# THE ROLE OF DOPAMINE OXIDATION IN DOPAMINE-INDUCED TOXICITY, INITIATION OF ENDOPLASMIC RETICULUM STRESS, AND POTENTIATION OF ROTENONE-INDUCED TOXICITY IN DIFFERENTIATED PC12 CELLS

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**THE ROLE OF DOPAMINE OXIDATION IN DOPAMINE-INDUCED TOXICITY, INITIATION OF ENDOPLASMIC RETICULUM STRESS, AND POTENTIATION OF ROTENONE-INDUCED TOXICITY IN DIFFERENTIATED PC12 CELLS**

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Parkinson's disease (PD) neurodegeneration of the dopaminergic cells of the substantia nigra has been linked to various types of cellular injury, including oxidative stress, mitochondrial dysfunction, and dysfunction of the ubiquitin proteasome system. Multiple genetic mutations and high prevalence of idiopathic disease conceals the unifying mechanism for PD. Given the selective vulnerability of dopaminergic cells, dopamine (DA) may play a major role in PD pathogenesis. DA metabolism and oxidation into DA quinone (DAQ) leads to the production of reactive oxygen species. In addition, DAQ can react with reduced sulfhydryls, covalently modifying cysteine residues. DAQ modification of free cysteines, glutathione, and cysteines in proteins could result in decreased antioxidant capacity and inactivation and/or misfolding of proteins. In this study, I measured the effect of DA treatment in differentiated PC12 cells, and found that DA exposure was toxic, lead to increased DAQ modified free cysteines, glutathione, and cysteines in proteins, and decreased ATP levels. I also demonstrated that metabolism of DA by monoamine oxidase did not influence DA-induced toxicity, but that DA uptake by the dopamine transporter was necessary for DA-induced cell death. Further, I demonstrated that activation of endoplasmic stress (ER) also occurred following DA exposure, with increases in ER chaperone proteins calreticulin, ERp29, ERp99, Grp58, Grp78, Grp94, and Orp150. Decreased mitochondrial levels of the glycolytic enzyme aldolase A and increased levels of

whole cell aldolase A were also observed following DA exposure, suggesting that DA may affect ATP levels by altering energy-related proteins. Finally, to determine the role of DA oxidation in the rotenone model of PD, I used the mitochondrial complex I inhibitor in DA depleted cells. I found no protection with DA depletion, but significant increases in rotenone toxicity when co-treated with methamphetamine, which leads to the cytoplasmic release of DA. Since I used sub-toxic levels of methamphetamine, this data suggests that the increased levels of DA oxidation lead to potentiation of rotenone-induced toxicity. Therefore, in this thesis I show that DA oxidation is linked to oxidative stress, ER stress activation, and mitochondrial dysfunction, and thus may play a role in the pathogenesis of PD.

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# PREFACE

When I thought about my growth as a scientist and began to mentally list all of the people who encouraged and guided me in some way or another, I started to appreciate how many individuals were involved in my transformation. I wouldn't have reached this point in my life, both personally and intellectually, without the advice, support, and assistance of numerous family members, friends, teachers, and colleagues. I am so grateful to each of you, and want you to know that I greatly appreciate everyone who has helped me along the way. To name everyone would require a book in itself, but I would like to take a moment to thank a few individuals who have helped me tremendously and have made some of the greatest contributions to the development and completion of these studies.

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# 1.0 INTRODUCTION

## 1.1 PARKINSON'S DISEASE

### 1.1.1 Pathology, Clinical Features, and Epidemiology

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of pigmented dopaminergic neurons in the substantia nigra pars compacta (SNpc), the loss of SNpc projections to the caudate and putamen, as well as the formation of proteinaceous cytoplasmic inclusions called Lewy bodies (Lang and Lozano, 1998; Samii et al., 2004). Significant damage to the nigrostriatal system, including the loss of 80% of striatal dopamine (DA) and 50% of nigral neurons, occurs before the clinical symptoms of PD typically manifest (Samii et al., 2004). Symptoms associated with PD include a range of movement disorders such as postural and gait deficits, bradykinesia, rigidity, and resting tremor. However, autonomic dysfunction, sensory disturbances, and cognitive symptoms, including depression and occasionally dementia, are also prevalent in PD (Samii et al., 2004). This debilitating disorder afflicts approximately one million North Americans, with a prevalence of 1% in people age 60 and older (Lang and Lozano, 1998; Twelves et al., 2003; Samii et al., 2004; Lester and Otero-Siliceo, 2006). Despite years of study, the cause of the degeneration in most cases of PD remains unknown. A number of genes have been identified in connection with rare familial forms of PD, providing insights to the potential

mechanisms of the disease. However, 85-90% of PD cases are considered sporadic (Nussbaum and Polymeropoulos, 1997; Bajaj et al., 1998; Gasser, 2001), with little understanding of the underlying causes of neurodegeneration.

