrij *et al.* 2004).

In vivo, 20S proteasomes exist not only as a part of the 26S complexes but also as free particles (Kisselev *et al.*, 1998) and comprise about 1 % of cell proteins (Lee and Goldberg, 1998). Indeed, although initial studies suggested that the 26S was the major proteasomal form *in vivo*, subcellular localization and stochiometrics of the different complexes showed that the number of free 20S particles exceeds that of the 19S and 11S (free or bound to 20S) by 3 to 4-fold. Moreover, the 20S proteasome itself degrades proteins without a requirement for ubiquitin or ATP, including oxidized and damaged proteins (Davies *et al.*, 2001; De Vrij *et al.*, 2004). Oxidised proteins are particularly relevant to neurodegenerative disorders, such as PD, and seem to be degraded via the 20S in an ubiquitin-independent manner (Davies, 2001; Grune *et* al., 2003).

A growing body of evidence suggests that proteasome activity declines with age and is involved in certain pathologies (Bulteau *et al.*, 2001; Carrard *et al.*, 2002; Farout *et al.*, 2006). This has been attributed, at least in part, to oxidative stress since it has been shown that the proteasome can undergo modification by 4-hydroxy-2-nonenal (HNE), a lipid peroxidation product, during these situations (Bulteau *et al.*, 2001; Carrard *et al.*, 2002). Moreover, recent data have been published reporting that HNE oxidatively modified specific subunits of the 20S proteasome (Farout *et al.*, 2006).

4.1.2 Aims of chapter

As introduced in the last chapter there is an increasing number of studies which link complex I inhibition to proteasomal impairment. However, limited data is available about the possible direct effects of complex I toxins on the proteasome itself. Data presented in last chapter indicates that direct incubation of SH-SY5Y cell lysates with MPP^+ and DA leads to reduced CLA; however, recent work suggests no direct effects of some mitochondrial toxins, such as MPP^+ or rotenone, on the proteasome (Höglinger *et al.*, 2003; Betarbet *et al.*, 2006; Wang *et al.*, 2006). It is important to note that the approach of these authors was incomplete since relatively low concentrations of toxins with short time exposures were utilised when assaying 20S activity of cell lysates.

Therefore it was considered important to further monitor the direct effects of the neurotoxins MPTP, MPP^+ and DA on the activities of the commercial 20S proteasome purified from human red blood cells and in particular on TLA, which was shown to be less sensitive to the toxins in the previous chapter. Indeed, the use of purified 20S proteasome will assure that substrates (particularly TLA substrate) are specifically measuring proteasomal activity. In addition, the protective capacity of vitamin C (ascorbic acid) was examined to establish whether the neurotoxins caused free radical damage. Additionally, for comparison, the effects of these toxins on purified trypsin enzyme were also monitored to establish whether a general protease was affected in a similar manner to the proteasome. Finally, 2D SDS-PAGE techniques were utilised in order to separate the 20S subunits and analyse whether specific proteasome subunits were affected; this was monitored using silver-staining and antibodies directed against various β-subunits (to help define the exact catalytic subunits affected) and α-subunits on Western blots (Brooks *et al.* 2000).

4.2 RESULTS

4.2.1 Effect of MPP^+, MPTP and DA on 20S proteasomal activities

Figure 4.1 (A) shows that all toxins reduced CLA in a dose dependent manner, with the effects of 2 mM MPTP and MPP^+ being similar, resulting in 72 % and 54 % reductions, respectively. On the other hand, DA had a greater effect and completely abolished activity at 2 mM whilst a low concentration (10 μM) reduced CLA by 70 %.

On the other hand, TLA was not affected by MPTP at all concentrations tested (Figure 4.1B). However, MPP^+ significantly reduced TLA by 30% and 42% at 500μM and 2mM respectively. DA had a more potent effect on this activity, and completely abolished activity when used at 500 μM or 2 mM. Again, the pattern shown by MPP^+ and DA was dose dependent.

As for CLA, PLA was reduced by all toxins (Figure 4.1C). Indeed, PLA was more sensitive to high doses of MPP^+ and MPTP than CLA since PLA was completely abolished at the highest toxin concentration. Although, DA was the most damaging, abolishing PLA at 500 μM, the effects of the three toxins were very similar.

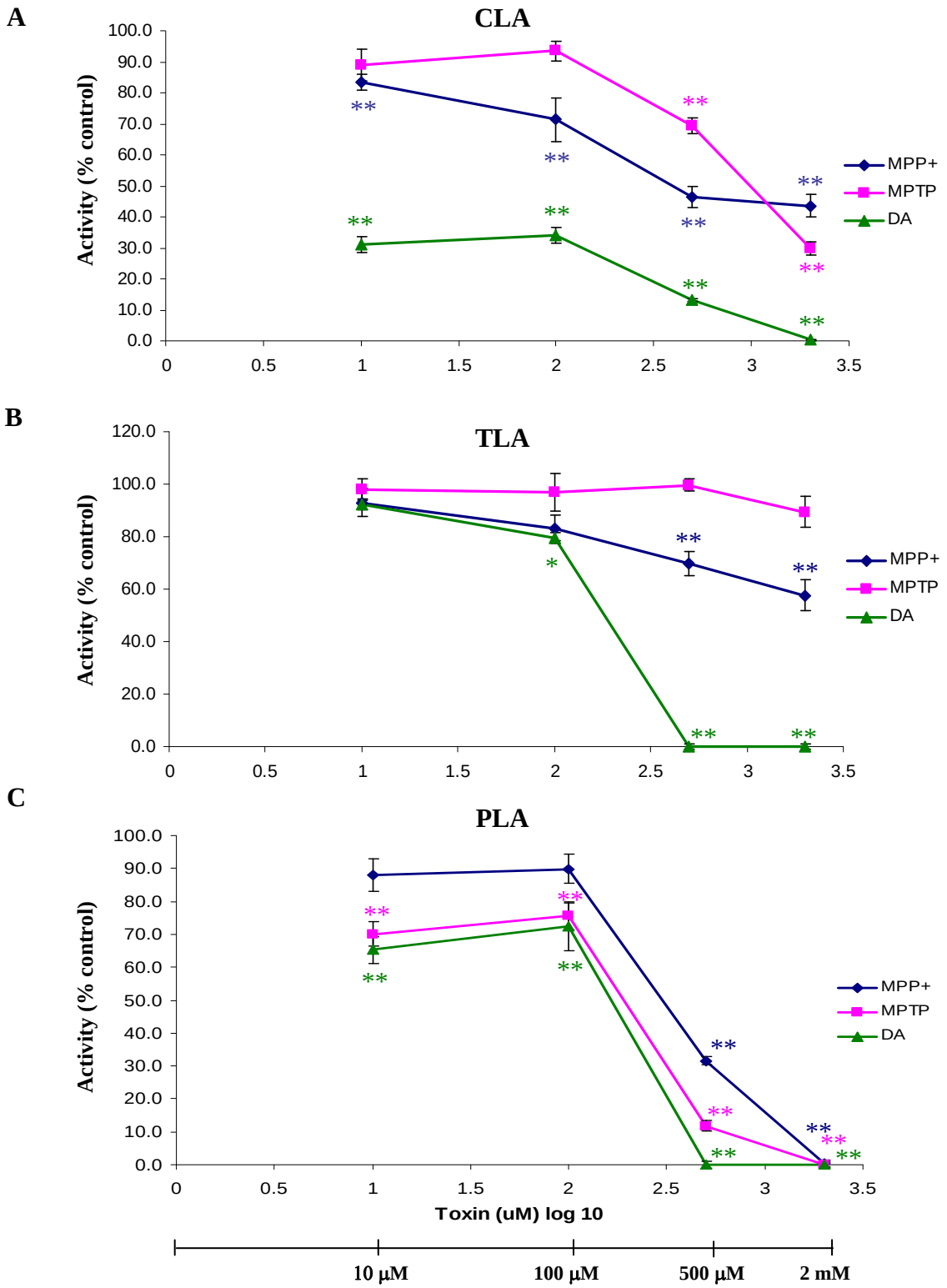


Figure 4.1. ***Effect of MPP+, MPTP and DA on CLA (A), TLA (B) and PLA (C) of commercial 20S proteasome.*** *Suc-LLVY-AMC, Boc-LRR-AMC and Z-LLE-AMC were used to monitor proteasomal CLA, TLA and PLA, respectively as described in section 2.2.6.1. Results were calculated as ΔFU/s/µg ± SEM and converted to mean % in comparison to controls. The data are from three independent experiments with assays replicated four times. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when ** = p< 0.01 cf. control.*

4.2.2 Effect of the antioxidants on toxin induced 20S proteasome impairment

4.2.2.1 Screening for the most suitable antioxidant

Different antioxidants were used: glutathione (GLUT), N-acetyl cysteine (NAC), vitamin E (α-tocopherol) and vitamin C. Firstly, the effect of these antioxidants against DA toxicity towards proteasomal chymotrypsin- like activity was screened, in order to select a suitable antioxidant for further studies. Table 4.1 shows that 100 μM DA reduced CLA to 39 % of controls and this reduction was partly reversed by100 μM vitamin C (68 % in comparison to vitamin C control). In contrast, none of the other antioxidants tested offered protection to the proteasome against DA toxicity. Therefore, vitamin C was selected for further studies with the other toxins.

	ΔFU/s/μg	**SEM**	**% Activity cf. control**
Control	2.215	0.031	100
GLUT 100 μM	2.266	0.109	102.3
NAC 100 μM	2.225	0.056	100.4
VI T E 100 μM	2.050	0.092	92.5
VIT C 100 μM	2.663	0.115	120.2
DA 100 μM	0.870	0.075	39.3 **
DA/ GLUT	1.042	0.074	46.0 **
DA/ NAC	0.736	0.076	33.1 **
DA/ VIT E	0.883	0.124	43.1 **
DA/ VIT C	1.811	0.119	68.0 **/ ••

Table 4.1. ***Assessment of the effect of different antioxidants on DA toxicity to CLA from purified 20S proteasome.*** *DA and antioxidants were directly added to the 20S (0.05 μg) proteasome in a black 96-well plate and preincubated for 15 min before the addition of the CLA substrate, Suc-LLVY-AMC. CLA was monitored as described in section 2.2.6.1. Results were calculated as ΔFU/s/μg ± SEM and converted to mean % in comparison to controls or antioxidant controls. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when ** = p< 0.01 cf. untreated and antioxidant-treated control or •• = p< 0.01 cf. DA (n=3).* <u>*Important note*</u>*: variations in activity between control 20S are due to the fact that different batches of commercial purified proteasome produce different basal activities due to source differences (ie. different individuals). This is specified in the data sheet of the product (code PW8729) purchased from Biomol International, Exeter, UK.*

4.2.2.2 Protective effect of vitamin C on CLA from purified 20S proteasome treated with various toxins

Figure 4.2 shows vitamin C protected the 20S proteasome from 2 mM MPTP, MPP^+ and DA to different extents. 100 μM vitamin C was required to protect CLA against DA, restoring activity from 1 % of controls to 46 % of controls; with 500 μM and 1 mM vitamin C, CLA was increased to 62 % and 70 % of controls, respectively. Low doses of vitamin C (10μM) protected CLA against MPP^+ and MPTP, restoring CLA from 47 % and 7.5 % to 67 % and 62 % of controls, respectively; protection improved when the vitamin C dose was increased to 100 μM (to 78 % and 87 % for MPP^+ and MPTP, respectively).

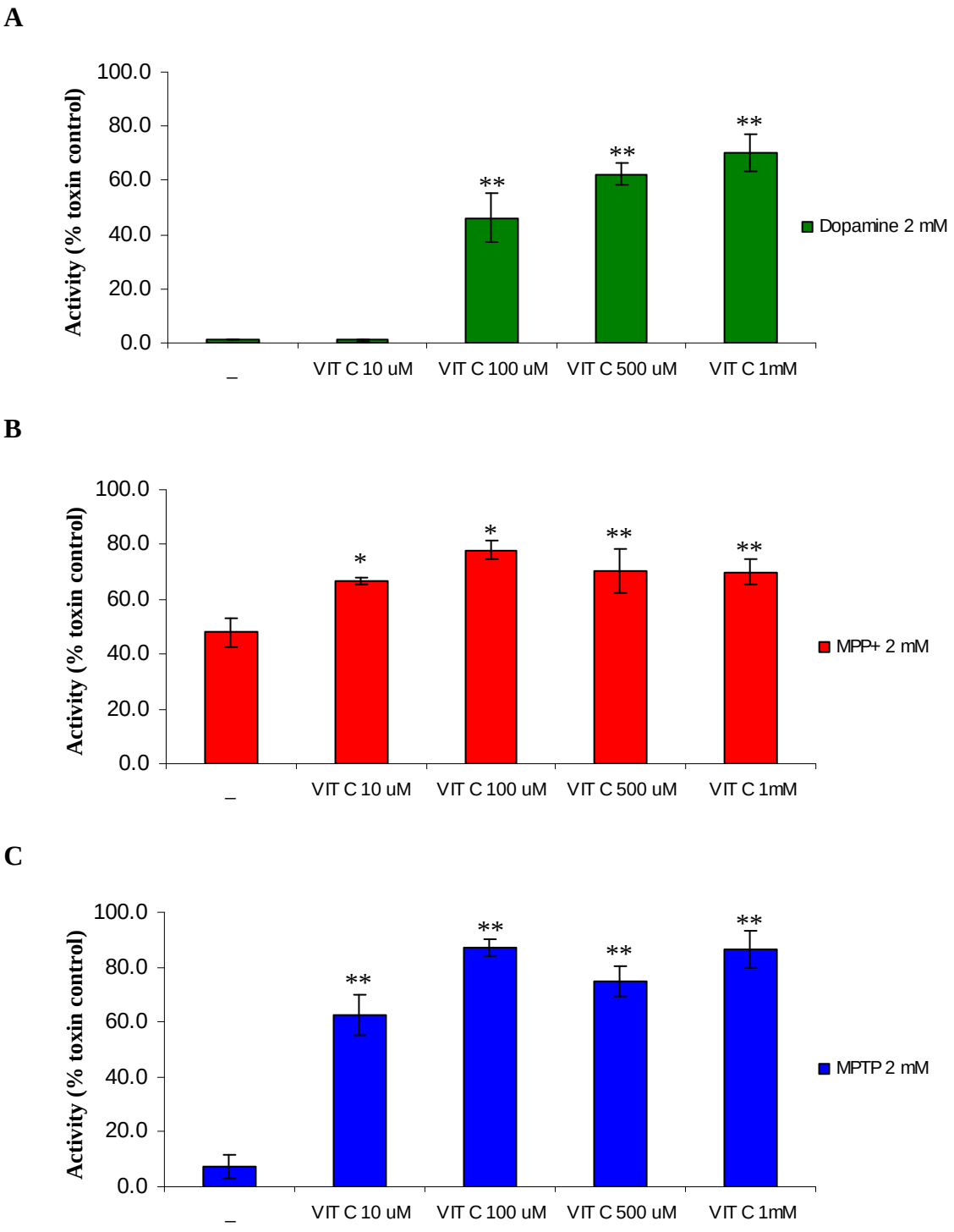


Figure 4.2. ***Effect of vitamin C on toxin induced proteasome impairment.*** *DA (A), MPP$^+$ (B) or MPTP (C) were added directly to 0.05μg commercial 20S with or without 10, 100, 500 μM and 1 mM vitamin C. Suc-LLVY-AMC was used to monitor CLA (as explained in section 2.2.6.1). Results were calculated as ΔFU/s/μg ± SEM and converted to mean % in comparison to controls or antioxidant controls. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when * = p<0.05; ** = p< 0.01 cf. toxins (n=3).*

4.2.3 Effects of MPP^+, MPTP and DA on trypsin enzyme activity

Next, the effect of toxins on trypsin enzyme (EC 3.4.21.4) were compared to the effects on its proteasomal counterpart, 20 S trypsin-like activity.

Firstly, different dilutions (1:5000; 1:10000; 1:20000; 1:40000 and 1:80000; in 50 mM HEPES, 5mM EGTA, pH 8.0) of trypsin enzyme (1mg/ml) were tested to determine an amount of the pure enzyme that provided a fluorescence rate similar to the 20S proteasome (section 4.2.1). 10 μl of 1:10000 dilution per assay (ie. 1 ng pure trypsin) were found to be suitable for the experiment. The procedure was as described previously for the 20S proteasome except 10μl of the diluted enzyme were added as opposed to commercial 20S.

The data presented in Table 4.2 show 20S TLA to be more sensitive to DA than trypsin enzyme, with 500 μM DA completely inhibiting 20S-TLA whilst reducing trypsin activity by only 71 %. In contrast, MPP^+ had a similar effect on both activities. Consistent with previous data (see section 4.2.1) MPTP had no significant effect on proteasomal TLA and similarly had no adverse effect on trypsin enzyme activity.

	20S		Trypsin	
Treatments	**ΔFU/s/μg ± SEM**	**% Activity ca. control**	**ΔFU/s/μg ± SEM**	**% Activity ca. control**
Control	4.504 ± 0.176	100.0	8.679 ± 0.351	100.0
MPP^+ 100 μM	4.135 ± 0.280	91.8	8.192 ± 0.425	94.4
MPP^+ 500 μM	3.658 ± 0.209	81.2*	7.208 ± 0.420	83.1*
MPP^+ 2 mM	3.473 ± 0.118	77.1**	6.690 ± 0.310	77.1**
MPTP 100 μM	4.090 ± 0.065	90.8	7.781 ± 0.664	89.7
MPTP 500 μM	3.842 ± 0.259	85.3	9.135 ± 0.198	105.3
MPTP 2 mM	4.156 ± 0.111	92.3	7.881 ± 0.444	90.8
DA 100 μM	1.914 ± 0.355	42.5**	4.558 ± 0.332	52.5**
DA 500 μM	0.000 ± 0.408	0.0**	2.483 ± 0.437	28.6**/ •
DA 2 mM	0.000 ± 0.489	0.0**	1.594 ± 1.594	18.4**/•

Table 4.2. ***MPP^+, MPTP and DA effect on pure trypsin enzyme.*** *1 ng of pure trypsin enzyme/HEPES-EGTA and 0.05 μg of 20S proteasome were used in a fluorogenic substrate assay, as described before, to compare the effect of the toxins on both enzymes. The data are from a representative experiment with assays replicated three times. Results were calculated as ΔFU/s/μg protein ± SEM and then converted to % reduction in comparison to control samples. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when * = p<0.05; ** = p< 0.01 ca. controls or • = p<0.05 cf. the same DA treatment on TLA vs. trypsin enzyme.*

4.2.3.1 Effect of vitamin C on trypsin enzyme activity after toxic insult

Figure 4.3 shows that concentrations of vitamin C ≥100 μM almost completely reversed the DA-mediated effect on trypsin enzyme activity.