### 1.1.2 Idiopathic Disease

Most cases of idiopathic and familial PD have similar pathological hallmarks, suggesting that both forms of the disease have a common biological pathway (Gandhi and Wood, 2005). Although PD cases are primarily idiopathic; several environmental and genetic predispositions are thought to contribute to disease pathogenesis, including pesticide exposure, well water exposure, heavy metal exposure, dietary factors, and polymorphisms in monoamine oxidase-A (MAO-A), monoamine oxidase-B (MAO-B), the dopamine transporter (DAT), tyrosine hydroxylase (TH), the D2 receptor, the D3 receptor, and the D4 receptor (Gorell et al., 1997; Bandmann et al., 1998; Marsden and Olanow, 1998; Anderson et al., 1999b; Gandhi and Wood, 2005; Benmoyal-Segal and Soreq, 2006; Lester and Otero-Siliceo, 2006). Thus, finding common underlying mechanisms between both idiopathic and inheritable forms of PD is necessary for understanding the disease and for the development of new therapeutics.

### 1.1.3 Genetic Disease

Only a small percent of PD is attributed to inheritable genetic mutations, and this makes up 20% of early onset PD and 1-3% of late-onset PD (Farrer, 2006; Lorincz, 2006). There are currently 9 genes linked to PD, 7 of which follow Mendelian inheritance, and 2 (PARK10 and PARK11) that are gene loci associated with PD susceptibility (Lester and Otero-Siliceo, 2006).

The protein products of 6 of these genes have been identified. These proteins include α-synuclein, parkin, ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), PTEN induced putative kinase 1 (PINK1), DJ-1, and leucine-rich repeat kinase 2 (LRRK2). Mutations in a few of these proteins link PD pathogenesis to protein aggregation and dysfunction of the ubiquitin proteasome system (UPS), and many proteins are linked to mitochondria.

### 1.1.4 Alpha-synuclein

There are three confirmed mutations in the Park1 gene for α-synuclein, resulting in the protein amino acid substitutions A53T, A30P, and E36K, and duplication or triplication of the gene is also linked to PD (Hardy et al., 2006; Klein and Schlossmacher, 2006; Lester and Otero-Siliceo, 2006). Although the primary function of α-synuclein is not known, it has been shown to bind to lipids, be involved in vesicle function, have possible chaperone capabilities, and affect DA synthesis (Perez and Hastings, 2004; Hardy et al., 2006). The most obvious link between α-synuclein and PD is that α-synuclein is one of the main components of Lewy bodies, regardless of whether PD was sporadic or inherited. In addition, mutant forms of α-synuclein increase sensitivity to PD-linked toxins. Over-expression of mutant α-synuclein increases the sensitivity of cells to 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), ubiquitin proteasome dysfunction-induced apoptosis, and mitochondria-dependent apoptosis (Tanaka et al., 2001; Sun et al., 2005; Zhou et al., 2006b). Also, rotenone treatment can lead to α-synuclein accumulations *in vivo* and *in vitro* (Betarbet et al., 2000; Sherer et al., 2002; Sherer et al., 2003c; Testa et al., 2005; Betarbet et al., 2006). Additionally, the UPS and other cellular pathways may be affected by α-synuclein, since it is known to interact with

numerous proteins and can aggregate, even in its non-mutant form (Ghee et al., 2000; Snyder et al., 2003).

### 1.1.5 Parkin

Parkin (Park2) is linked to early onset PD. Parkin is an E3 ubiquitin ligase, which is thought to bind specific proteins to target them for degradation by the UPS (Moore et al., 2005; Hardy et al., 2006; Lester and Otero-Siliceo, 2006). Patients with parkin-related PD differ from others with PD pathology, since they do not develop Lewy bodies. *In vitro* studies of parkin have found multiple binding partners, including parkin-associated endothelin receptor-like receptor (Pael-R), an ER stress associated protein (Imai et al., 2001); synaptic proteins CDCrel-1 (Zhang et al., 2000), synphilin-1 (Chung et al., 2001), and synaptotagmin XI (Huynh et al., 2003); protein biosynthesis proteins, p38 subunit of the aminoacyl-tRNA synthetase complex (Corti et al. 2003) and the far upstream element (FUSE)-binding protein 1 (FBP1) (Ko et al., 2006); proteins involved in inherited neurodegenerative diseases, polyglutamine (Tsai et al., 2003) and O-glycosylated α-synuclein (Shimura et al., 2001); structural proteins α- and β-tubulin (Ren et al., 2003); DAT (Jiang et al., 2004); RanBP2, an E3 SUMO ligase (Um et al., 2006); and cyclin E, an apoptosis and cell cycle regulation protein (Staropoli et al., 2003). How parkin confers neuroprotection to DA neurons remains unanswered, but recent studies have linked parkin to mitochondrial function and to another inheritance-linked PD protein, PINK1 (Yang et al., 2005; Clark et al., 2006; Park et al., 2006). Also, oxidative modification of parkin by nitric oxide (NO) and dopamine quinone (DAQ) have been found in *in vitro* studies and in human brain (LaVoie et al., 2005; Klein and Schlossmacher, 2006). Thus deficiencies in parkin could also occur in idiopathic PD due to oxidative modifications.