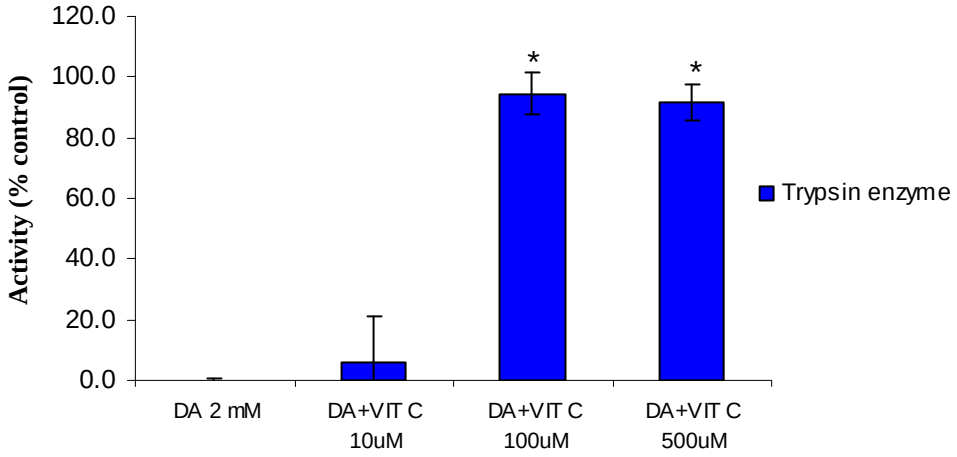


Figure 4.3. ***Effect of vitamin C on trypsin enzyme activity after toxic insult with 2 mM DA.*** *1 ng of pure trypsin enzyme/HEPES-EGTA was used in a fluorogenic substrate assay, as described in section 2.2.6.1., to compare the effect of the toxins on both enzymes. The data are from a representative experiment with assays replicated four times. 2 mM DA was added directly to the trypsin enzyme in the presence or absence of (10-500 μM) vitamin C. Results were calculated as ΔFU/s/μg ± SEM and converted to mean % in comparison to controls or antioxidant controls. Statistical analysis was carried out using a two-tailed t-test. Statistical significance was accepted when * = p<0.05 cf. toxins.*

4.2.4 Effect of toxins on purified 20S proteasomal subunits

DA and MPP^+ have been shown to reduce 20S proteasome activity when either added directly to purified proteasome (section 4.2.1) or to SH-SY5Y cells (section 3.3.2). This section investigated whether any specific core subunit of the 20S proteasome is particularly affected by MPP^+ and DA.

Purified 20S proteasome (2 μg in 50 mM HEPES, 5mM EGTA pH 8.0) was incubated with 2mM MPP^+ or DA in phosphate buffer saline (PBS) for 2 h at 37º C prior to fractionation by 2D-SDS PAGE as described in section 2.2.9.5. Gels were then silver stained (see section 2.2.9.5) or analysed by Western blotting/ immunoprobing (see sections 2.2.10.1 and 2.2.10.2) using specific antibodies against 20S proteasome core subunits. Membranes were probed/ stripped several times as detailed in section 2.2.10.5.

Table 4.3 gives a summary of the molecular masses and isoelectric points (pI) of the fourteen 20S subunits whilst Figure 4.4 is a 2D electrophoretic reference map for 20S subunits (blots taken from Claverol *et al.*, 2002). As can be seen, the pI values range from 4.8 to 8.7 and the molecular masses are from 22 to 30 kDa.

20S subunit	Theoretical molecular mass (Da)	Theoretical pI	Function
α5	26,469	4,86	Catalyse a proteasome RNAase activity
β1	21,862	5,15	PLA active site
α7	28,302	5,43	Unknown
β2	25,295	6,09	TLA active site
β7	24,379	5,76	PLA active site complementary subunit
α6	29,556/ 30,108[a]	6,90/ 7,28	Unknown
α1	27,339	7,01	Unknown
β3	22,930	6,81	Unknown
α3	29,483	7,95	Unknown
α2	25,767	7,74	Unknown
β4	22,836	7,31	CLA active site complementary subunit
α4	22,458	8,67	Unknown
β6	27,887	8,38	TLA active site complementary subunit
β5	23,548	8,66	CLA active site

Table 4.3. ***Subunit identification of human 20S proteasome purified from erythrocytes.*** *The above table shows the theoretical molecular weights and isoelectric points of the 14 subunits of the human proteasomal 20S catalytic core. Table taken from Claverol et al. (2002) with modifications. Most of the subunits (12 of 14) exhibit several isoforms (from 2 to 4). Only subunits α5 and β3 appear as one single spot (see Figure 4.4).* [a] *= short and long isoform of α6 subunit.*

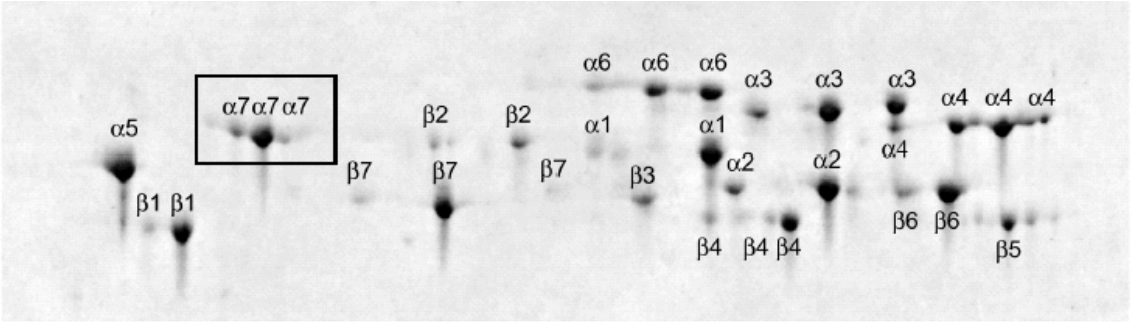


Figure 4.4. ***Two- dimensional electrophoretic reference map of human 20S proteasome (Picture taken from Claverol et al., 2000).*** *20S proteasome from erythrocytes (40μg) was separated using a pH 3-10 non linear IPG strip in the first dimension followed by a 12.5 % polyacrylamide SDS gel in the second dimension. The 2D gel was stained with Coomassie Brilliant Blue. All labelled spots were identified by MALDI-TOF mass spectrometry and database search.*

Figure 4.5 and Table 4.4 show some of the spots which were identified in the 2D-gel by MALDI-TOF mass spectroscopy and data base search (performed by Kevin Bailey and John Kyte from School of Biomedical Sciences, University of Nottingham Medical School, Nottingham, UK). The rest of the spots mapped in Figure 4.6 were identified by theoretical molecular weights and pI of the different subunits together with a comparison with the refence map available from Claverol *et al.,* (2002; see Figure 4.4).

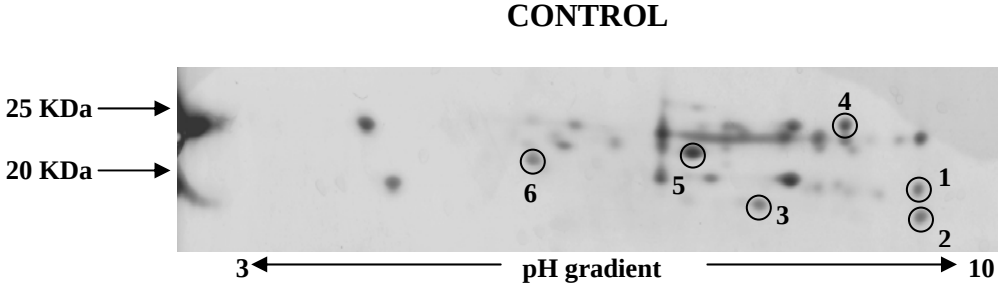


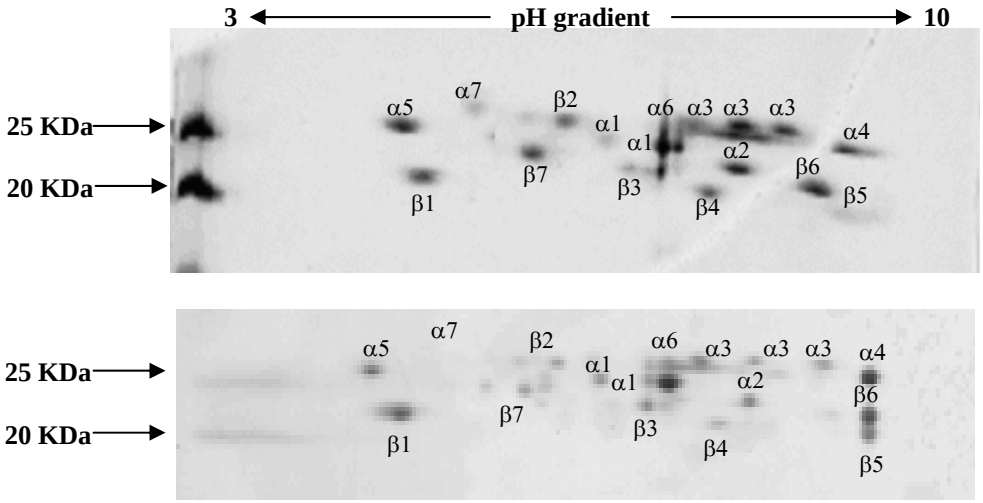
Figure 4.5. Two-dimensional SDS-PAGE of 20S purified proteasome from human erythrocytes. *2μg of 20S proteasome were separated using a pH 3-10 non-linear IPG strip (1st dimension) followed by a 15 % polyacrylamide SDS gel electrophoresis (detailed in section 2.2.9.4). The 2D gel was silver stained as detailed in section 2.2.9.5. and all labelled spots were identified by MALDI-TOF mass spectrometry and database search.*

Spot number	Identification	Tryptic fragments (Number of fragments/ matched fragments)	% Sequence coverage	Score	Theoretical pI	Theoretical MW (Da)
1	β6	8/8	46 %	93	8.27	26,472
2	β5	9/10	44 %	101	7.33	23,606
3	β4	4/4	26 %	51	6.51	22,882
4	α3	4/6	19 %	36	7.57	29,465
5	α1	7/8	29 %	68	6.34	27,382
6	β7	4/4	21 %	49	5.70	25,893

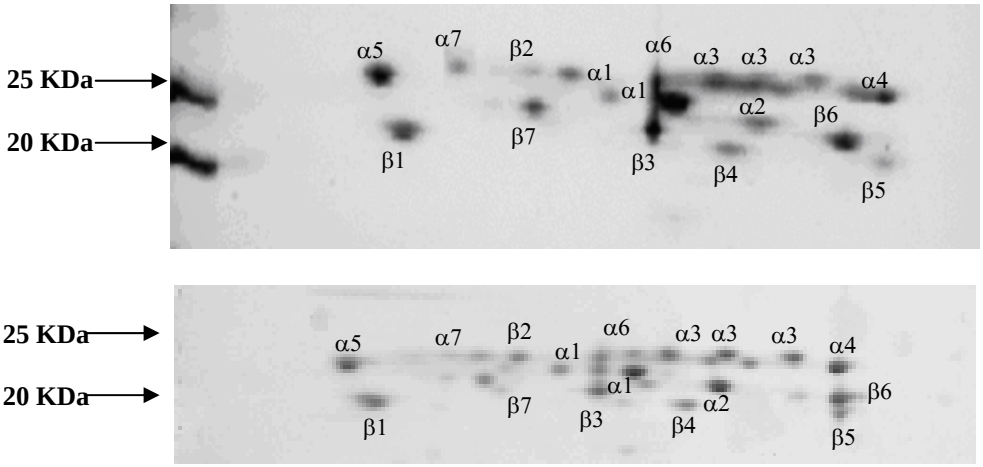
Table 4.4. Subunit identification of human 20S proteasome purified from erythrocytes. *The above table shows the identification of the spots labelled in figure 4.5. Identification was performed by MALDI-TOF mass spectrometry and database search. Protein score is -10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 64 are significant, p< 0.05.*

Figure 4.6 shows there is no obvious difference in the 20 S subunits when the proteasome was treated with 2 mM MPP^+ (panel B) or DA (panel C) in comparison to control (A). The change in position of the β5 subunit observed in the gels might be due to differences between sample batches since it occurred in both, controls and toxin-treated samples (see discussion section 4.3.2). Further analysis using Western blotting and immunoprobing with specific antibodies against the different proteasome core subunits showed that treatment with DA led to a loss of detection of β4 (CLA active site complementary subunit), β2 (TLA active site complementary subunit) and β6 (TLA active site complementary subunit; panels B, C and D, respectively). On the other hand, anti-core subunits antibody revealed that no change occurred in the PLA-associated subunits β1 and β7 (panel E). Finally, detection of the α-subunits 1, 2, 3 and 6 was also decreased after treatment of the proteasome with DA although no changes in α5/7 subunits were observed (panel F). MPP^+ did not appear to significantly change the pattern of any of the subunits in comparison to controls (panels A-F).

A) Control



B) 2 mM MPP^+



C) 2 mM DA

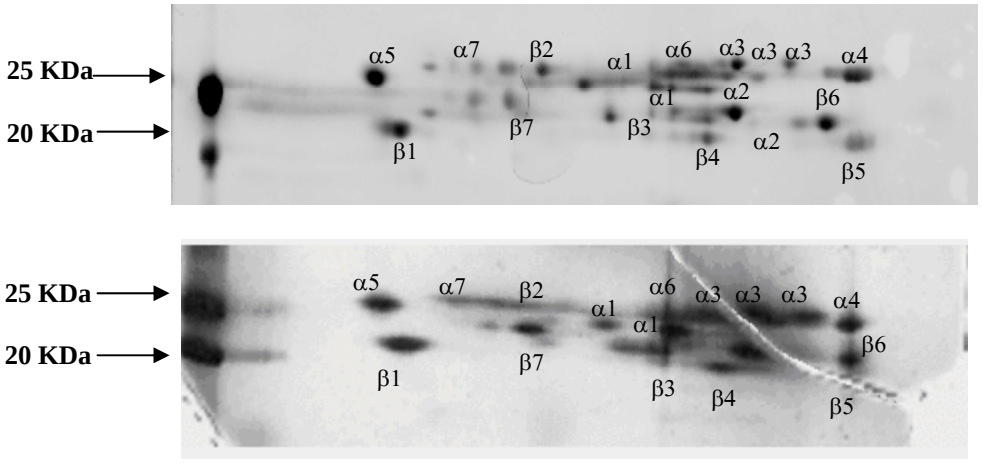
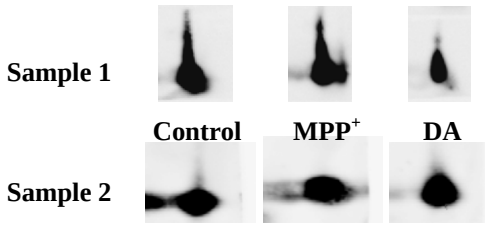
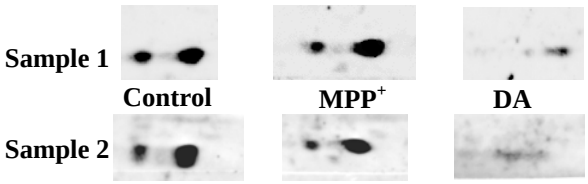


Figure 4.6. ***Two dimensional SDS-PAGE of 20S purified proteasome from human erythrocytes treated with 2 mM MPP$^+$ and DA.*** *2 μg of untreated (A), 2 mM MPP$^+$ treated (B) and DA treated (C) 20S proteasome were separated using a pH 3-10 non-linear IPG strip (1st dimension) followed by a 15 % SDS polyacrylamide gel electrophoresis (detailed in section 2.2.9.4). The 2D gel was silver stained as detailed in section 2.2.9.5. All labelled spots were identified by MALDI-TOF mass spectrometry and database search or by comparison with established reference maps of human 20S proteasome. The experiment was repeated three times obtaining similar spot patterns; samples presented in duplicates to demonstrate the reproducibility obtained.*

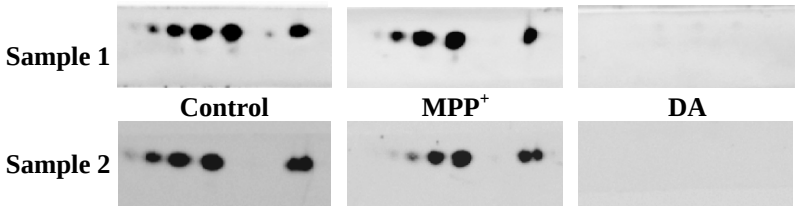
A) β5 SUBUNIT (CLA active site)



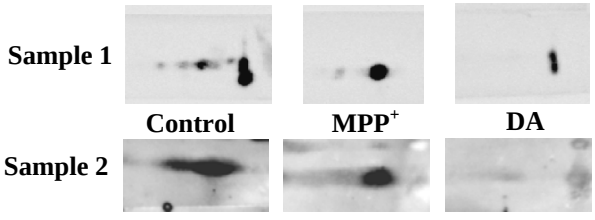
B) β4 SUBUNIT (CLA active site supplementary subunit)



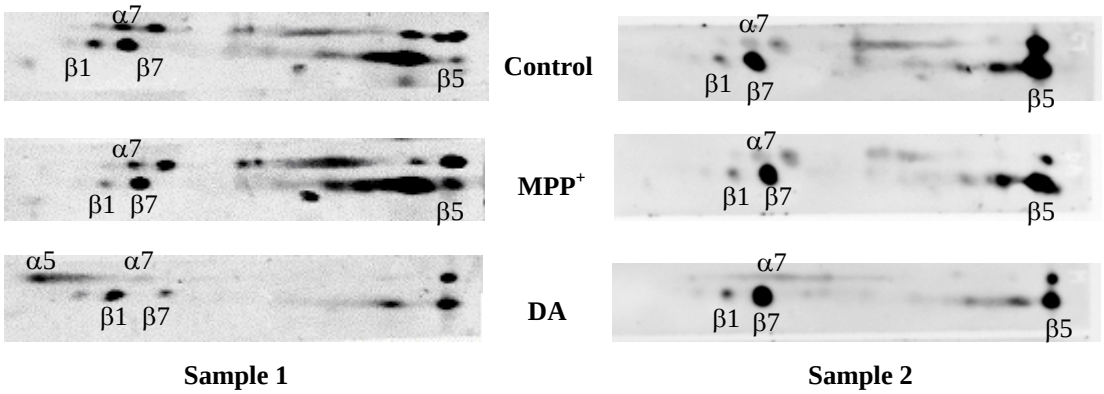
C) β2 SUBUNIT (TLA active site)



D) β6 SUBUNIT (TLA active site supplementary subunit)



E) CORE SUBUNITS α5/α7, β1, β5i, β5, β7



F) ALPHA SUBUNITS α1, 2, 3, 6, 5/7

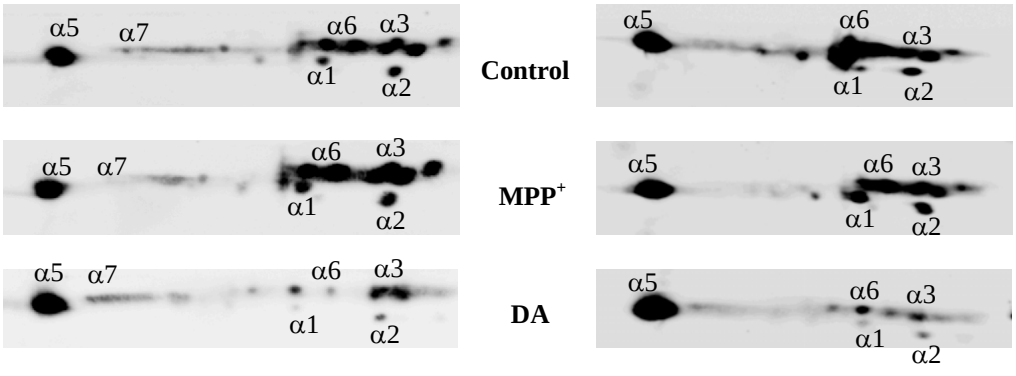


Figure 4.7. Western blot analysis of 20S core subunits after treatment with 2 mM MPP+ and DA. *2 μg of 20S proteasome (untreated or treated with 2 mM MPP+ /DA) were separated using a pH 3-10 non-linear IPG strip (1st dimension) followed by a 15 % SDS polyacrylamide gel electrophoresis (detailed in section 2.2.9.4). Then protein was transferred to nitrocellulose membrane by Western blotting and probed with antibodies against 20S subunits β5, β4, β2, β6, core subunits (α5/α7, β1,β5i, β5 β7) and alpha subunits (α1, 2, 3, 5, 6 and 7). For further information on the antibodies utilised in this section see Table 2.5 in section 2.2.10.2.2. Primary antibody binding was detected by ECL method as detailed in section 2.2.10.4. All labelled spots were identified by comparing antibodies staining with the theoretical molecular weight and pI from established reference maps of human 20S proteasome. The experiment was repeated three times obtaining similar spot patterns.*

4.3 DISCUSSION

4.3.1 MPTP/ MPP^+ and DA have a direct effect on 20S proteolytic activities

Table 4.5 shows a summary of the relative effects of MPP^+, MPTP and DA on the different 20S proteasomal activities with full statistical analysis. Data confirm that toxins can directly affect 20S activity. Overall, the relative sensitivities of the activities on the proteasome are as follows:

MPP^+: CLA > PLA > TLA

MPTP: PLA > CLA > TLA

DA: CLA > PLA > TLA

However, PLA was more sensitive to high concentrations of MPP^+ (ie. 0.5-2 mM) than CLA. Importantly, TLA appeared to be the least sensitive activity to the toxins. This is consistent with results in the last chapter (see section 3.2.2) which show a lower sensitivity of TLA activity in SH-SY5Y cells when treated with MPP^+ and DA over a period of 72 h. Indeed, TLA from purified 20S was unaffected by MPTP. Overall, toxins decreased proteasomal activity in a dose-dependent manner and DA appeared more damaging than MPP^+ or MPTP to the purified enzyme. In fact, the highest dose of DA tested ie.2 mM abolished the three proteasomal activities. This is not surprising since DA and its derivates from autooxidation are strong oxidants and it is well-known that proteins, and therefore enzymes, can easily be modified resulting in a loss of function (Szweda *et al.,* 2002; Grune *et al.,* 2003; Elkon *et al.,* 2004). In contrast, the effects of MPP^+ and DA on CLA and PLA were similar in the SH-SY5Y cell model and overall MPP^+ had a greater effect on these activities than DA, suggesting that the non-direct effects of MPP^+on the proteasome are very deleterious to the cells (see section 3.2.2).

To date, this is the first study showing that DA and MPP^+ added directly to the 20S proteasome can cause a reduction in proteasomal activity. Several studies refute direct effects of certain toxins on the proteasome. For instance, Höglinger *et al.* (2003) excluded a direct inhibition of the proteasome by MPP^+ and rotenone by measuring CLA in cell extracts exposed for 10 min to low doses of these toxins. However, their approach seems incomplete, since the high content of other proteins in the cell extract

may be quenching the effect of the toxins on the proteasome ie. the actual amount of free toxin in contact with the proteasome might be too low and also the exposure time of the lysate to the toxin was very short. This is also the case in very recent study, which reports that treatment of a human neuroblastoma cell line (containing a 26S reporter system) with several pesticides, including rotenone, reduced 26S proteasome but none of these pesticides result in direct impairment of 20S activity (Wang *et al.,* 2006). However, the authors again used cell lysates to monitor the direct effect of these pesticides. In addition to the fact that the pesticides were used at low concentrations (ie. 1-10μM), one could also argue that since cell lysates will contain 26S proteasomes, they might not truly be checking the direct effects on the 20S proteasome. Betarbet *et al.* (2006) also found that direct incubation of SK-N-MC cell lysates to 10 μM rotenone had no direct effect on proteasomal activity.

	Concentration (μM)	% CLA	% TLA	% PLA	t-Test CLA vs. TLA	t-Test CLA vs. PLA	t-Test TLA vs. PLA
MPP^+	**10**	83.4	92.9	88.0	0.1225	0.4138	0.5139
	100	71.4**	83.3	89.8	0.2014	0.0534	0.3497
	500	46.3**	69.9**	31.6**	**0.0022**	**0.0043**	**3.985E-05**
	2000	43.6**	57.6**	0.3**	0.0665	**2.039E-06**	**1.437E-05**
MPTP	**10**	88.9	97.9	65.2**	0.2205	**0.0201**	**0.0010**
	100	93.4	97.0	75.5**	0.6576	**0.0074**	**0.0314**
	500	66.1**	99.6	11.8**	**1.231E-05**	**1.01E-07**	**1.132E-09**
	2000	29.7**	89.4	0.0**	**3.690E-06**	**3.653E-07**	**3.085E-07**
DA	**10**	31.1**	92.2	65.2**	**2.299E-08**	**4.019E-05**	**0.0003**
	100	34.0**	79.4*	72.5**	**3.443E-07**	**0.0004**	0.3989
	500	13.2**	0.0**	0.0**	**1.214E-10**	**8.592E-11**	0.1091
	2000	0.3**	0.0**	0.0**	0.4199	1.0110	0.8661

Table 4.5. Comparison of the effects of toxins on the different proteasomal activities *from commercial 20S proteasome.* *Results of effects of MPP^+, MPTP and DA on CLA, TLA and PLA from 20S proteasome purified from human red blood cells. Reductions in activity in comparison to controls were considered statistically different to controls when * = $p<0.05$ or ** = $p< 0.01$ after analysis using a two-tailed t-test. Analyses of the differences in the rate values between the three proteasomal activities after toxic insult was also performed using a two-tailed t-test. Statistical values are presented in the table and values were considered significantly different when $p< 0.05$.*

4.3.1.1 Direct effect of the toxins on 20S proteasome is partly due to oxidative modification of the proteasome

It is plausible that the direct effect of the toxins on the 20S proteasome could be due to oxidative damage to the proteasome. This was confirmed by incubating the proteasome with the toxins in the presence of vitamin C, a well known antioxidant. Since vitamin C protected CLA from MPTP, MPP^+ and DA toxic insult it is hypothesised that the direct effects of the toxins on 20S proteasome is partly due to direct oxidative damage to the proteasome itself. Our findings support the study by Shamoto-Nagai *et al.* (2003), who found a decrease in proteasome activity in rotenone-treated cells together with oxidative modification of the proteasome itself and aggregation with other proteins. Nevertheless, this study did not look at the direct effect of rotenone on 20S activity *per se.* Moreover, Reinheckel *et al.* (1998) found that several oxidants that appear to play major roles in biological systems inhibited the activity of the 20S proteasome in a concentration-dependent manner. It is noteworthy that in our studies, low doses of vitamin C (10 μM) were enough to protect the proteasome from MPTP/MPP^+ toxicity whilst higher doses (ie. 100 μM) were required to protect the 20S from DA, indicating that DA is a stronger oxidant.

4.3.1.2 The proteasome is slightly more sensitive to DA than a general protease

As discussed before, proteins and therefore proteases are inheritently susceptible to oxidative damage but, is the proteasome more susceptible to toxic insult and oxidative damage than other proteases? Our results comparing proteasomal TLA with its counterpart, trypsin enzyme, indicates that although MPP^+ had a similar effect on both enzymes, the proteasome was more susceptible to DA insult than trypsin. Since DA is a stronger oxidant than MPP^+ and MPTP, it seems that the proteasome is more sensitive to oxidative damage than the trypsin enzyme. Studies also showed that vitamin C protected trypsin enzyme from DA toxicity. It is possible that either the β-subunits themselves, the α−subunits, or local factors surrounding the trypsin-like active centre of the 20S proteasomal core are more affected by oxidative damage than trypsin.

4.3.2 Structural modification of the 20 S α and β subunits by the toxins

Further analysis using 2D-SDS-PAGE fractionation and silver staining did not reveal major changes in the position of subunits following exposures to toxins *in vitro*. Some changes were found in the relative position of the β5 subunit, however this change also occurred in the control sample suggesting that different batches of 20S proteasome to be responsible (as stated in the data sheet of the product (code PW8729; purchased from Biomol International, Exeter, UK) batches will vary in their exact composition and activities).

The subunit mapping performed in our 2D gels was initially purely theoretical and based on previous data available regarding isoelectric points and molecular weights of the different 20S proteasomal subunits. However, the identity of some of the spots was confirmed by MALDI-TOF mass spectroscopy (ie. subunits β4-7, α1 and α3).

Western blotting analysis using specific antibodies against the different subunits of the core proteasome complex revealed that several α- and β-subunits were modified by DA in a way that the antibodies no longer recognised the epitopes of the proteins. Table 4.5 summarises the changes in immunoreactivity of the proteasomal subunits following toxin treatment. Treatment of the 20S proteasome with 2 mM DA caused a loss in the detection of the CLA-related subunit β4; in the TLA-related subunits β2 and β6 but not in the PLA subunits (β1 and β7). In contrast, MPP^+ caused no change in detection of any of the subunits. Thus loss of reactivity did not match loss of activity since all three activities were abolished by 2 mM DA and CLA and PLA were abolished by MPP^+. DA decreased the detection of α1, α2, α3 and α6, whilst no change occurred on α5 and α7. Since the α-subunits are thought to play an important role in maintaining the stability of the enzymatic complex (DeMartino and Slaughter, 1999; Ferrell *et al.*, 2000), modification of these subunits might also be responsible for the loss of activity of the 20S proteasome. On the other hand, no change in any α-subunit was observed after treatment of the proteasome with MPP^+.

The above data suggest that the approach used might either not be detecting more subtle changes which may be occurring in the subunits or, such changes occurred in epitopes that are not recognised by the antibodies,. Moreover, it was shown in section 4.2.2.2

	Subunit	DA	MPP^+
CLA active site	**β5**	No effect	No effect
CLA complementary subunit	**β4**	Loss	No effect
TLA active site	**β2**	Loss	No effect
TLA complementary subunit	**β6**	Loss	No effect
PLA active site	**β1**	No effect	No effect
PLA complementary subunit	**β7**	No effect	No effect
α-subunits (stability and scaffolding functions)	**α1**	Loss	No effect
	α2	Loss	No effect
	α3	Loss	No effect
	α5	No effect	No effect
	α6	Loss	No effect
	α7	No effect	No effect

Table 4.6. Summary of changes in immunoreactivity of 20S proteasomal subunits following toxin treatment revealed by immunoblotting.

CHAPTER V

EFFECTS OF PD MIMETICS ON NEUROFILAMENT TURNOVER IN SHSY-SY CELLS

5. EFFECTS OF PD MIMETICS ON NEUROFILAMENT TURNOVER

5.1 INTRODUCTION

5.1.1 Presence of NFs in LB

As indicated in the main introduction, LBs contain α- synuclein (Giasson *et al.,* 2000), ubiquitin and ubiquitinated proteins (Lowe *et al.,* 1988) and also misfolded tubulin (Galloway *et al.,* 1992), components of the UPS (e.g. proteasomal subunits, ubiquitination/ de-ubiquitination enzymes and proteasome activators; McNaught *et al.,* 2002a) and all three NF subunits (Forno *et al.,* 1986, Galloway *et al.,* 1988; Galvin *et al.,* 1997). Of the three NF proteins, NF-H and NF-M are most commonly associated with LBs (Pollanen *et al.,* 1993), with an abundance of the phosphorylated forms of these proteins (Forno *et al.,* 1986; Smith *et al.,* 1991).

Axonal transport is essential for normal neuronal function, therefore its impairment may be damaging to the cell. Indeed, blockage of axonal transport can lead to the development of aberrant inclusions within the cell body, typical of several neurodegenerative disorders such as dementia with Lewy bodies, AD or PD (Petzold, 2005). The presence of both cytoskeletal proteins and ubiquitin / ubiquitinated proteins within the LB suggests that impaired protein catabolism (ie. the UPS) might also be linked to the abnormal accumulation of these proteins in neurological diseases and in particular PD. Indeed, it has been found that normal segregation of highly phosphorylated NFs in axons is disrupted in some neurons in pathological states associated with perikaryal accumulation of neurofilaments (Hirokawa and Takeda, 1998; Nixon R.A. 1998; Yabe *et al.* 2001). Interestingly, phosphorylation of NFs promotes their dissociation from kinesin motors (Yabe and Shea, 2000).

A direct link between aberrations in the cytoskeleton and PD was provided by the identification of a point mutation in the gene coding for NF-M in a patient with early-onset severe PD (Lavedan *et al.,* 2002). However, the relevance of this mutation in PD is not clear since Kruger *et al.* (2003) reported that mutations in the NF-M gene may increase susceptibility to develop PD but do not play a major role in the disease.

5.1.2 Effect of neurotoxins on NF expression and post-translational modifications

As introduced earlier, aberrant cytoskeletal expression and altered post-translational modifications in cytoskeletal proteins are associated with neuropathological processes (discussed by D'Andrea *et al.,* 2001) linked with changes in cell morphology and possibly with the appearance of protein aggregates (reviewed by Xiao *et al.,* 2006). Most current *in vitro* studies investigating LB-like inclusions are focused on α-synuclein (Gomez-Santos *et al.,* 2002, 2003, 2005; Matsuzaki *et al.,* 2004) or parkin accumulation (Zhao *et al.,* 2003; Muquit *et al.,* 2004); however, very little data are available regarding aberrant NF accumulation and phosphorylation in *in vitro* PD models.

Nevertheless, several *in vitro* studies have reported that certain toxins can induce abnormal accumulation and phosphorylation of NFs. For example, treatment with acrylamide and 2,5-hexadione resulted in the accumulation of NFs in the perikarya of non-differentiated SH-SY5Y cells (Hartley *et al.,* 1997). Shea and his co-workers reported that aluminium treatment of neuronal cells led to extensive NF-H phosphorylation (Shea *et al.,* 1995) forming filamentous inclusions in the perikaryon of the cell (Shea *et al.,* 1997).

Of relevance to PD, Masaki *et al.* (2000) reported that proteasome inhibition with lactacystin over a period of 24 h resulted in an increase in phosphorylated NF-H and NF-M, which was associated with increased activities of the stress activated kinases JNK but no change in CDK-5 activity in PC12h cells. In addition, in our laboratory, De Girolamo *et al.* (2000) showed that treatment of differentiated mouse N2a neuroblastoma cells with subcytotoxic concentrations of MPTP (ie. 10 μM) led to increased levels of pNF-H; moreover immunofluorescence analyses revealed that pNF-H accumulate in the perikaryon suggesting that MPTP treatment altered NF-H distribution concomitant with a change in cell morphology. More recently, this has been reported to be associated with a transient increase in JNK activity in the presence of sustained ERK activity (De Girolamo and Billett, 2006). However, with high concentrations of MPTP (ie. 5 mM) JNK activation is sustained whilst ERK is inhibited (De Girolamo and Billett, 2006).