### 1.1.6 UCH-L1

An autosomal dominant form of PD can be inherited from an ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1; Park5) mutation. UCH-L1 is a very abundant protein; its mRNA is found throughout the brain with especially high expression in the DAergic neurons of the SN (Solano et al., 2000). UCH-L1 hydrolyzes polyubiquitin chains into monomeric ubiquitin, aiding in the recycling of ubiquitin for the proteasome (Wilkinson et al., 1989). The UCH-L1 mutation leads to decreased activity (approximately 50%), which would limit the availability of free ubiquitin for normal protein clearance (Betarbet et al., 2005; Barrachina et al., 2006). UCH-L1 has been shown *in vitro* to have an additional function as an ubiquityl ligase, but whether this additional function occurs *in vivo* has yet to be determined (Liu et al., 2002). Decreased levels of UCH-L1 have been observed *in vitro* following oxygen-glucose deprivation and iron-dependent oxidation (Drake et al., 2002; Shen et al., 2006), and have been observed *in vivo* after administration of DAergic toxins, including methamphetamine and MPTP (Kuhn et al., 2003; Liao et al., 2005; Iwazaki et al., 2006). Additionally, UCH-L1 has been identified as a component of Lewy bodies and has also been found to be down-regulated and oxidized in brain tissue from sporadic PD patients, and thus may also be involved in idiopathic mechanisms of PD degeneration (Solano et al., 2000; Nishikawa et al., 2003; Choi et al., 2004; Barrachina et al., 2006).

### 1.1.7 PINK1

PTEN induced putative kinase 1 (PINK1, Park6) is a nuclear encoded mitochondrial serine/threonine kinase related to autosomal-recessive early-onset PD (Greenamyre and Hastings, 2004; Hardy et al., 2006). PINK1 has also been linked to parkin, since parkin over-expression in PINK1 knockout (KO) mice attenuated the PINK1 KO phenotype (Clark et al., 2006; Park et al., 2006; Tan and Dawson, 2006). PINK1 KO Drosophila were more susceptible to paraquat, rotenone, osmotic stress, and the ER stress inducer dithiothreitol, suggesting that PINK1 is protective against mitochondrial dysfunction induced by stress (Greenamyre and Hastings, 2004; Clark et al., 2006; Gosal et al., 2006). Thus PINK1 may also be related to idiopathic PD, since mitochondrial complex I deficits have been observed in sporadic PD patients (Blandini et al., 1998).

### 1.1.8 DJ-1

DJ-1 (Park7) mutations are linked with autosomal-recessive early-onset PD. Normal DJ-1 is an abundant protein found throughout the brain as a dimer and is thought to respond to and protects against oxidative stress (Gosal et al., 2006; Klein and Schlossmacher, 2006). DJ-1 has been shown to inhibit α-synuclein aggregate formation *in vivo* and *in vitro* (Shendelman et al., 2004; Meulener et al., 2005b; Zhou et al., 2006a). Over-expression of DJ-1 protects cells against mitochondrial damage, and sulfoxidation of Cys106 leads to an acidic shift of the protein necessary for translocation of DJ-1 to the mitochondria and for a protective response to oxidative stress, suggesting that it could be an intracellular sensor of oxidative stress (Bonifati et al., 2003; Miller et al., 2003; Bandopadhyay et al., 2004; Canet-Aviles et al., 2004; Kim et al., 2005). DJ-1

has also been shown to protect against oxidative stress *in vitro* by up-regulating glutathione synthesis (Zhou and Freed, 2005). There is also *in vivo* evidence for DJ-1 as a protector against oxidative stress in Drosophila and mouse models. DJ-1 deficient Drosophila have been shown to be more sensitive to various oxidative stressors, including $H_2O_2$, paraquat, and rotenone (Menzies et al., 2005; Meulener et al., 2005a; Park et al., 2005; Yang et al., 2005). DJ-1 KO mice are more susceptible to MPTP toxicity (Kim et al., 2005). In addition, DJ-1 KO mice were less sensitive to signaling by the DA D2-receptor, indicating that dopaminergic neurotransmission was altered in these mice (Goldberg et al., 2005). Therefore, DJ-1 may play a role in PD pathogenesis through its effects on mitochondria, DA receptor signalling, and oxidative stress.

### 1.1.9 LRRK2

Leucine-rich repeat kinase 2 (LRRK2; Park8) mutations are associated with early- to late-onset autosomal dominant forms of PD. Lewy body formation does not necessarily occur in all cases of LRRK2-related PD, and in some patients tauopathy and ubiquitin-positive inclusions also occur (Wszolek et al., 1997; Hardy et al., 2006; Taylor et al., 2006). LRRK2 mutations are much more frequent than other PD mutations, occurring even in sporadic PD (Gilks et al., 2005; Hardy et al., 2006; Mata et al., 2006). LRRK2 mRNA is expressed throughout the brain at low levels (Zimprich et al., 2004). Although the function of LRRK2 remains unknown, it is a large protein with several predicted protein domains, including an ankyrin domain, a leucine-rich repeat domain, a Roc GTPase domain, a COR (C terminal of Roc) domain, a tyrosine kinase domain, and a WD40 domain (Bosgraaf and Van Haastert, 2003; Paisan-Ruiz et al., 2004; Zimprich et al., 2004; Mata et al., 2006). The combination of the predicted protein kinase and

GTPase activities with the multiple predicted protein interaction domains suggests that LRRK2, might be involved in a multi-protein signaling complex (Mata et al., 2006). Some binding partners of LRRK2 have been found, including heat-shock protein 90 and its co-chaperone, Cdc37, while parkin has also been found to interact with over-expressed LRRK2 *in vitro* (Smith et al., 2005b; Gloeckner et al., 2006). Also, over-expressed mutant LRRK2 leads to cell death in both SH-SY5Y cells and primary neurons (Smith et al., 2005b). LRRK2 also seems to play a role in regulating neuronal axon and dendritic processes; over-expressed mutant LRRK2 reduced branching and length of neuronal processes *in vitro* and shRNA knockdown of LRRK2 increased neuronal branching *in vitro* (MacLeod et al., 2006). Therefore, the proposed functions of LRRK2 as a "master regulator" combined with the frequency of mutations in inheritable and sporadic PD suggest that LRRK2 may be an important key to understanding PD pathology (Mata et al., 2006).