5.1.3 Degradation of NFs

Estimates of the half life of NFs vary between approximately 20 days (Nixon and Logvinenko, 1986) and 0.5-1.0 year (Lee and Cleveland, 1996). Their long life makes NFs more prone to a build up of damage by oxidative stress and possibly their accumulation in pathological states. Phosphorylation of NFs has been reported to decrease the susceptibility of NFs to degradation (Sternberg and Sternberg, 1983), perhaps due to the fact that pNFs are are poorer substrates for calpain (Pant *et al.,* 1988).

Indeed, calcium-activated neutral proteases (i.e. calpains) that locate along the axon are thought to be the proteolytic enzymes which primarily mediate NF breakdown, suggesting that calpains might play a crucial role in neurodegeneration (Kupina *et al.,* 2003; Kunz *et al.,* 2004). Both μ- and m-calapins are present in axons (Stys *et al.,* 2002; Kupina *et al.,* 2003) and are involved in NF degradation (Nixon, 1986). This is further supported by studies suggesting that NFs are degraded by calpains in axons undergoing Wallerian degeneration (discussed by Nixon and Logvinenko 1986).

Lysosomal degradation of NF proteins has also been reported. Indeed, cathepsin D has been shown to degrade NFs in rat, bovine and human tissue (Nixon and Marotta, 1984). Moreover, it has also been reported that NFs can be cleaved by trypsin and α-chymotrypsin (Chin *et al.,* 1983; Fasani *et al.,* 2004).

Finally, limited evidence also involves the UPS with NFs degradation. Gou and Leterrier (1995) showed that incubation of purified radiolabeled NFs with a soluble rat brain fraction containing the enzymes necessary for ubiquitin dependent degradation of proteins resulted in degradation of NFs when ATP and ubiquitin were added.

5.1.4 Aims of chapter

Since NFs are one of the major components of LBs and the activity of the UPS is reduced in PD, the first aim of this study was to determine whether NFs undergo proteasomal degradation, either under normal or under stressed conditions in human dopaminergic SH-SY5Y cells. The limited data available about the possible proteasomal degradation of NFs were achieved using rat brain homogenates (Gou and Leterrier, 1995) and no information is available in human tissues/cells.

The role of the proteasome in NF turnover was examined using the specific proteasome inhibitor lactacystin and Western blotting. In order to simplify the analysis new protein synthesis was inhibited by cycloheximide. The effects of artificially reducing glutathione levels to promote a more oxidative environment on NF turnover were also studied.

In previous chapters it is clear that complex I inhibition leads to proteasome dysfunction. Previous published work has indicated that (a) complex I inhibition alters NF phosphorylation and distribution in a manner that may be relevant for PD (De Girolamo *et al*, 2000; De Girolamo and Billett, 2006) and (b) proteasome inhibitors can also affect NF phosphorylation (Giasson and Mushynski, 1995; Masaki *et al.,* 2002). Thus the second aim was to study the effect of different concentrations of MPP^+ and also DA on NF levels and phosphorylation in my human cell model. Moreover, since calpain is reported to be the major protease involved in the degradation of NF proteins (Stys and Jiang, 2002; Kupina *et al.,* 2003; Kunz *et al.,* 2004) the effect of calpain inhibition on mitotic SH-SY5Y cells exposed to high doses of MPP^+ and DA was also studied.

Finally, since neurodegeneration is a chronic process and recent work with rotenone indicates that, following long-term exposure, insoluble inclusions of synuclein accumulate (Sherer e*t al.* 2001), it is of interest to study the effect of chronic exposure to low levels of DA and MPP^+ on NF profiles and post-translational modifications.

5.2 RESULTS

5.2.1 Neurofilaments can partly be degraded by the proteasome

Before this study could commence, the effect of treatment of cells with a proteasome inhibitor (lactacystin) on endogenous proteasomal CLA was investigated. Although recent data suggests that epoxomicin is the most specific proteasomal inhibitor (Kisselev and Goldberg, 2005) literature at the time the experiment was performed, suggested that lactacystin was the "gold standard" proteasome inhibitor for the purpose of the experiment (Fenteany *et al.;* 1995; Craiu *et al.;* 1997; Fenteany and Schreiber, 1998; David *et al.,* 2002).

Cells were grown in 6-well plates and treated with 1 and 2.5 μM lactacystin for 24h prior to extraction and measuring CLA (see sections 2.2.4.4 and 2.2.6.1). Lactacystin at 2.5 μM virtually abolished CLA and was thus used in subsequent experiments (see Table 5.1).

Lactacystin	% inhibition	SEM
1 μM	65 % **	4.235
2.5 μM	95 % **	4.881

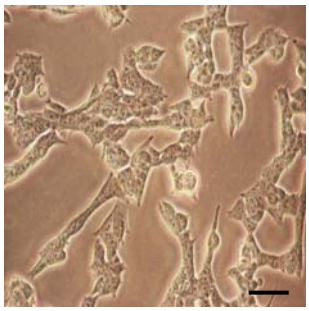
Table 5.1. Inhibitory effect of lactacystin on endogenous CLA in SH-SY5Y cells. *Cells were grown in 6-well plates and incubated with growth medium supplemented with/ without 1 and 2.5 μM lactacystin for 24 h prior to extraction and measuring CLA as described in methods sections 2.2.4.2 and 2.2.6.1. Results were calculated as ΔFU/s/μg and converted to mean % inhibition in comparison to control ± SEM. Statistical analysis was carried out using a two-tailed t-test and statistical significance accepted when ** = p<0.01 cf. controls (n=3).*

The effects of lactacystin and BSO on NF turnover were then investigated, using cycloheximide to inhibit new protein synthesis. Cells were grown in T-25 flasks to 80% confluence prior to treatment with fresh growth medium supplemented with 10 μg/ml cycloheximide, with or without 2.5 μM lactacystin and 2 mM BSO for 24 h, as described in Table 5.2. Cells were then extracted (see section 2.2.6.2) their protein content estimated (section 2.2.5.1.) and 30 μg protein subjected to SDS-PAGE electrophoresis and NFs examined by Western blot analysis (see sections 2.2.8 and 2.2.10).

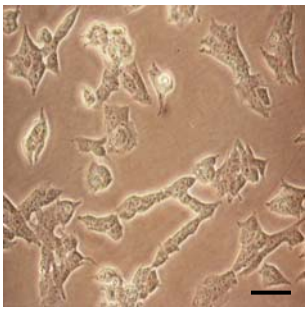
	TREATMENTS		
Sample name	**Cycloheximide 10 μg/ml**	**BSO 2 mM**	**Lactacystin 2.5 μM**
CONTROL	+	-	-
BSO	+	+	-
LACT	+	-	+
LACT + BSO	+	+	+

Table 5.2. ***Treatments of SH-SY5Y cells for analysis of NF degradation via the proteasome.*** *Cells were incubated for 24 h with growth media supplemented with different treatments as summarised above.*

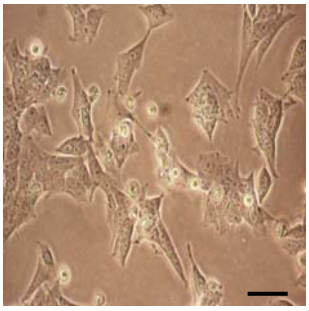
Figure 5.1 shows that BSO, lactacystin or a combination of both had little effect on cell morphology and cell growth, compared to the cycloheximide treatment alone after 24 h.



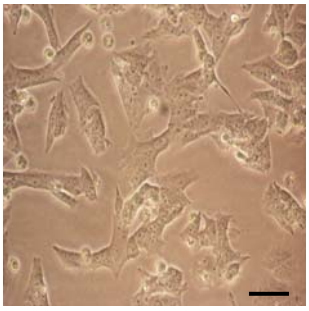
A) Cycloheximide 10 μg/ml



B) Cycloheximide 10 μg/ml + BSO 2mM



C) Cycloheximide 10 μg/ m l + Lactacystin 2.5 μM



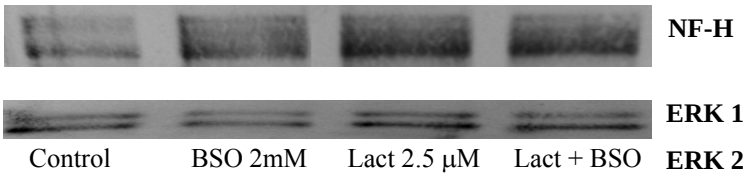
B) Cycloheximide 10 μg/ ml + Lactacystin + BSO 2mM

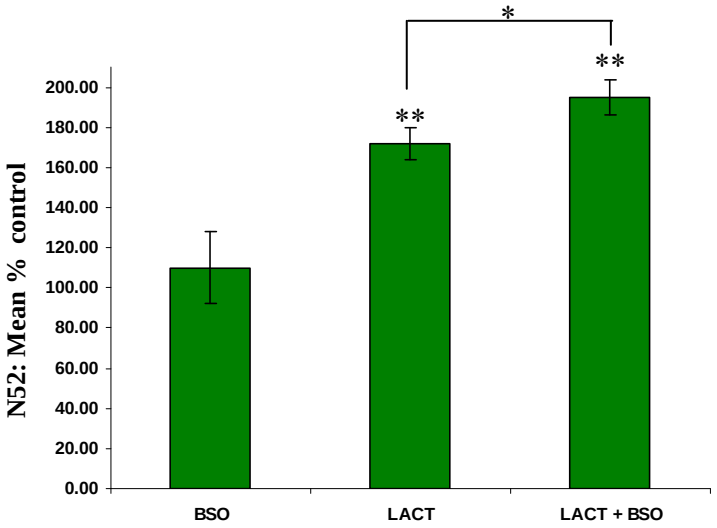
Figure 5.1. Morphological assessment of SH-SY5Y cells after 24 h treatment with cycloheximide (10 μg/ml), lactacystin (2.5 μM) and BSO (2mM). *Images were taken with a Nikon eclipse TS100 inverted microscope (x 400 magnification). Scale bar represents 20 μm.*

Total NF-H status was studied using the commercial neurofilament 200 monoclonal antibody (clone N52). It is said to be phosphorylation independent and therefore does not discriminate between phosphorylated and de-phosphorylated epitopes of NF-H. The N52 antibody gives rise to a doublet in which the two bands do not always separate. On the other hand, specific neurofilament phosphorylation was assessed using SMI 31, which detects hyperphosphorylated NF-H and NF-M epitopes (pNF-H and pNF-M, respectively).

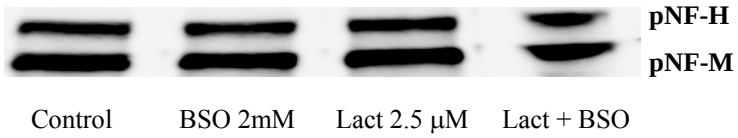
Proteasome inhibition by lactacystin led to an increase in total NF-H after 24 h; such accumulation was further increased when both lactacystin and BSO were added (Figure 5.2A). However, proteasome inhibition with lactacystin in the presence or absence of BSO did not increase phosphorylated NF-M and NF-H levels or change significantly the ratio between these two phosphorylated subunits. In mitotic SH-SY5Y cells the normal ratio of pNF-H to pNF-M in control cells averaged at 0.7 (panel B).

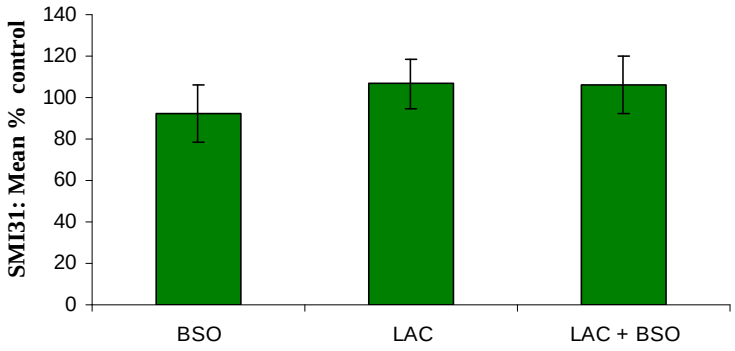
A) N52 and ERK





B) SMI 31





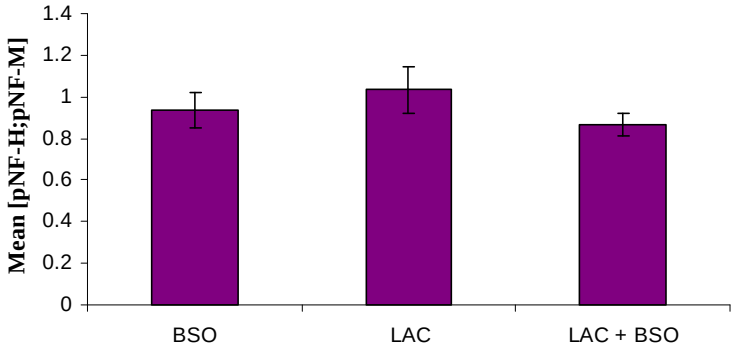


Figure 5.2. ***Western blot analysis of NF proteins in mitotic cells after 24 h exposure to proteasome inhibitors and/or BSO.*** *Cells were grown in T-25 flasks, treated as detailed in table 5.2 for 24h and extracted as detailed in section 2.2.4.1. Proteins were separated by SDS-PAGE, transferred to nitrocellulose by western blotting (see sections 2.2.8 and 2.2.10) and probed with N52 (1:500), SMI 31 (1:1000) and anti total ERK 1/ 2 (1:500). Primary antibody binding was detected by alkaline phosphatase(N52 and total ERK; see section 2.2.10.3) and ECL (SMI 31; see section 2.2.10.4, respectively) Bands were corrected against total ERK (N52 blot) or protein copper staining (SMI31 blot) for differences in protein loading and band intensity was quantified as detailed in section 2.2.10.6. Results are presented as mean % of controls ± SEM or ratio between pNF-H: pNF-M ± SEM. Statistical analyses were carried out using a two-tailed t-test and statistical significance accepted when * = p<0.05 cf. controls (n=3). Control samples were assigned an intensity value of 100 % for N52 or SMI 31 reactivity or 1 for the ratio of pNF-H: pNF-M.*

5.2.2 Changes in NF profiles following exposure to MPP^+

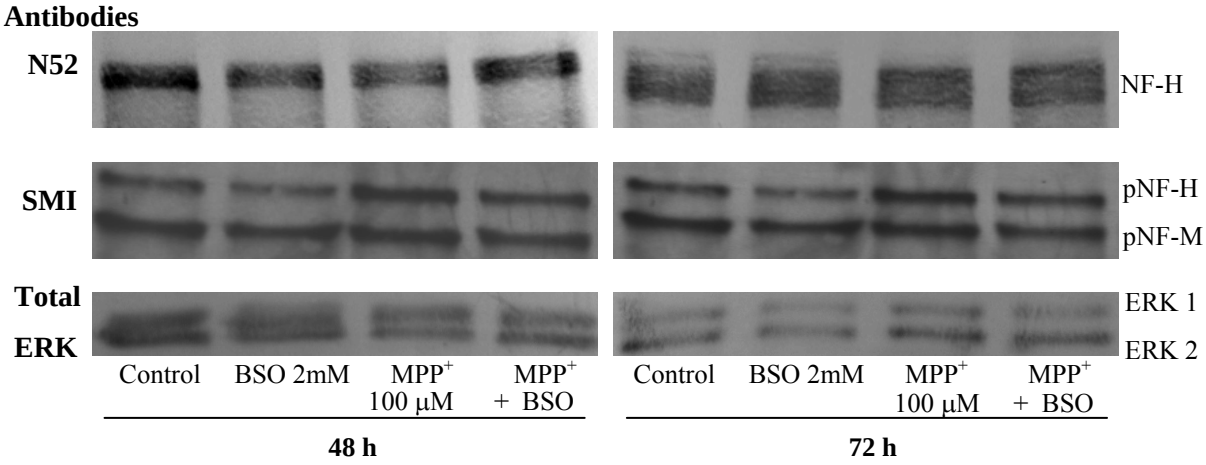
In this section, the effects of different concentrations of MPP^+ on NF profiles are analysed by Western blotting (see sections 2.2.8 and 2.2.10) and immunoprobing of the samples with antibodies against NF proteins (N52 and SMI 31, see sections 2.1.3.1 and 3.3.10.2). BSO was also used together with the toxin in order to mimic the glutathione depletion that occurs in PD. In these experiments cycloheximide was included in the growth medium.

Figure 5.3A shows that BSO, 100 μM MPP^+ or both together did not affect either total NF-H levels or total phosphorylated NF-H + NF-M levels in comprison to controls at any time tested (ie. 48 and 72 h). However, 100 μM MPP^+ increased the ratio of pNF-H: pNF-M in comparison to controls; the addition of BSO did not further increase this ratio in comparison to the MPP^+ treatment.

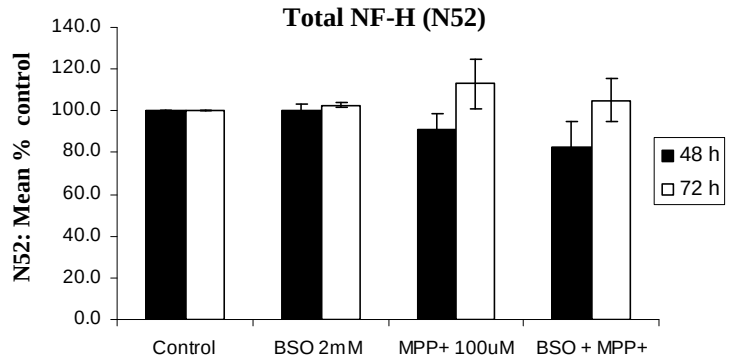
When MPP^+ was used at 500 μM (Figure 5.3B), in the presence or absence of BSO, there was no significant change in total NF-H in comparison to control over 72h. As before, BSO alone did not affect NF profiles. However, 500 μM MPP^+ with and without BSO increased the levels of total phosphorylated NF-H in comparison to controls after 72h exposure and also the ratio between pNF-H: pNF-M after 48h and 72h.

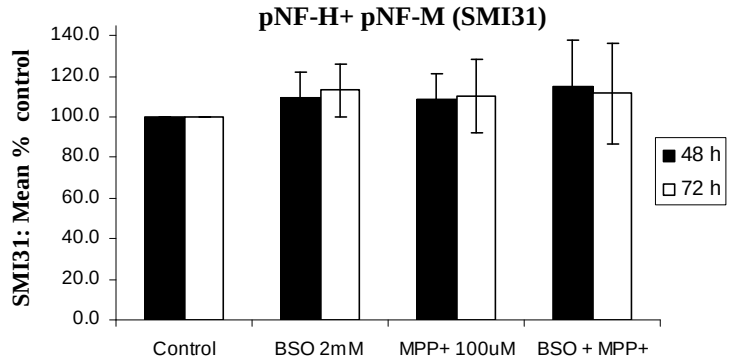
A) 100 μM MPP^+

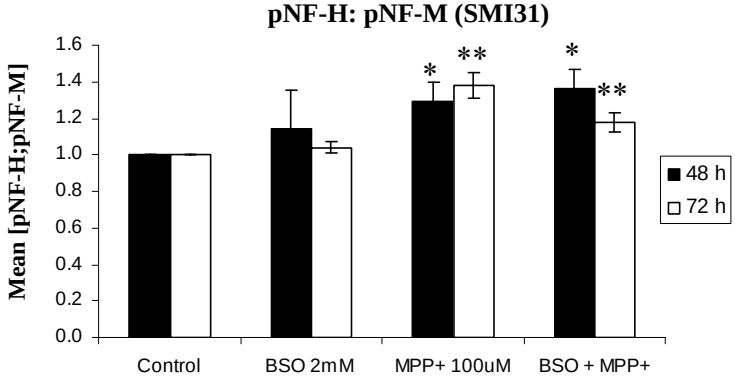
i) BLOTS



ii) QUANTIFICATION

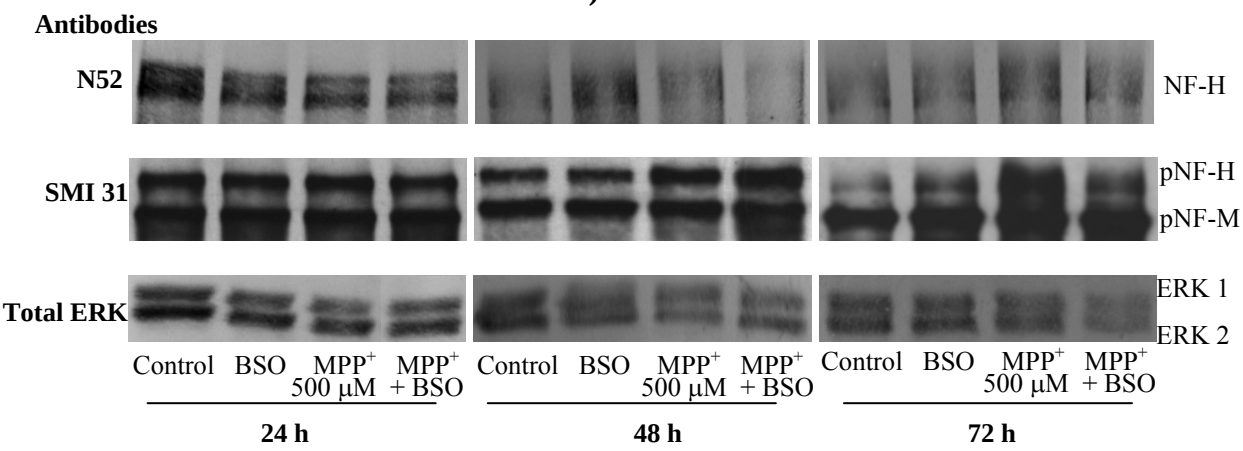






B) 500 μM MPP^+

ii) BLOTS



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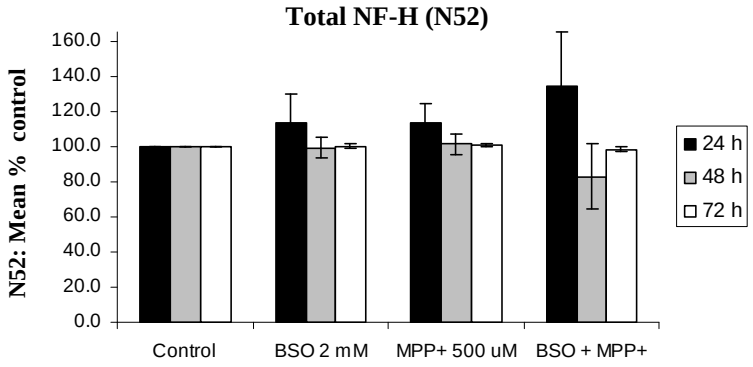
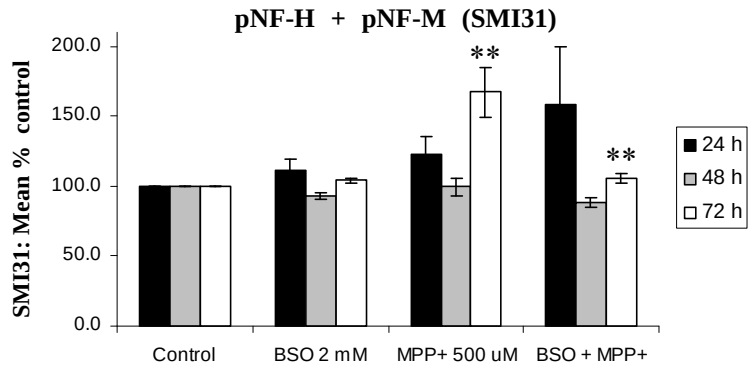




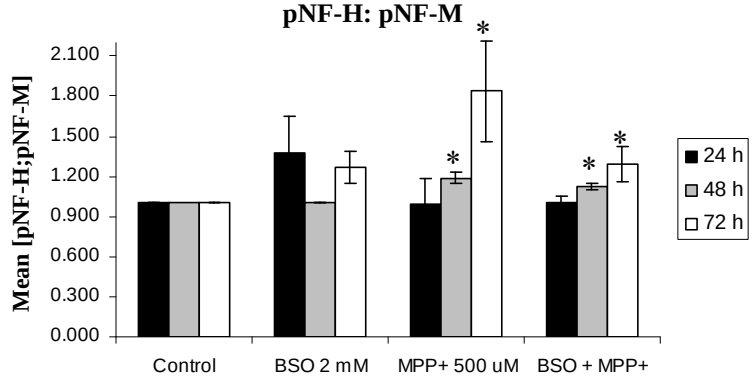


Figure 5.3. Changes in NF profiles after 24, 48 and 72 h exposure to 100 μM and 500 μM MPP$^+$ in SH-SY5Y cells. *Cells were grown in T-25 flasks, treated with cycloheximide (10 μg/ml; control) +/- 100 μM (panel A) or 500 μM (panel B) MPP$^+$ and/ or BSO for 24, 48 and 72 h. Proteins were extracted (section 2.2.4.1) and separated by SDS-PAGE, transferred to nitrocellulose by western blotting (see sections 2.2.8 and 2.2.10) and probed with N52 (1:500), SMI 31 (1:1000) and anti total ERK 1/ 2 (1:500). Primary antibody binding was detected by alkaline phosphatase (N52 and total ERK) and ECL (SMI31; section 2.2.10.3 and 2.2.10.4, respectively) and band intensity was quantified as detailed in section 2.2.10.6. Bands were corrected against total ERK for differences in protein loading. Results are presented as mean % of controls ± SEM or ratio between pNF-H: pNF-M ± SEM. Statistical analyses were carried out using a two-tailed t-test and statistical significance accepted when * = p<0.05 or ** = p< 0.01 cf. controls (n=3).Control samples were assigned an intensity value of 100 % for N52 or SMI31 reactivity or 1 for the ratio of pNF-H: pNF-M .*

5.2.3 Changes in NF profiles following exposure to MPP^+, DA and protease inhibitors

In this section, the effects of proteasome and calpain inhibitors on NF profiles treated with high doses of MPP^+ and DA were investigated. As before, cycloheximide was included in the growth medium.

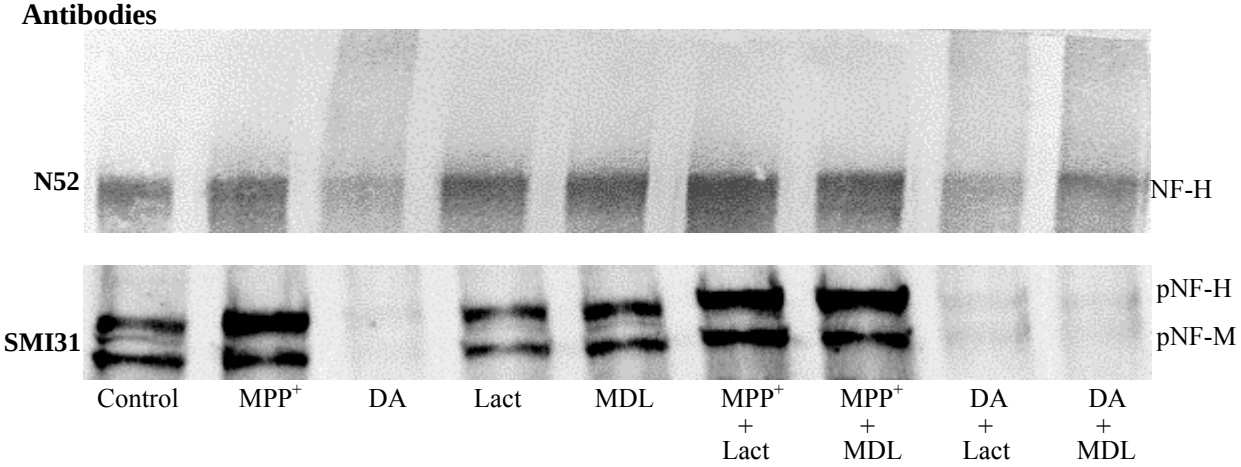
Figure 5.4 shows that inhibition with 2.5 μM lactacystin led to the accumulation of total NF-H, this time, after a 72 h treatment. As before, phosphorylated NF-M and NF-H did not accumulate with lactacystin and the ratio between pNF-H: pNF-M did not change either. Since it was not possible to optimise the fluorogenic assay for measuring calpain activity in our cell system, MDL 28,170 (10 μM) was utilised to inhibit endogenous calpain activity (based on Wang *et al.*, 1996; who used MDL 28,170 to inhibit calpain in SH-SY5Y cells). As for lactacystin, calpain inhibition with MDL 28,170 resulted in accumulation of total NF-H but not phosphorylated NF-M and NF-H.

Figure 5.4 also shows that 2 mM MPP^+ not only increased total levels of pNF-M + pNF-H but also the ratio between pNF-H: pNF-M as seen previously with 500 μM MPP^+. However, 2 mM MPP^+ also increased the levels of NF-H, contrary to the situation with 500 μM MPP^+. Treatment with 500 μM DA decreased both phosphorylated and total NF levels. Interestingly, with N52, a smear of NF-H can be seen up to the top of the gel in the DA lane, probably due to protein aggregation.

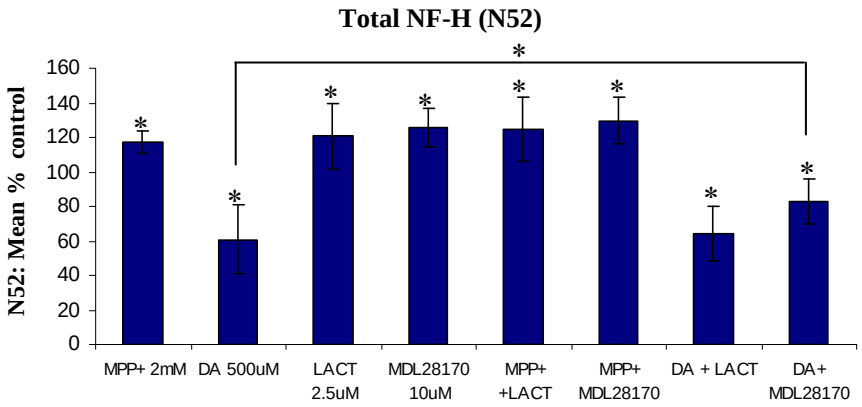
Addition of lactacystin or MDL 28,170 did not further increase total NF-H , [pNF-H + pNF-M] or the ratio between pNF-H:pNF-M in MPP^+ treated-cells. However, in DA-treated cells addition of MDL 28,170 increased total NF-H levels in comparison to the DA control.

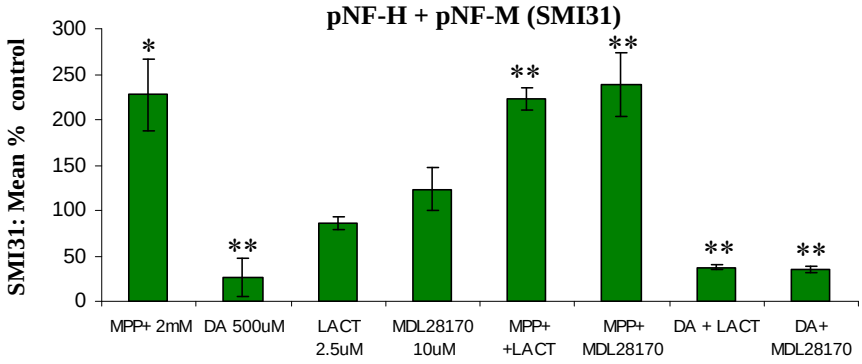
A) BLOTS

Antibodies



B) QUANTIFICATION





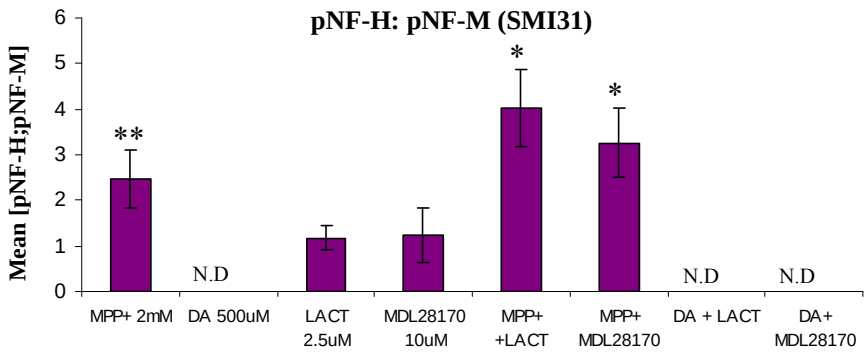


Figure 5.4. ***Effect of proteasome and calpain inhibition on NF profiles after toxic insult.*** *Cells were grown in T-25 flasks, treated with cycloheximide 10 μg/ml (control cells) and 2 mM MPP^+ or 500 μM DA with or without 2.5 μM lactacystin or 10 μM MDL 28,170 for 72 h and extracted as detailed in section 2.2.4.1. Proteins were separated by SDS-PAGE, transferred to nitrocellulose by western blotting (see sections 2.2.8 and 2.2.10) and probed with N52 (1:500) and SMI 31 (1:1000). Primary antibody binding was detected by alkaline phosphatase (N52) and ECL (SMI31; section 2.2.10.3 and 2.2.10.4, respectively) and band intensity was quantified as detailed in section 2.2.10.6. Bands were corrected against copper staining of the proteins for differences in protein. Results are presented as mean % of controls ± SEM or ratio between pNF-H: pNF-M ± SEM. Statistical analyses were carried out using a two-tailed t-test and statistical significance accepted when * = p<0.05 or ** = p< 0.01 cf. untreated or toxin-treated controls (n=3). Control samples were assigned an intensity value of 100 % for N52 or SMI31reactivity or 1 for the ratio of pNF-H: pNF-M . N.D= not detected*

5.2.4 Effects of 100 μM and 2 mM MPP^+ on NFs distribution

Immunocytochemistry was performed on cells treated with MPP^+ for 72 h. Cells were fixed in methanol and then permeabilised using triton X-100 as detailed in section 2.2.12. The distribution of phosphorylated NF-H and NF-M was visualised by probing with SMI 31.

Before visualising the NF proteins on the confocal microscope (section 2.2.11), cell morphology was studied using a phase contrast microscope. As Figure 5.5A shows, there were no gross changes in morphology between control cells (panel A, top) and cells remaining following MPP^+ treatment (panel B middle and bottom).

Immunocytochemical analysis of cells stained with SMI31 revealed that phosphorylated NF-M and NF-H were evenly distributed in both the cell body and axon-like processes (Figure 5.5, top right panel). However, treatment of the cells with either 100 μM or 2 mM MPP^+ resulted in a greater staining in the cell body compared to axon-like processes and concentration around the nucleus (Figure 5.5B middle and bottom right panel, yellow stain indicates NFs and nuclei to be closely associated).