## 1.2 EVIDENCE FOR OXIDATIVE STRESS IN PD

### 1.2.1 Definition of Oxidative stress

Oxidative stress is typically denoted by an overabundance of highly reactive oxygen-based molecules, known as reactive oxygen species (ROS), compared to the antioxidant system designed to remove ROS in the cellular environment (Halliwell, 1992). ROS is detrimental to tissue since most cellular components, including DNA, proteins, lipids, and carbohydrates, are susceptible to damaging oxidative modification by various forms of ROS (Sayre et al., 2005). However, ROS production is a regular part of biological systems, since normal aerobic

mitochondrial respiration which is necessary for cell survival, results in the formation of ROS (Sayre et al., 2005). Peroxynitrite, which is formed from NO and superoxide anion, and other reactive nitrogen species (RNS) have recently been shown to modify lipids, DNA, and proteins on the tyrosine and tryptophan residues, resulting in "nitrosative stress" (Alvarez and Radi, 2003; Pacher et al., 2007). Cells have endogenous defense mechanisms against ROS, namely the antioxidant system. The antioxidant system protects against excess ROS by binding promoters of ROS formation or by enzymatically degrading or directly scavenging ROS, forming lower-oxidizing products (Cui et al., 2004). Changes in the levels of antioxidants and antioxidant-related proteins, through increased ROS formation, are used as markers of oxidative stress (Gutteridge, 1995; Barnham et al., 2004).

Oxidative stress has been implicated in many neurodegenerative diseases, including Alzheimer's disease (Butterfield et al., 2001; Honda et al., 2004), amyotrophic lateral sclerosis (Carri et al., 2003), Huntington's disease (Butterfield and Kanski, 2001), and PD. Evidence for oxidative stress in disease can be obtained through directly measuring modification of biomolecules by ROS, in addition to measuring changes in the antioxidant systems. Although these changes are typically measured late in the development of disease, evidence from living PD patients, experimental models of PD, and studies of genetic mutations associated with PD have lead to an increasing body of evidence supporting oxidative stress as a contributor to PD pathogenesis (Hastings and Berman, 2000; Dawson and Dawson, 2003; Fiskum et al., 2003; Greenamyre and Hastings, 2004). The evidence that links oxidative stress to PD, as well as current thoughts on potential pathways of oxidative stress in PD will be discussed below.

### 1.2.2 Antioxidant Enzymes

Several indicators of increased oxidative stress have been observed in PD brain, including changes in antioxidant enzymes and levels of antioxidants. Antioxidant enzymes are proteins with functions that detoxify ROS, by converting high oxidizing ROS species into a lower oxidizing species. Enzymes such as catalase and glutathione peroxidase catalyze the reduction of hydrogen peroxide to water, and superoxide dismutase (SOD) catalyzes the reduction of superoxide anion into hydrogen peroxide. There is some question of whether levels of catalase and glutathione peroxidase are changed, with evidence for deficits (Ambani et al., 1975; Kish et al., 1985) and no change (Marttila et al., 1988; Sian et al., 1994a) in enzyme levels, in addition to up-regulated mRNA (Duke et al., 2007; Moran et al., 2007) reported in PD. However, there is consistent evidence that the activity of SOD is increased selectively in the SN of PD patients (Marttila et al., 1988; Saggu et al., 1989; Basso et al., 2003). Elevation in the manganese-dependent mitochondrial form of SOD has been observed in the cerebrospinal fluid (CSF) of PD patients, and elevated levels of the copper/zinc-dependent cytosolic form of SOD in lymphocytes were found in PD patients on L-DOPA treatment alone (Yoshida et al., 1994; Blandini et al., 2003; Blandini et al., 2004). Increases in the mitochondrial form of SOD observed in PD may be compensatory for increased ROS production by impaired mitochondria. The increase in cytosolic SOD observed in PD patients has recently been linked to increased cell death due to the L-DOPA treatment, since increased caspase-3 was found in lymphocytes of L-DOPA treated patients only (Blandini et al., 2004). Thus, changes in antioxidant enzymes may partially be the result of PD treatments, and not necessarily due to changes caused by the disease.

### 1.2.3 Glutathione

In addition to changes in antioxidant enzymes, the major cellular antioxidant glutathione (GSH) is also altered in PD. GSH plays a vital role in a variety of cellular processes in addition to the antioxidant system, including metabolism, synthesis of DNA and protein, gene expression, immunity, cell proliferation, and cell death (Wu et al., 2004). GSH is present in cells at high concentrations, ranging from 0.5–10 mM. The cysteine residue on GSH molecules can be oxidized by ROS, creating a disulfide bond between two GSH molecules to form oxidized glutathione (GSSG). GSSG can then be converted back to GSH via the enzyme glutathione reductase, which is necessary to keep the GSH balance in cells.

Although levels of other antioxidants like ascorbic acid and alpha-tocopherol are unchanged in PD, an estimated 40% decrease in GSH has been observed selectively in the SN of PD brain (Perry et al., 1982; Perry and Yong, 1986; Riederer et al., 1989; Dexter et al., 1992; Sian et al., 1994b). Decreased GSH is thought to be an early event in PD since reduced GSH levels occur in incidental Lewy body disease, which in some cases is thought to be a pre-parkinsonian state (Jenner and Olanow, 1996). Surprisingly, the decrease in total GSH is not associated with concurrent increases in GSSG (Sofic et al., 1992). An increase in the levels of gamma-glutamyl transpeptidase, an enzyme associated with the translocation and degradation of GSH, was observed in PD SN, which may be a compensatory mechanism to import GSH precursor molecules or to breakdown excess GSSG (Sian et al., 1994a; Jenner and Olanow, 1996). However, the levels of most other enzymes involved in GSH synthesis and GSH-dependent reactions remain unchanged in PD, such as gamma-glutamylcysteine synthetase (the rate-limiting enzyme for GSH synthesis), GSH reductase (the enzyme that reduces GSSG), or GSH transferase (enzymes that catalyze the conjugation of GSH to various electrophilic

compounds, including quinones), suggesting a failed antioxidant response occurs in PD (Sian et al., 1994a; van Bladeren, 2000; Prigione et al., 2006).

Loss of GSH can lead to the reduction of S-thiolation of enzymes such as mitochondrial complex I and the E1 and E2 ubiquitin enzymes, linking mitochondrial function and the UPS to GSH loss (Jahngen-Hodge et al., 1997; Hurd et al., 2005). Although most GSH is synthesized in the cytoplasm, it is transported into mitochondria, helping to detoxify superoxide anion produced by aerobic respiration (Zeevalk et al., 2005). Another important role for GSH in the antioxidant system is the removal of $H_2O_2$ via GSH peroxidase and GSH reductase, protecting neurons from further ROS-production (Zeevalk et al., 2005). GSSG formed from this reaction can then be reduced by GSH reductase, which is also found in mitochondria, recycling GSH and helping to maintain a proper GSH/GSSG ratio (Hurd et al., 2005; Zeevalk et al., 2005). In addition, GSH loss can lead to damage to mitochondrial complexes I, II, and IV, which can then result in the increased formation of ROS (Zeevalk et al., 2005). It has also been suggested that reduced GSH levels precede increased iron levels observed in PD, and thus may contribute to increased iron oxidation (Jenner and Olanow, 1996).

### 1.2.4 Iron

Reduced transition metals, iron (II) in particular, can catalyze the Fenton reaction during which $H_2O_2$ is oxidized to the highly reactive and toxic hydroxyl radical, leading to the production of iron (III) and ROS (Riederer et al., 1989; Youdim et al., 1989). Increased amounts of iron have been found in PD SNpc (Dexter et al., 1989b; Dexter et al., 1991; Hirsch et al., 1991; Sofic et al., 1991; Hirsch and Faucheux, 1998; Griffiths et al., 1999), including increases in iron (III) (Riederer et al., 1989; Yoshida et al., 2001). Iron accumulates in brain during

normal aging, even to levels associated with oxidative stress and cell death (Thomas and Jankovic, 2004; Zecca et al., 2004). Since PD cases are rare before the age of 50, and because the incidence of PD increases with age, aging is seen as risk factor for PD and may be related in part to iron accumulation (Samii et al., 2004). Iron accumulation occurs in other disorders as well, including Alzheimer's disease, multiple system atrophy, progressive supranuclear palsy, and Huntington's disease (Valberg et al., 1989; Dexter et al., 1991; Connor et al., 1992; Jenner, 2003). However, iron levels in SN in incidental Lewy body disease are not different from age-matched control, suggesting that iron accumulation is not necessary for cell death and does not occur in all neurodegenerative disease (Dexter et al., 1994b).

In addition to increased iron, decreased ferritin (the protein that binds and stores iron) and decreased copper (a metal found in antioxidant enzymes such as SOD) were also found in PD SN (Sofic et al., 1988; Dexter et al., 1989b; Dexter et al., 1989a; Dexter et al., 1991; Connor et al., 1995; Zecca et al., 2001; Zecca et al., 2002b). Oxidative stress due to iron exposure has also been shown to promote α-synuclein aggregation, and iron has been shown to be a component of Lewy bodies (Hashimoto et al., 1999; Castellani et al., 2000; Hsu et al., 2000; Ostrerova-Golts et al., 2000; Uversky et al., 2001; Manning-Bog et al., 2002; Cole et al., 2005). Iron-mediated oxidative stress has also been shown to inhibit complex I activity (Harley et al., 1993; Jenner, 2003). These findings link iron accumulation to oxidative stress, mitochondrial dysfunction, and Lewy body pathology, which are all hallmarks of PD.