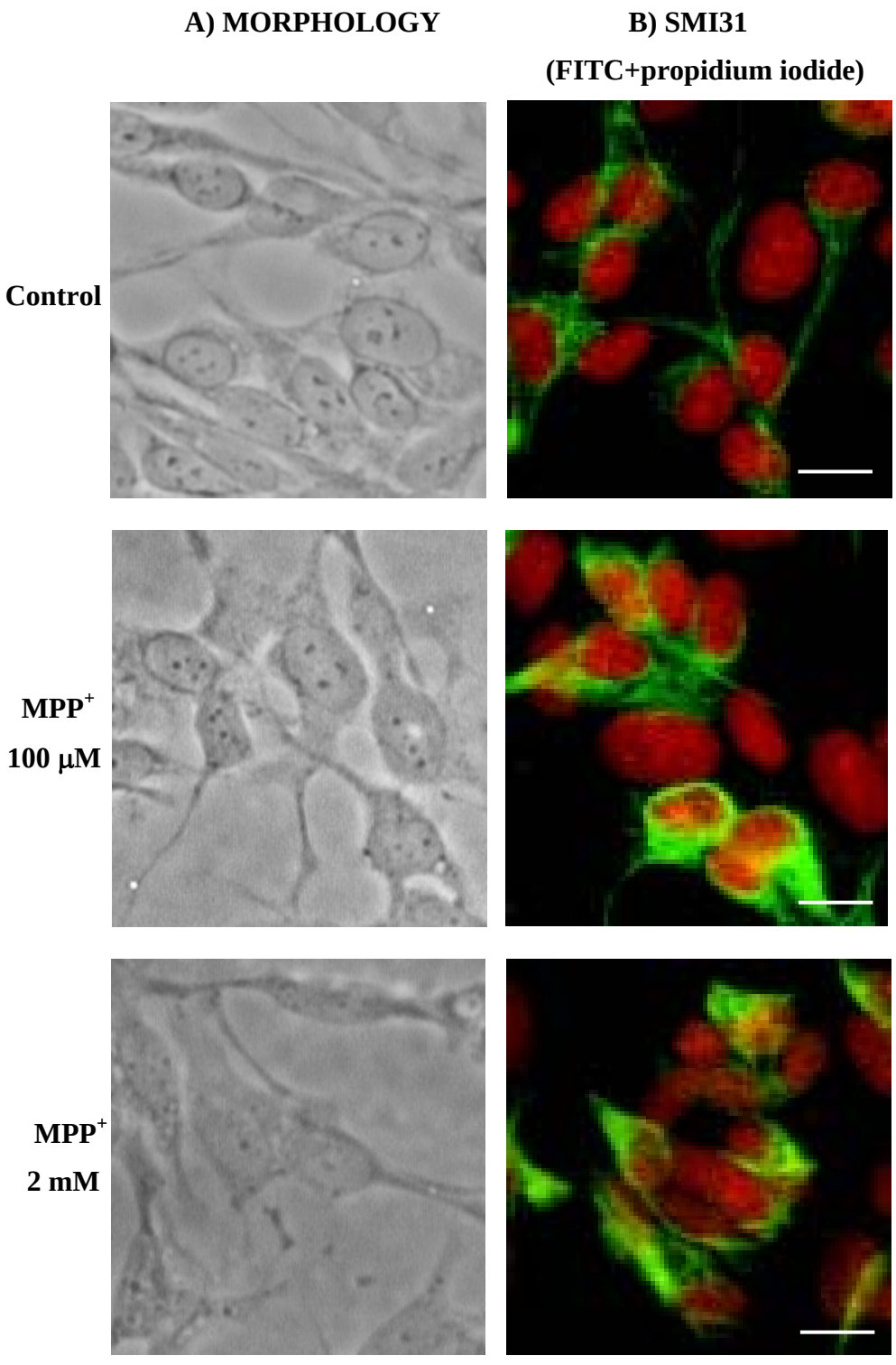

A) MORPHOLOGY
B) SMI31
(FITC+propidium iodide)
Control
MPP+
100 μM
MPP+
2 mM

Figure 5.5. Immunocytochemical analysis of NF proteins in SH-SY5Y cells following 72 h treatment with MPP^+. *Figure 5.5A represents cells visualised using phase contrast microscopy whilst Figure 5.5B represents confocal laser microscopy analysis. Cells were grown and treated with 100 μM or 2 mM MPP^+ for 72 h in permanox 8-well chamber slides at a density of 10,000 cells per well. Following treatment cells were fixed, permeabilised and immunocytochemistry performed as detailed in section 2.2.12 using SMI 31 (1:500). Scale bar represents 10 μm.*

5.2.5 Changes in NF proteins in SH-SY5Y cells following chronic exposure to MPP^+ and DA

In the previous section the effects of short exposure to mildly and highly cytotoxic concentrations of MPP^+ and DA on NF proteins profiles were investigated.

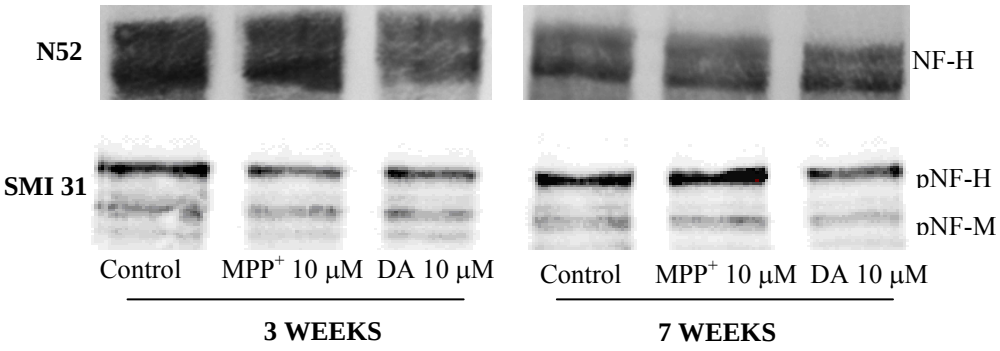
In this section, the effects of chronic exposure (3 and 7 weeks) to low concentrations (10 μM) of MPP^+ and DA on the expression and post-translational modification of NF proteins are analysed. This may provide a greater resemblance to neurodegeneration *in-vivo*. Western blotting/ immunoprobing and immunocytochemical techniques were employed to provide both quantitative analysis of protein expression and distributional changes in proteins.

5.2.5.1 Western blotting analysis of NF profiles after 3 and 7 weeks exposure to MPP^+ and DA

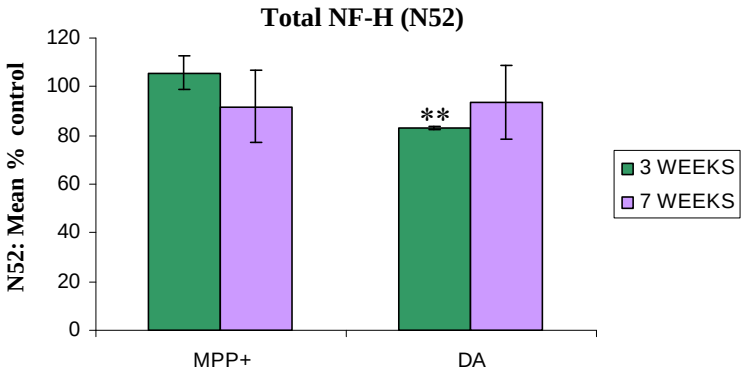
The results obtained were different from those reported earlier with shorter exposure times.

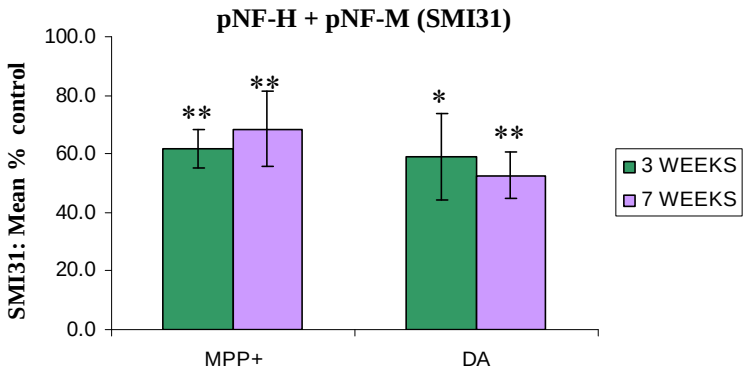
Figure 5.6 shows no significant change in total NF-H levels (N52) following exposure to 10 μM MPP^+ over 7 weeks, whilst DA led to a transient decrease (~17 % in comparison to control) in total NF-H levels (N52) after 3 weeks. On the other hand, both toxins led to a significant decrease in pNF-M + pNF-H (SMI 31). MPP^+ decreased total pNF-M + pNF-H to 62 % and 68 % in comparison to control after 3 and 7 weeks respectively. Similarly, DA reduced total NF-H to 59 % and 52 % after 3 and 7 weeks, respectively. Finally, no change in the ratio of pNF-H: pNF-M was observed after 3 or 7 weeks treatment with either toxin. The observed ratio between pNF-H and pNF-M was 1.4 and 2.3 for 3 and 7 week control cells, respectively.

A) BLOTS



A) QUANTIFICATION





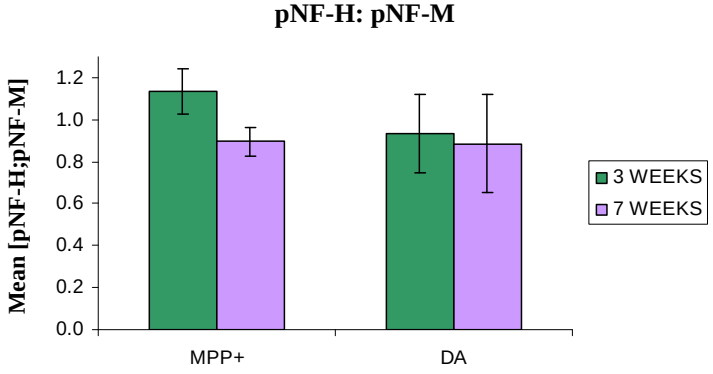


Figure 5.6. Effect of chronic exposure to MPP^+ and DA on NF turnover in SH-SY5Y cells. *Cells were grown in T-25 flasks, treated with 10 μM MPP^+ and DA for 3 and 7 weeks and extracted as detailed in section 2.2.4.1. Proteins were separated by SDS-PAGE, transferred to nitrocellulose by western blotting (see sections 2.28 and 2.2.10) and probed with N52 (1:500) and SMI 31 (1:1000). Primary antibody binding was detected by alkaline phosphatase (N52) and ECL (SMI31; section 2.2.10.3 and 2.2.10.4, respectively) and band intensity was quantified as detailed in section 2.2.10.6. Bands were corrected against copper staining of the proteins for differences in protein. Results are presented as mean % of controls ± SEM or ratio between pNF-H: pNF-M ± SEM. Statistical analyses were carried out using a two-tailed t-test and statistical significance accepted when * = $p<0.05$ or ** = $p< 0.01$ cf. controls (n=3). Control samples were assigned an intensity value of 100 % for N52 or SMI31reactivity or 1 for the ratio of pNF-H: pNF-M.*

5.2.6 Immunocytochemical analyses of NF proteins after 3 and 7 weeks exposure to MPP^+ and DA

Immunocytochemistry was performed on control, MPP^+- and DA- treated cells (7 weeks treatment) fixed in methanol and then permeabilised using triton X-100 as detailed in section 2.2.12. The distribution of phosphorylated NF-H and NF-M was visualised by probing cells with SMI 31.

Figure 5.7A and B represent cells visualised using phase contrast microscopy and confocal laser analysis, respectively. There were no significant changes in morphology between control cells (Figure 5.7A top panel) and cells treated with MPP^+ (Figure 5.7A panels middle and bottom), all containing some axon-like structures.

Immunocytochemical analysis of cells showed that distribution of pNF-M and pNF-H were uniform in both the cell body and axon-like structures in control cells (Figure 5.7B top panel), whilst in cells treated with 10 μM MPP^+ and DA, pNF-M and pNF-H accumulated in the cell body and there was virtually no staining within the axon-like processes (Figure 5.7B middle and bottom panels).

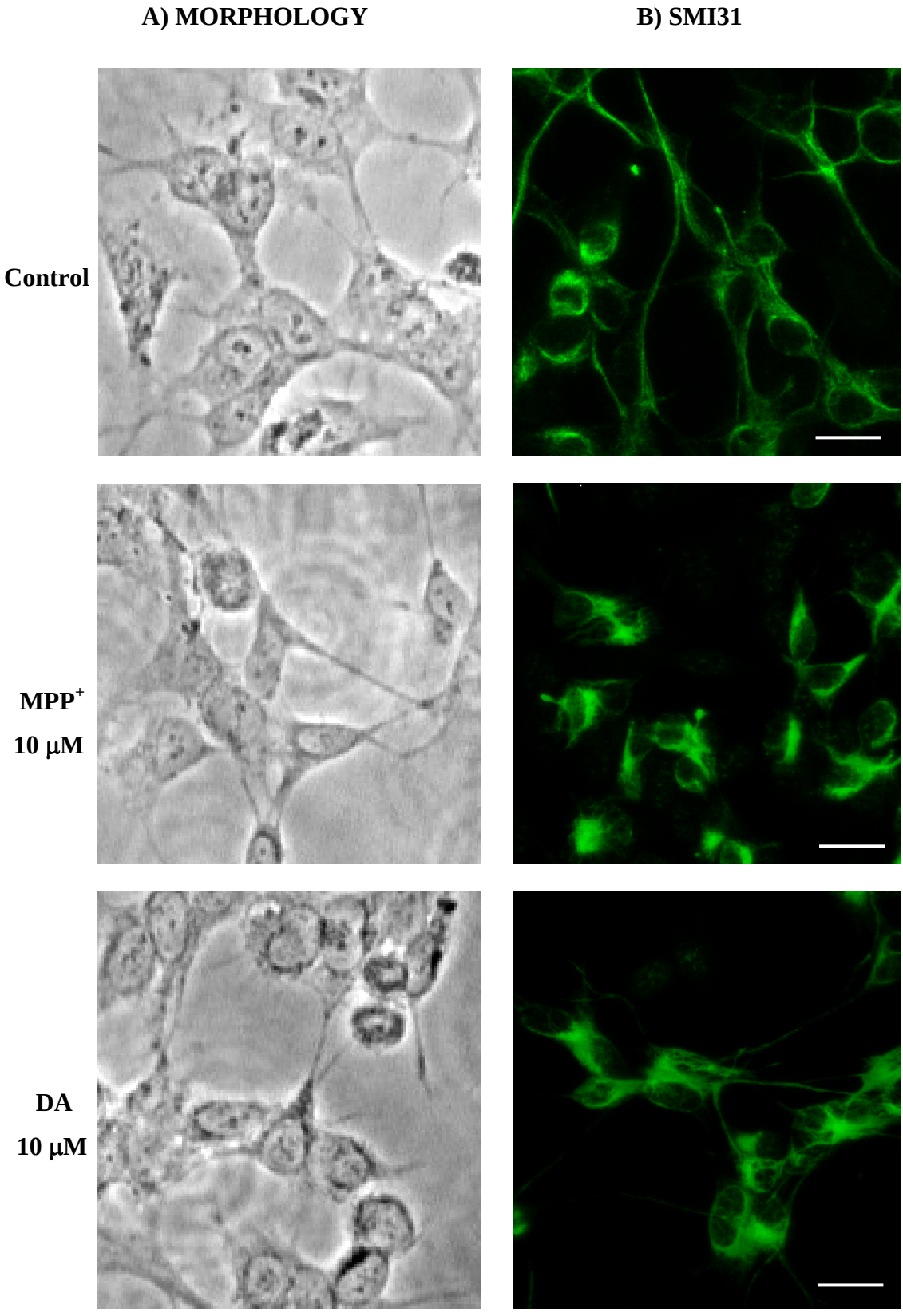

A) MORPHOLOGY
B) SMI31
Control
MPP+
10 μM
DA
10 μM

Figure 5.7. ***Immunocytochemical analysis of NF proteins in SH-SY5Y cells following chronic treatment with MPP$^+$ and DA.*** *In fig 5.8A mitotic cells were visualised using phase contrast microscopy. Fig 5.8B represents confocal laser microscopy analysis of mitotic cells. SH-SY5Y cells were grown in T-25 flasks and treated for 7 weeks with 10 μM MPP$^+$ and DA and then transferred to permanox 8-well chamber slides at a density of 10,000 cells per well. After overnight recovery cells were fixed, permeabilised and immunocytochemistry performed as detailed in section 2.2.12 using SMI 31 (1:500). Scale bar represents 10 μM.*

5.3 DISCUSSION

5.3.1 NFs can partly be degraded by the proteasome

In this study we report that inhibition of proteasomal activity in human dopaminergic SH-SY5Y cells led to an accumulation of total NF-H levels suggesting that NF can be degraded via the proteasome. Furthermore, depletion of glutathione, an event which is been widely reported to occur within the dopaminergic neurons of PD patients, led to further accumulation of total NF-H levels after proteasome inhibition. Thus, gluthatione depletion might generate high levels of ROS within the cells which in turn may be modifying NFs making them better substrates for the proteasome. In fact, it has been reported that the proteasome complex is responsible for the selective degradation of modified cytoplasmic, nuclear and endoplasmic reticulum proteins (Davies 2001) and that oxidised non-ubiquitinated proteins might be degraded by the 20S proteasome (Grune *et al.,* 2003). Thus my data would support the involvement of the UPS in the degradation of NFs, in agreement with Gou and Leterrier (1995). Figure 5.8 is an attempted overview of the effects of proteasome inhibition on the NF system in SH-SY5Y cells.

Phosphorylated NF-M and NF-H (pNF-M and pNF-H, respectively) did not accumulate after proteasome inhibition with lactacystin nor when glutathione levels were depleted, suggesting that these phosphoepitopes are not degraded via the proteasome, at least in control cells. Previous reports indicated that the main proteolytic pathway involved in the degradation of NF proteins to be calpains (Schlaepfer *et al.,* 1985; Ray *et al.,* 2000; Stys and Jiang, 2002). However, in my system the calpain inhibitor MDL 28,170 had no effect on phosphorylation, suggesting that the phosphoepitopes are not degraded via the calpain system either. Indeed, it has been reported that calpains degrade phosphorylated NFs at slower rates than dephosphorylated NFs (Pant *et al.,* 1988). Nevertheless my results show an increase in total NF-H when calpain activity was inhibited with MDL 28,170. This was expected since it is well-documented that NFs undergo calpain degradation *in vivo* (Schlaepfer *et al.,* 1985; Ray *et al.,* 2000; Stys and Jiang, 2002; Kunz *et al.,* 2004).