### 1.2.5 DNA Oxidative Damage

The four nucleotide bases that comprise DNA (purines, adenine and guanine, and pyrimidines, cytosine and thymine) are highly susceptible to oxidative damage by ROS, leading

to mutations, strand breakage, and ultimately cell death (Halliwell and Gutteridge, 1999). Increased levels of oxidized DNA have been found in many neurodegenerative disorders, including Alzheimer's disease (Gabbita et al., 1998; Migliore et al., 2005), Huntington's disease (Browne et al., 1997), amyotrophic lateral sclerosis (Ferrante et al., 1997), and PD (Halliwell, 2001). Guanine is the most sensitive to oxidative modification, and evidence for oxidative modification in PD is currently limited to the purines, guanine in particular. Increases in oxidized purine in PD lymphocytes and leukocytes have been reported, suggesting that oxidative stress is systemic in patients (Petrozzi et al., 2001; Migliore et al., 2002). In addition, increased levels of oxidized deoxyguanosine, 8-hydroxy-2'-deoxyguanosine, have been found in the caudate, putamen, SN, and cerebral cortex of PD patients as compared to control patients (Sanchez-Ramos et al., 1994). Increases in the oxidized form of the intermediate 8-hydroxy-guanine radical, 8-hydroxyguanine, combined with decreases its reduced form, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, in PD SN suggest that there is an increased oxidative environment in diseased brain (Alam et al., 1997b). The oxidation of DNA bases leads to DNA damage, and the levels of DNA strand breakage are increased in PD lymphocytes and leukocytes, (Petrozzi et al., 2001; Migliore et al., 2002), and in PD midbrain, caudate nucleus/putamen, thalamus, and hippocampus (Hegde et al., 2006). Imprecise antisense base pairing, which can lead to unstable DNA, was also found in PD midbrain, caudate nucleus/putamen, thalamus, and hippocampus (Hegde et al., 2006).

In addition to cytoplasmic DNA oxidation, evidence for increased cytoplasmic RNA oxidation has also been observed in the SN of PD patients, and, to a lesser extent, in patients with multiple system atrophy-Parkinsonian type and dementia with Lewy bodies (Zhang et al., 1999). In patients with PD and other neurodegenerative disorders, mitochondrial DNA oxidation

was increased in the SN and in other brain areas (Zhang et al., 1999; Gu et al., 2002). In response to mitochondrial DNA damage, increased levels of mitochondrial 8-oxo-dGTPase, the enzyme that removes oxidized deoxy-guanosine-triphosphate from the DNA precursor pool, were measured in PD SN (Shimura-Miura et al., 1999; Fukae et al., 2005). In addition, levels of mitochondrial DNA deletions, which can be the result of free radical damage, are increased in aged SN and occur more often in PD SN (Bender et al., 2006; Burton, 2006; Kraytsberg et al., 2006). The increased amount of DNA oxidation, both nuclear and mitochondrial, and increased oxidation of RNA lends evidence to support the role of oxidative stress in PD pathology.

### 1.2.6 Lipid Peroxidation

The oxidation of unsaturated lipids is another indication of oxidative stress. Highly reactive lipid peroxyradicals are formed from the reaction of ROS with the double bond of unsaturated fatty acids. These lipid peroxyradicals are themselves reactive and can oxidize other fatty acids, leading to the formation of lipid peroxidation by-products such as 4-hydroxy-2,3-nonenal (HNE), acrolein, $F_2$-isoprostanes, and thiobarbituric acid reactive substances (TBARS) including malondialdehyde (Barnham et al., 2004). Increased HNE levels have been observed in many neurodegenerative disorders, including Alzheimer's disease and amyotrophic lateral sclerosis (Pedersen et al., 1998; Butterfield et al., 2002). High levels of HNE were also found in PD plasma and CSF (Selley, 1998). HNE reactions with cysteine, lysine, and histidine residues form HNE-protein conjugates, which have also been found to be increased in PD midbrain (Yoritaka et al., 1996). Levels of TBARs were higher in PD red blood cells compared to control (Serra et al., 2001), and increases in the TBAR malondialdehyde have also been observed in PD (Dexter et al., 1989b). Increased levels of isofurans, which are formed by lipid peroxidation,

were measured in PD and incidental Lewy body disease patients (Fessel et al., 2003). Increases in other markers of lipid peroxidation, such as fatty acid hydroperoxides and cholesterol lipid hydroperoxides were also observed in PD SN (Dexter et al., 1994a). Lipid peroxidation, in addition to producing reactive peroxyradicals that can damage other cellular components, and specifically HNE has been shown to lead to DNA fragmentation and caspase-dependent apoptosis (Liu et al., 2000). In addition, lipid peroxidation affects membrane fluidity and permeability, which leads to dysfunction of membrane-associated ion channels, changes in the number and affinity of membrane receptors, and alterations in organelles, leading to problems like lysosomal membranes breakage and the release of their proteolytic enzymes (Farooqui and Horrocks, 1998; Halliwell, 2006). Therefore, oxidative damage to lipids may have serious effects that could contribute to the cell death observed in PD.

### 1.2.7 Protein Oxidative Damage

Oxidative damage to proteins has been well documented in neurodegenerative disease, including PD and AD (Beal, 2002; Castegna et al., 2003). The most common measurement of protein oxidation is the formation of protein carbonyls. Increased levels of protein carbonyls have been found in PD SN, basal ganglia, cortex, globus pallidus, and cerebellum (Alam et al., 1997a; Floor and Wetzel, 1998). Recently, advances in proteomic techniques have allowed investigators to detect and identify specific proteins oxidatively modified in disease. In PD, proteins like DJ-1 and Cu,Zn-SOD have been identified as oxidatively modified in PD brain (Choi et al., 2005; Choi et al., 2006). Increased levels of oxidized lipoproteins in CSF and plasma of PD patients have also been observed (Serra et al., 2001; Buhmann et al., 2004). In addition to protein damage by ROS, there is also evidence for protein damage by RNS in PD;

increases in levels of CSF nitrates and increased brain nitrosyl adducts were found in PD (Giasson et al., 2000). In addition, 3-nitrotyrosine-modified proteins were detected in Lewy bodies and other protein aggregates in PD brain SN (Good et al., 1998), indicating that aggregated protein was oxidized by peroxynitrite. Specifically, increased amounts of nitrated Mn SOD have been found in the CSF of PD patients (Aoyama et al., 2000). DJ-1, Cu,Zn-SOD, and Mn SOD are all proteins involved in protecting against oxidative stress, and if inactivated, could lead to even more oxidative stress and cell death.

Another form of oxidized protein shown to be potentiated by oxidative stress, advanced glycation end-products, are proteins that have been post-translationally modified by sugars and then oxidized in a process called glycoxidation (Baynes, 1991). Glycoxidated proteins have also been shown to be increased in PD (Castellani et al., 1996). Protein oxidation and glycoxidation may lead to protein dysfunction and aggregation, which can be detrimental to normal cellular processes, especially in the case of antioxidant proteins.

## 1.3 MITOCHONDRIAL DYSFUNCTION, OXIDATIVE STRESS, AND PD

### 1.3.1 Mitochondrial Dysfunction and Oxidative Stress

There is a strong relationship between mitochondrial dysfunction and oxidative stress. Mitochondrial complexes I and III of the electron transport chain (ETC) form superoxide anion during normal respiration, and both ROS and RNS can inhibit mitochondrial complexes (Halliwell, 2001; Orth and Schapira, 2001, 2002; Fiskum et al., 2003; Turrens, 2003). Inhibition of mitochondrial complex I has also been shown to generate even more ROS in isolated

mitochondria, suggesting that ROS-induced mitochondrial dysfunction can lead to further ROS production and increased damage (Pitkanen and Robinson, 1996; Votyakova and Reynolds, 2001). Mitochondria, through the Bcl-2 family of proteins, play a unique role in cell death signaling, and it has been shown that increased superoxide anion formation can lead to cytochrome c release, one of the key initiators of apoptosis (Atlante et al., 2000; Rego and Oliveira, 2003). Therefore, mitochondrial dysfunction and oxidative stress, both of which are observed in neurodegenerative diseases like PD, likely contribute to the pathogenesis of disease.

### 1.3.2 Evidence for Mitochondrial Dysfunction in PD

Decreased mitochondrial complex I activity has been observed directly in the SN and systemically in platelets, lymphocytes, and muscle tissue of PD patients (Schapira et al., 1990b; Shoffner et al., 1991; Janetzky et al., 1994; Blandini et al., 1998; Winkler-Stuck et al., 2005). Further, defects in paired complex I/III have been observed in lymphocytes, and deficits in complex IV have been observed in both lymphocytes and muscle of PD patients (Winkler-Stuck et al., 2005; Shinde and Pasupathy, 2006).

The genetic component of mitochondrial dysfunction in PD is being explored through the utilization of cybrids, cell lines in which endogenous mitochondrial DNA has been removed and replaced with mitochondrial DNA from PD and control patients. Cybrid studies have shown that there is likely a genetic component to PD complex I dysfunction, since cybrids from PD patients have complex I deficiencies and abnormal mitochondrial morphology (Swerdlow et al., 1996; Sheehan et al., 1997; Trimmer et al., 2000). In addition, cybrid experiments comparing maternal and paternal mitochondrial DNA of PD patients found that the maternal cybrid lines had reduced complex I activity, increased production of ROS, and more mitochondrial morphology

abnormalities compared to the paternal lines (Swerdlow et al., 1998). Since the source of the majority of mitochondrial DNA is from the mother, this suggests complex I deficiencies seen in PD may be inherited from the mother and thus may be present over the patient's entire life. Although maternal mitochondrial deficits could play a role in some cases of PD or be due to an environmental affect on mitochondria and not through inheritance, there is currently no strong evidence that maternal inheritance of mitochondrial deficits occurs commonly in PD (Zweig et al., 1992; Wooten et al., 1997; de la Fuente-Fernandez, 2000).

Many other studies are looking for polymorphisms in mitochondria DNA that are associated with PD to determine which genes are linked to decreased mitochondrial function. Cybrids made from mitochondrial DNA containing two polymorphisms associated with increased risk of PD contained mitochondria with altered matrix pH and calcium signaling (Kazuno et al., 2006), associating mitochondrial abnormalities with PD-risk. Polymorphisms in complex I have been identified that result in a reduced risk of PD, although the reason for this protection has yet to be elucidated (van der Walt et al., 2003).