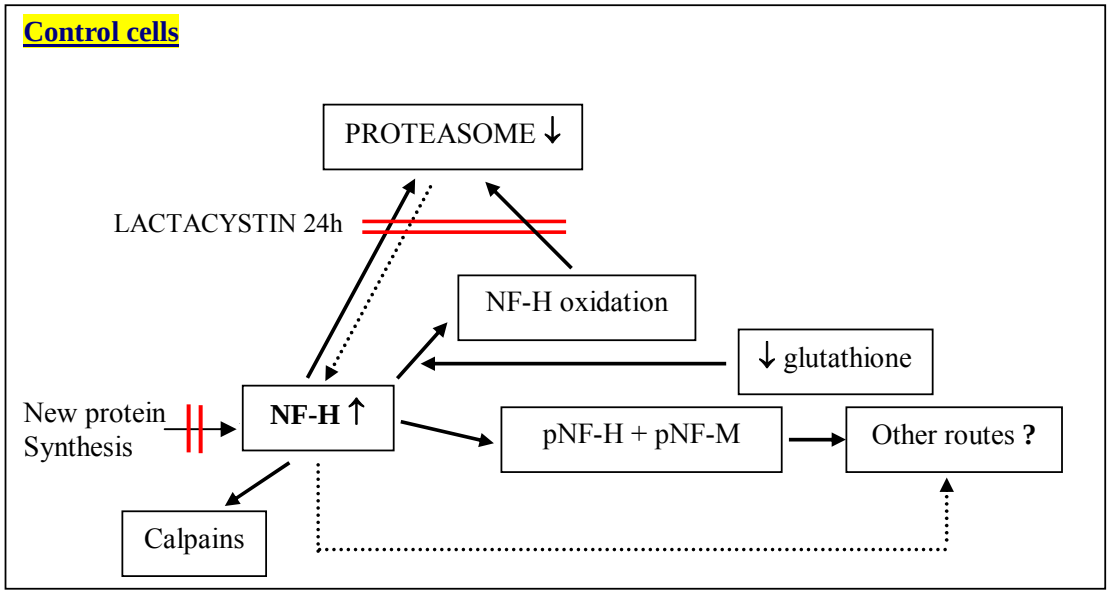


Figure 5.8. Overview of the effects of proteasome and calpain inhibition on the NF system in control SH-SY5Y cells.

5.3.2 Changes in NF profiles after treatment with toxins

Table 5.3 summarises the effects of toxins and/or protease inhibition on NF profiles.The main finding in this section is an increase in the ratio between pNF-H and pNF-M after treatment with 100-500 µM MPP^+ by 48 h. Generally, total NF-H levels and phosphorylated NF-M and NF-H levels did not increase with these concentrations except for the 72 h-treatment with 500 µM MPP^+ which led to a 60 % increased in pNF-M and pNF-H. The increase in the ratio of pNF-H: pNF-M was mainly due to an increase in pNF-H, with NF-M levels being maintained. These changes may be a result of activation of stress kinases known to occur in response to both complex I inhibition (De Girolamo and Billett, 2006) and proteasome inhibition (Masaki *et al.,* 2000). The findings that NF phosphorylation and the ratio between pNF-H and NF-M increased with doses ≥ 500 µM MPP^+ suggest that the relative phosphorylated state of these subunits is important in MPP^+ toxicity (see Figure 5.9). Indeed, several toxins have been reported to induce aberrant phosporylation of NFs *in vivo* (in LB; Forno *et al.,* 1986) and *in vitro* (De Girolamo *et al.,* 2000; Hartley *et al.,* 1997). For example, increased NF-H phosphorylation and NF accumulation has been reported in SH-SH5Y cells following treatment with acrylamide or 2,5-hexanedione (Hartley *et al.,* 1997) or in neuronal cells after treatment with aluminium (Shea *et al.,* 1995).

TIME	TREATMENT	NF-H	pNF-H + pNF-M	[pNF-H:pNF-M]	Conclusion
48h	**100μM MPP^+**	=	=	↑ (30%)	100μM MPP^+ increases ratio of pNF-H: pNF-M; no additional effect with BSO
	100μM MPP^+ + BSO	=	=	↑ (40%)	
72 h	**100μM MPP+**	=	=	↑ (42%)	
	100μM MPP^+ + BSO	=	=	↑ (20%)	
24 h	**500μM MPP^+**	=	=	=	500μM MPP^+ increases [pNF-H:p NF-M] by 48 h; no additional effect with BSO. It also leads to an increase in phosphorylation of NF-H + NF-M after 72 h.
	500μM MPP^+ + BSO	=	=	=	
48 h	**500μM MPP^+**	=	=	↑ (20%)	
	500μM MPP^+ + BSO	=	=	↑ (15%)	
72 h	**500μM MPP^+**	=	↑ (60%)	↑ (80%)	
	500μM MPP^+ + BSO	=	=	↑ (35%)	
72 h	**2 mM MPP^+**	↑ (18%)	↑ (127%)	↑ (246%)	2mM MPP^+ increased total NF-H levels, pNF-H + pNF-M and [pNF-H: pNF-M]; no additional effect by the addition of inhibitors; proteasome activity might be involved in NF degradation after MPP+ treatment but not calpains. With DA, increased NF-H with calpain inhibition, thus calpain is involved in degrading NF-H
	LACT + 2mM MPP^+	↑ (21%)	↑ (123%)	↑ (400%)	
	LACT	↑ (19%)	=	=	
	MDL 28,170	↑ (22%)	=	=	
	MDL 28,170 + 2 mM MPP^+	↑ (30%)	↑ (138%)	↑ (325%)	
	DA 500 μM	↓ (40%)	↓ (75%)	N.D	
	DA 500 μM + LACT	↓ (35%)	↓ (63%)	N.D	
	DA 500 μM + MDL 28,170	↓ (15%) ↑ca DA	↓ (65%)	N.D	

Table 5.3. Summary of effects of toxins and/or protease inhibitors on NF profiles. *Accumulation or decrease in NF are symbolised as ↑ or ↓ respectively with the % change indicated in brackets. No change in NFs is symbolised as =. N.D refers to not determined.*

Furthermore, in this study, treatment of mitotic SH-SY5Y cells with 2mM MPP^+ also increased total NF-H levels after 72 h, this increase might be due to impaired proteolysis of NFs via the proteasome since (a) NF-H has been shown to undergo proteasomal degradation (see section 5.2.1) and (b) 2 mM MPP^+ has been shown to reduce proteasome activity by 48 h (46 % reduction ca. controls; see section 3.2.4). Although it was found that phosphorylated NF-H and NF-M might not undergo proteasomal degradation in control cells, an increase in these phosphorylated epitopes was evident after 72 h treatment with both 500 μM and 2 mM MPP^+. Thus it is hypothesised that MPP^+ leads to the aberrant phosphorylation of NFs, as it has been previously reported (De Girolamo *et al.,* 2000), and that aberrantly phosphorylated NFs may undergo proteasomal degradation. Alternatively, these epitopes are degraded via another protease system whose activity is also reduced following MPP^+ treatment (see Figure 5.9A). Neither proteasome nor calpain inhibition of cells treated with 2 mM did not further accumulate NFs in comparison to MPP^+ treatment on its own. This is not surprising with the proteasome inhibition (since proteasome activity has been shown to be virtually abolished with lactacystin by 24 h) but the lack of effect with the calpain inhibitor suggest that calpain is not actively degrading NFs in cells treated with MPP^+.

Following MPP^+ treatment (100 μM and 2 mM) pNF-M and pNF-H accumulated in the cell, predominantly in the perinuclear site. Since Western blot analysis after 72 h treatment with 100 μM MPP^+, revealed no change in total pNF-H + pNF-M, changes in the distribution of phosphorylated NF are very subtle. My findings are consistent with Beck (2004) who also found that phosphorylated NF-H and NF-M were located within the cell body in differentiated SH-SY5Y cells following 24 h exposure to 5 mM doses of MPP^+. Indeed, NF phosphorylation is linked to the slowing of NF axonal transport and also promotes their dissociation from kinesin motors (Yabe and Shea, 2000). This is of interest since it has been found that normal segregation of highly phosphorylated NFs in axons is disrupted in some neurons in pathological states associated with perikaryal accumulation of neurofilaments (Hirokawa and Takeda, 1998; Nixon R.A. 1998; Yabe *et al.* 2001).

Finally, the effects of 500 μM DA on the expression and post-translational modifications of NFs were also studied. Results show that DA led to a decrease in phosphorylated and dephosphorylated NFs. Proteasome inhibition with lactacystin did

not change levels of total NF-H and [pNF-H + pNF-M] in DA treated cells. However, when calpain was inhibited an increase in total NF-H levels was observed in cells treated with DA in comparison to DA control. This suggests that calpains may be activated by DA and thus make NF-H a preferable substrate for calpain. This hypothesis is supported by the finding that that oxidative stress and certain toxins can increase intracellular calcium levels thereby increasing calpain activity in cultured cells (Lee M.S. *et al.*, 2000). In section 3.2.5.2, it was shown that 100 µM DA increased ROS levels in SH-SY5Y cells to a great extent, thus it is likely that DA would activate calpains via ROS production. Since 2 mM MPP^+ raised ROS levels to a much lesser extent than DA, it is hypothesised that MPP^+ might not activate calpains as much as DA and therefore NFs in MPP^+-treated cells might preferably undergo proteasomal degradation.

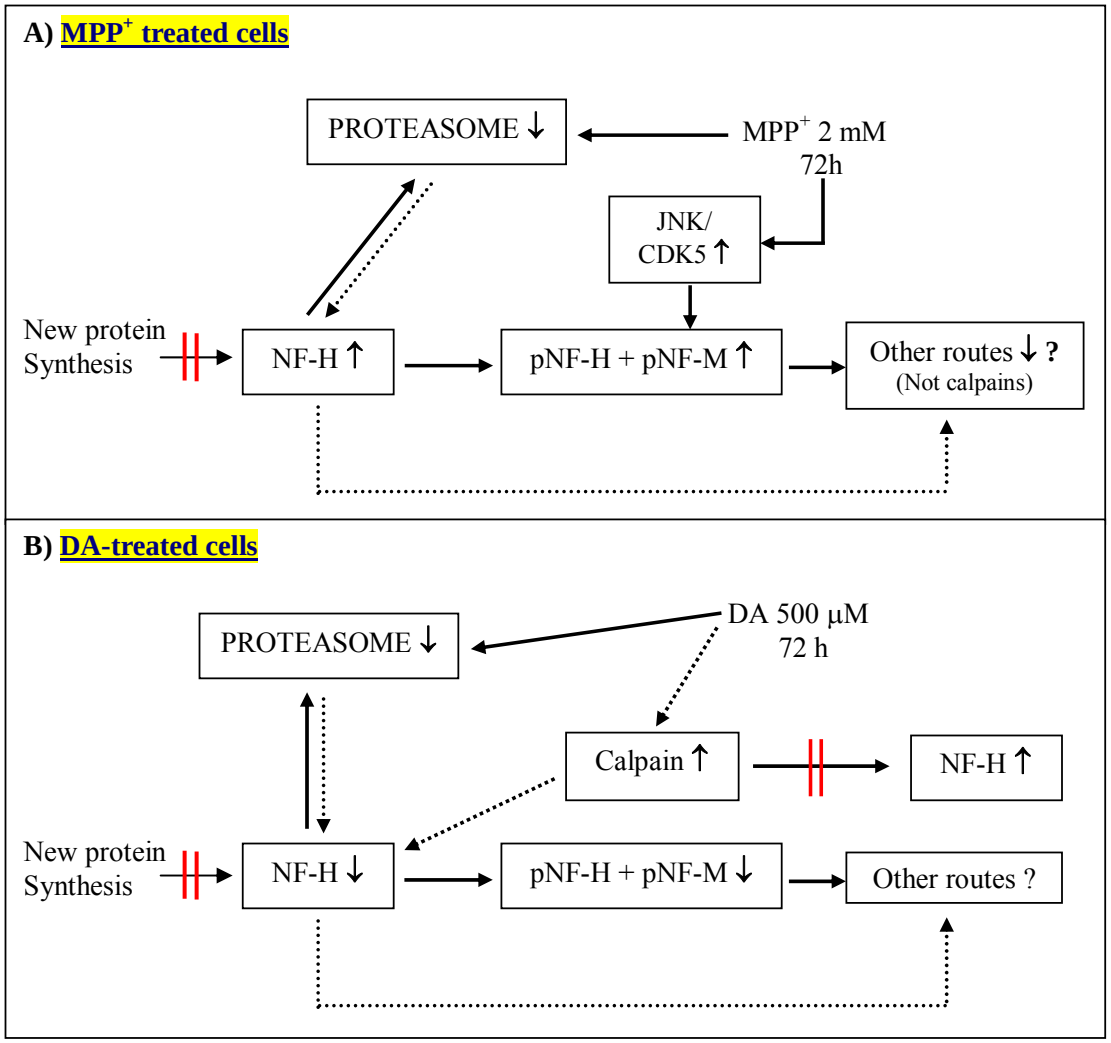


Figure 5.9. Overview the effects of high levels of MPP^+ (A) and DA (B) on the NF system.

5.3.3 Effect of chronic exposure to low levels of MPP^+ and DA on NFs levels and distribution

Finally, since PD is a chronic disorder, the effect of chronic exposure to 10 μM MPP^+ and DA was also estudied. Table 5.4 shows a summary of the effects of chronic treatment with the toxins on NF levels and phosphorylation linked to the effects of these treatments on proteasome activity (data from section 3.3.4).

Total levels of NF-H were transiently decreased after 3 weeks treatment with DA; at this time CLA and PLA were markedly reduced, therefore it is hypothesised that NF-Hs were degraded by calpains. This hypothesis is supported by (a) some evidence that DA may activate calpains (see section 5.3.2) and (b) the fact that apparent TLA is greatly activated after 3 weeks treatment and, as discussed in chapter III, could be due to the fact that the substrate for TLA is able to measure calpain activity. However, NF-H levels were not affected after 7 weeks treatment with DA.

No significant changes in total NF-H were observed with MPP^+. However, both neurotoxins reduced the levels of pNF-M + pNF-H; this could be explained in a number of ways. Firstly, as for control cells, phosphorylated NFs might not undergo proteasomal degradation (see section 5.2.1) and other routes capable of degrading these phosphoepitopes are more active. Secondly, toxins could induce aberrant phosphorylation and oxidation of NFs, thus making them more suitable proteasomal substrates. This is also a possible explanation since proteasome activity is only reduced by 10- 30 % by the toxins over a period of 7 weeks, thus NF-H can still be normally degraded by the proteasome.

Finally, no change in the ratio of pNF-H:pNF-M was found after 3 or 7 weeks treatment with these low levels of toxins. However, it is important to note that the ratios in control samples were 1.4 and 2.3 for cells cultured for 3 and 7 weeks, respectively. This is the opposite of the situation with short term control cells where the mean control ratio was 0.7. Thus, it appears that the ratio of pNF-H: pNF-M increases with time of culture. It remains to be checked whether the activity of stress kinase enzymes were altered following chronic exposure.

Weeks	Treatment (10 μM)	Total NF-H	pNF-H + pNF-M	[pNF-H: pNF-M]	ca. Controls		
					% CLA	% TLA	% PLA
3	MPP^+	=	↓ (38%)	=	91.2	91.5	91.9
	DA	↓ (15%)	↓ (32%)	=	51.0	426.16	25.7
7	MPP^+	=	↓ (40%)	=	70.0	86.2	52.96
	DA	=	↓ (46%)	=	81.9	178.7	80.4

Table 5.4. Summary of the effects of 3 and 7 weeks treatment with 10 μM MPP^+ and DA on NF levels and phosphorylation. *Accumulation or decrease in NF are symbolised as ↑ or ↓ respectively with the % change indicated in brackets. No change in NFs is symbolised as =. The data is compared with the effects of chornic exposure to toxins on proteasome activity (see section 3.2.6) for a better understanding of the data.*

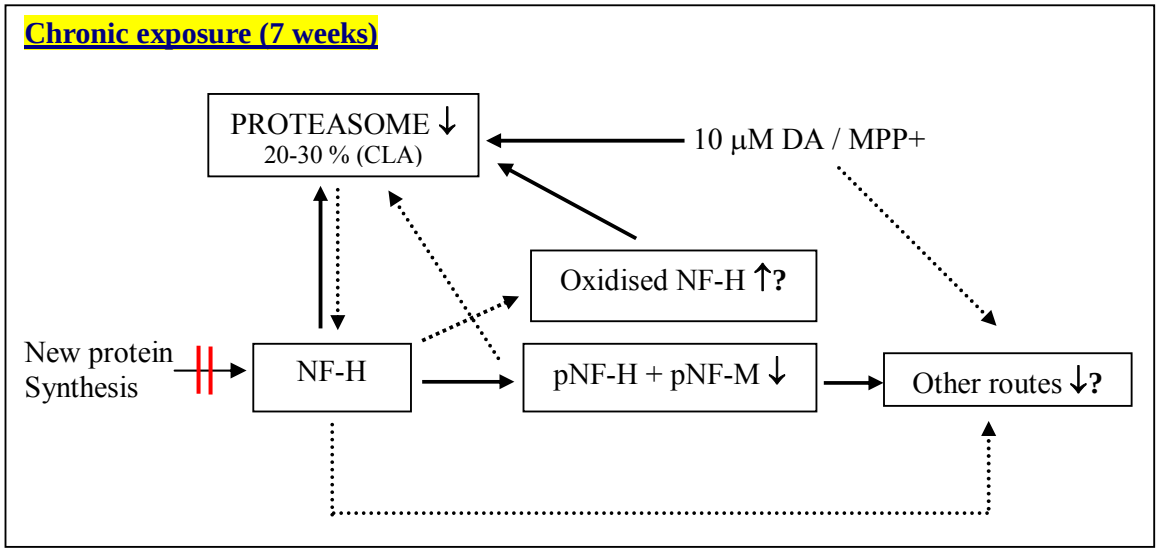


Figure 5.10. Overview of the effects of chronic exposure to 10 mM MPP^+ and DA on the NF system in SH-SY5Y cells.

The distribution of phosphorylated NF-H and NF-M after chronic treatment (7 weeks) of the cells with toxins was also studied. As stated before, it is important to note that although my study was performed with mitotic cells, some axon-like structures could still be observed in control cells. These axon-like structures were not altered after 7 weeks treatment with either toxin. Immunocytochemical analysis revealed that in control cells, SMI31 staining occurred within the axon-like processes and also in the

perikarya. However, after treatment with the toxins SMI31 staining was intense and restricted to the cell body and was predominantly perinuclear. These results are consistent with De Girolamo *et al.* (2000) and De Girolamo and Billett (2006) who found accumulation of phosphorylated NF-H within the cell perikaryon after treatment of mouse neuroblastoma cells with sub-cytotoxic concentrations of MPTP. The changes in the distribution of pNFs might be due to impaired transport of NF proteins within the cell, and may be involved in the formation of inclusion bodies seen in several neurodegenerative disorders (Petzod, 2005).

CHAPTER VI

GENERAL DISCUSSION

6. GENERAL DISCUSSION

This thesis has focused in two areas of research regarding the pathogenetic mechanisms underlying PD, altered proteasomal activity and changes in NFs after mimicking some of the biochemical features of PD by using MPP^+ and DA in an *in vitro* model.

6.1 EFFECTS OF MPP^+ AND DA ON PROTEASOME ACTIVITY

6.1.1 Role of ATP and ROS in toxin-mediated proteasome impairment

The causes underlying PD have not yet been fully elucidated, thus a wide amount of research is being undertaken in order to determine the specific mechanisms of nigral cell death. Recently, UPS impairment is gaining importance in the field as a key event contributing to the pathogenesis of both sporadic and familial PD. Moreover, impairment of this proteolytic pathway might also be involved in the formation of protein aggregates typical of the disease (reviewed by McNaught and Olanow, 2006).

In this thesis, the effects of MPP^+ and DA on proteasomal activity from human dopaminergic SH-SY5Y cells were investigated since both toxins are relevant in PD; Indeed, MPP^+ can reproduce some of the biochemical deficits of PD (reviewed by Przedborsky and Vila, 2001) and DA containing neurons are specifically lost in PD. Moreover, DA metabolism creates a highly oxidative environment thus increasing the vulnerability of these neurons (reviewed by Blum *et al.,* 2001). The contributions of ROS and ATP on proteasome impairment were studied since both parameters are thought to be important in PD and most previous *in vitro* work was performed in rat cells. This is important since different proteasome subtypes exist in different species, tissues and cells (Rivett *et al.,* 2001).

This study revealed that complex I inhibition by MPP^+ and the presence of DA led to a reduction in CLA and PLA in the cells. However, TLA activity was not reduced by the toxins; in fact, activation of this activity was observed following treatment with low doses of MPP^+ and high doses of DA, suggesting a compensatory effect of the cells in response to oxidative stress. This is the first time that differential proteasome sensitivity to the toxins has been reported despite the assessment of the three activities in a limited number of human post-mortem PD samples (McNaught and Jenner, 2001; McNaught *et al.,* 2003), in rats or SK-N-MC cells chronically treated with rotenone (Betardet *et al.,*

2006) and in rodent cells treated with rotenone, MPP^+ (Hoglinger *et al.,* 2003) and 6-OHDA (Elkon *et al.,* 2004).

The mechanisms triggering MPP^+- and DA-mediated proteasome impairment were found to be different in SH-SY5Y cells. MPP^+ decreased proteasome activity primarily via complex I inhibition (ie. ATP depletion) whilst, DA-mediated proteasome impairment was a major consequence of oxidative stress (ie. ROS generation) as confirmed by the fact that NAC, a precursor for glutathione synthesis and a mild antioxidant itself, could counteract the effects of DA on the proteasome linked to the recovery of cell morphology. These findings are in agreement with Elkon *et al.* (2004) who found that NAC alleviated proteasome impairment caused by 6-OHDA in an *in vitro* mouse cell model. Moreover, glutathione levels were transiently increased after DA treatment, as previously reported by Haque *et al.* (2003), possibly as a response of the cells to oxidative stress. In contrast, NAC did not protect the proteasome against MPP^+ toxicity. ATP has previously been claimed as the main factor driving proteasome impairment after MPP^+ and rotenone treatment in rat mesencephalic cell cultures (Hoglinger *et al.,* 2003).

Results in this thesis show that DA is a stronger oxidant than MPP^+, since low doses of DA generated greater amounts of ROS than high doses of MPP^+. However, NAC was able to withdraw the ROS generated by both toxins, thus it is tempting to hypothesise that ROS might also influence or exacerbate ATP depletion caused by MPP^+ thereby, further compromising proteasomal function. Indeed in dopaminergic cells, DA plus complex I inhibition would exacerbate each others effects.

It is important to note that short term glutathione depletion on its own might not be sufficient to impair CLA, thus other adverse events, notably ATP depletion, might act together with ROS in decreasing protesomal function.

Finally, since PD is a chronic disorder chronic treatment of the cells may give a better understanding of the situation *in vivo*. Interestingly, CLA was also impaired after 3 and 7 weeks treatment with 10 μM MPP^+ and DA. However, PLA and TLA were only significantly reduced after 7 weeks treatment suggesting that CLA is more sensitive than the other activities; this is consistent with our short exposure data. As before, TLA was the least sensitive proteasomal activity to the toxins. The relevance of this finding

remains uncertain, since the most important proteolytic activity for protesomal function has been reported to be CLA (Jäger *et al.*, 1999).

Since in this study proteasomal activity was monitored using fluorogenic peptides which measure ATP independent 20S activity (from both free 20S and 20S bound to regulatory particles), it remains to be determined whether the toxins differently affect the 26S proteasome. Furthermore, the effects of the toxins on other proteases, including calpains, also needs to be assessed.

6.1.2 Direct effects of toxins on proteasome activity

By studying the direct effects of the toxins on purified 20S proteasome and proteasomal activities of crude cell extracts, the primary and secondary effects of the toxins on proteasome activity were able to be discriminated. MPP^+ and DA were shown to directly decrease CLA when added to cell extracts, with MPP^+ having a greater effect than DA. This was the opposite situation to purified 20S samples where DA was more damaging than MPP^+. This suggests that MPP^+ direct effect is more specific towards the proteasome whilst DA might be randomly interacting with all the proteins present in the cell lysate. It is worth noting, that some the direct effects of the toxins detected via measurement via 20S activity in the cell lysates might possibly occurring via damage to the regulatory subunits, given that our extraction system contained ATP and would conserve at least some 26S particles (Coux *et al.*,1996).

Overall, 20S-CLA was more sensitive to MPP^+ and DA than 20S-PLA followed by 20S-TLA. Indeed, MPTP although unable to be metabolised to MPP^+ *in vitro* also reduced 20S-CLA and 20S-PLA (this time PLA being more sensitive than CLA) whilst TLA was unaffected. It appears than TLA is directly and indirectly less sensitive to the toxins than the other two activities. Since addition of an antioxidant could protect against toxic insult, the direct effect of the toxins to the proteasome is at least partly due to oxidative damage to the multiprotease itself. This is consistent with Shamoto-Nagai *et al.* (2003) who report decreased proteasomal activity in rotenone-treated cells associated with oxidative modification of the proteasome. Therefore, since TLA is the least affected activity by the toxins, either TLA catalytic subunits (β2 and β6) or TLA regulation (α-subunits) might be less sensitive to oxidative damage or the oxidative changes in this subunits might have less impact in the functionality of this activity.

The fact that the direct effect of the toxins on the proteasome might be partly due to oxidative damage could have consequential effects on the 26S proteasome since is four times more vulnerable to oxidative stress than the 20S (Reinheckel *et al.*,1998). This could be related to possible effects on the various regulatory subunits of the 26S proteasome. Indeed, McNaught *et al.* (2003) reported a loss in the α-subunits in SNpc of PD patients together with a reduction in the 19S activator and virtually undetectable levels of 11S. However, oxidative damage is not solely confined to the regulatory subunits since the β-subunits of the 20S are also reported to be targets in SH-SY5Y cells (Shamoto-Nagai *et al.*, 2003).

Indeed, this thesis shows that CLA (β4), TLA (β2 and β6) and some α-subunits (α1, α2, α3 and α6) of the 20S proteasome were modified after treatment with DA. However, although MPP^+ decreased proteasomal activity this was not detected by Western blotting, suggesting that changes in the subunits following MPP^+ treatment are either more subtle than the ones caused by DA or the epitopes modified by MPP^+ are different to the epitopes recognised by the antibodies.

Interestingly, whilst the direct effects of MPP^+ on 20S-TLA were very similar to their effects on trypsin enzyme, DA was more damaging to the proteasome suggesting that some of the oxidative damage could be affecting the non-catalytic subunits which can moderate the 20S-TLA. Indeed, as previously discussed, western blotting analysis showed that some of the α- subunits were affected by DA.

The fact that the three PD mimetics used in this study could directly affect 20S activity would suggest that chemicals with this type of structure may have direct deleterious effects on the UPS in vivo if their concentrations are sufficiently high. Indeed, it has been suggested that exposure to environmental toxins/pesticides may be contributing to the most common, sporadic form of PD (Liu and Yang 2005;Zhou *et al.* 2004) This is supported by a report that maneb leads to a reduction in proteasome activity (Zhou *et al.* 2004). Very recently a paper by Wang *et al.* (2006) looked at the direct effects of other pesticides (including rotenone) on 26S using human neuroblastoma cells with a 26S proteasome reporter system and found six pesticides which resulted in reductions in 26S activity, some at very low concentrations (10 nM for rotenone). Of the six pesticides, rotenone caused oxidative stress, but the others did not (e.g. benomyl, dieldrin, ziram), suggesting the involvement of other mechanisms. On the other hand none of the

pesticides had a direct effect on 20S proteasomal activity in cell lysates at the concentrations tested (maximum 10μM), as also concluded for rotenone in another recent study (Betarbet *et al.* 2006).

Thus this work adds further support to the suggestion that complex I inhibition/defects, whether due to the effects of toxins or due to genetic predisposition, leads to selective problems in dopaminergic neurons since the DA is released following ATP depletion, causing damage to the proteasome in these cells. It is likely that complex I inhibition in non-dopaminergic cells would be less damaging, and in this case ATP depletion rather than ROS would be the important determinant of 20S activity.

Further investigation is required to further confirm that the toxins oxidatively modify the 20S proteasomal subunits and to determine whether other possible modifications are also occurring. Moreover, it would be of interest to study the direct effects of the toxins on purified 26S proteasome.

6.2 EFFECTS OF MPP^+ AND DA ON THE NF SYSTEM

This thesis demonstrates that NFs can undergo proteasomal degradation in SH-SY5Y cells. Moreover, MPP^+ altered the expression and distribution of NF proteins. Short term exposure to high levels of the toxin led to the accumulation of total NF-H and increased NF-H and NF-M phosphorylation in my cell system. This could be a result of a decrease in proteasome activity caused by the toxin; moreover, MPP^+ might be causing aberrant phosphorylation of NF proteins. Indeed, an increase of the ratio in the ratio of pNF-H: pNF-M was also observed and might be important in the response of the cells to MPP^+ toxicity. Although this thesis did not study the kinases responsible for elevated NF phosphorylation following MPP^+ treatment, it is possible CDK-5 could be involved since it has been reported that oxidative stress can increase CDK-5 activity thereby increasing perikaryal NF phosphorylation (Shea *et al.*, 2004). Alternatively, NF phosphorylation could be increased by JNK since it has been reported that proteasomal inhibition increased NF-H phosphorylation and JNK activity in PC12h cells (Masaki *et al.*, 2000).

The effects of high doses of DA on NF proteins were also determined, contrary to MPP^+, DA decreased total NF-H levels and NF-H and NF-M phosphorylation after 72 h. It is hypothesised that DA is possibly activating calpains. Indeed, calpains are

reported to be the major pathway involved in NF degradation (reviewed by Petzold, 2005) along with further evidence presented in this thesis.

It is important to note that with high doses of the toxins (i.e 500 μM DA and 2 mM MPP^+) other factors different to the ones highlighted in this thesis might be also activated which might influence both the proteasomal and NF systems in my cell model, thus increasing the complexity of the analysis and extrapolation of these data to the in vivo situation.

Similarly to the short time exposure data, chronic exposure of the cells to DA resulted in a transient decrease of both total NF-H (again possibly, due to calpain activation), whilst no change was observed with MPP^+. However, both toxins decreased the levels of phosphorylated NFs by 3 weeks probably due to proteasomal degradation since proteasome activity was not greatly impaired by chronic exposure to the toxins.

Furthermore, immunocytochemical analyses revealed that both short and long term exposures to MPP^+ altered the distribution of NF which localised to the cell body. This was also the case in cells chronically treated with DA suggesting that both toxins might alter the axonal transport of these cytoskeletal proteins. This is of interest since abnormal NF aggregates are pathological hallmarks of many neurodegenerative disorders including PD (Julien, 1999). Moreover, NF proteins are found in LB together with ubiquitinated proteins, suggesting that disruption of the UPS might contribute to the formation of such aggregates and vice-versa, protein aggregates might also perturb proteasomal function by sequestering the UPS components or by overloading the proteasome capacity (reviewed by Betardet *et al.*, 2005). A link between LB and NFs accumulation or redistribution reported in this thesis is still uncertain and requires further investigation, for example immunocytochemical co-localisation studies of NFs, synuclein, proteasome subunits and ubiquitin need to be undertaken. To assess the significance of changes in NF distribution it will be useful to also study the status of other cytoskeletal elements (MTs and MFs) and proteins found associated with aggresomes (γ-tubulin and heat-shock proteins).

6.3 CONCLUSIONS

Figure 6.1 integrates the data presented in this thesis and suggests possible links to PD pathogenecity.

In conclusion, this thesis provides further evidence that the UPS in conjunction with complex I inhibition and ROS formation might play an important role in PD pathogenesis and possibly in protein aggregation which is a common feature not only of neurodegenerative disorders but also of ageing. The findings reported in this study can be summarised as follows:

- MPP^+ and DA decreased proteasomal activity, ATP and glutathione levels in SH-SY5Y cells. MPP^+ toxicity towards the proteasome is primarily caused by ATP depletion whilst ROS (glutathione depletion) appeared to be the determinant in the case of DA.
- MPP^+, MPTP and DA could directly impair the 20S proteasome partly due to oxidative damage to the multicatalytic complex.
- The relative sensitivity of the different proteasomal activities to the toxins in both the cells and the 20S was overall: CLA > PLA > TLA.
- The proteasome seemed more sensitive to DA than trypsin enzyme suggesting that the regulatory subunits of the 20S proteasome might also be critical in maintaining protesome activity.
- Several catalytic and non-catalytic subunits of the proteasomal core were modified by the toxins. Again TLA was the least sensitive activity to the direct effect of toxins.
- Proteasomal inhibition resulted in accumulation of NF-H levels suggesting that these proteins might undergo proteasomal degradation.
- MPP^+ and DA induced changes in the post-translational modification and distribution of the NF network.

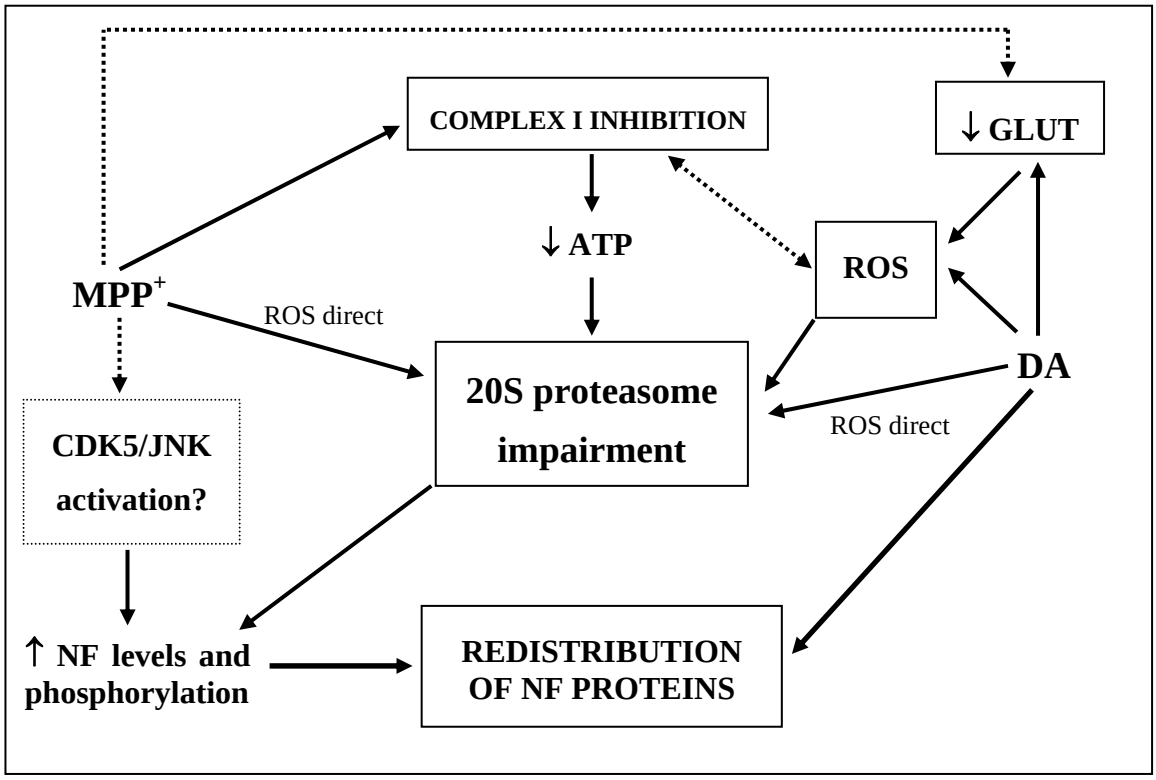


Figure 6.1. Overview of MPP+ and DA effects on UPS and NF system as detailed in this thesis and current literature. *MPP+ impairs proteasomal activity primarily via complex I inhibition whilst DA-mediated proteasome impairment is mainly due to ROS. In addition these toxins can directly reduce 20S proteasome activity. Reduced proteasomal function leads to accumulation of NFs (phosphorylated and dephosphorylated) in the cell body and are known to be found in LB with other aberrant proteins (Gai et al., 2000). Based on published work (reviewed by McNaught and Ollanow, 2006) it is likely that proteasome dysfunction leads to protein aggregation of other proteins also.*

CHAPTER VII

REFERENCE LIST

7. REFERENCE LIST

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