### 1.3.3 Models of PD and Mitochondrial Dysfunction

#### *Rotenone*

Rotenone is a potent complex I inhibitor (Degli Esposti, 1998), commonly used as a pesticide. Rotenone is a lipophilic molecule, able to cross membranes and penetrate the blood-brain barrier easily (Talpade et al., 2000). Rotenone was established by Betarbet et al., 2000 as a novel PD model in rat. Remarkably, using rotenone to systemically and partially inhibit complex I led to selective dopaminergic cell toxicity in the SN, dopaminergic striatal terminal

degeneration, and ubiquitin- and α-synuclein-positive protein inclusions, combined with PD behavioral symptoms like bradykinesia, rigidity, and postural deficits (Betarbet et al., 2000; Sherer et al., 2003a). Protein carbonyl formation and DNA oxidation has also been measured in rotenone models *in vivo* and *in vitro*, connecting mitochondrial dysfunction and oxidative stress (Sherer et al., 2002; Sherer et al., 2003c). GSH levels were significantly decreased by 40% *in vivo* and 57% *in vitro* following chronic rotenone exposure (Sherer et al., 2003c), showing that chronic mitochondrial dysfunction can lead to GSH deficits. Cell death and carbonyl formation due to rotenone inhibition of complex I *in vitro* was attenuated by transfecting cells with the rotenone-resistant NDI1, the single-protein equivalent of complex I from *Saccharomyces cerevisiae* (Sherer et al., 2003c). These data indicate that rotenone-induced toxicity results from the loss of complex I activity and possibly the increased oxidative stress resulting from mitochondrial inhibition, and not by some unknown secondary mechanism.

Rotenone-induced complex I inhibition can cause an electron leak (Hensley et al., 1998), which combined with $O_2$ forms superoxide, a potent modifier of lipids, proteins, and DNA. In the rotenone model a clear association between complex I deficits occurring before ROS production is observed, but whether this can be correlated to PD pathogenesis remains to be clarified. Nevertheless, the pathological and behavioral aspects of PD were replicated, including oxidative damage to DNA and proteins, with chronic, systemic rotenone exposure, connecting mitochondrial dysfunction to selective dopaminergic system vulnerabilities, suggesting the treatment with rotenone may be a useful model in which to study aspects of PD (Betarbet et al., 2000; Sherer et al., 2002; Sherer et al., 2003b; Sherer et al., 2003c; Sherer et al., 2003a).

***MPTP***

A botched synthesis of the meperidine analog, 1-methyl-4-phenyl-4 propionpiperidine (MPPP), containing the toxic impurity MPTP, led to the discovery of a new PD toxin model in the 1980s. Intravenous injection of MPTP along with the intended drug led to a reversible akinetic state, which was then proposed to be the result of damage to the SN (Langston et al., 1983). Later studies on MPTP found that it was not the toxic species directly, but when absorbed by cells, MPTP is converted to 1-methyl-4-phenyl-1,2-dihydroxypyridinium ion (MPDP+) by MAO-B, and then spontaneously oxidized into the poisonous metabolite, methyl-4-phenylpyridinium (MPP+), with both steps leading to the formation of superoxide (Chiba et al., 1984; Markey et al., 1984; Zang and Misra, 1992, 1993). Although MPTP is lipophilic and can pass through cell membranes in the brain, MPP+ is not, and must be released by a transporter from astrocytes and then selectively taken up into cells through the DAT (Chiba et al., 1985; Javitch et al., 1985; Russ et al., 1996). Inside dopaminergic cells MPP+ can bind and inhibit complex I, be taken up by the vesicular monoamine transporters (VMAT), or remain in the cytosol, leading to ATP loss and ROS formation (Nicklas et al., 1985; Mizuno et al., 1987; Singer et al., 1987; Chan et al., 1991; Ramsay et al., 1991; Chan et al., 1992; Teismann et al., 2001).

Other than people who inadvertently exposed themselves to MPTP, there is no evidence that MPTP-like toxins play a role in PD patients (Ikeda et al., 1992; Goodwin and Kite, 1998). Most animal models of PD using MPTP utilize non-human primates and C57black/6 strain mice, but not rats, because they are not sensitive to MPTP (Boyce et al., 1984; Heikkila et al., 1984; Sahgal et al., 1984; Gerlach and Riederer, 1996). *In vitro* models, including non-dopaminergic

lines (such as cerebellar granule cells and pituitary cells) and dopaminergic cell lines (such as PC12 cells, SK-N-MC cells, SH-SY5Y cells, MES 23.5 cells, and primary mesencephalic cultures) typically utilize the metabolite MPP+ so that the conversion of MPTP to MPP+ is not a factor in toxicity (Blum et al., 2001). Thus, many studies looking at dopaminergic cell death mechanisms have utilized MPTP or MPP+.

Mitochondrial function is essential for the supply of cellular ATP, which in turn is necessary for calcium homeostasis, mitochondrial membrane potential, cellular membrane potential, and transporter function (Di Monte et al., 1988; Hollinden et al., 1988; Wu et al., 1990; Schmidt and Ferger, 2001). MPTP treatment in mice has been shown to inhibit complex I, lead to 10-20% reductions in ATP, and lead to a 2- to 5-fold increase in the measurement of hydroxyl radical, one of the most reactive ROS (Mizuno et al., 1988; Chan et al., 1991, 1992; Desai et al., 1996; Smith and Bennett, 1997; Teismann et al., 2001). Over-expressing SOD protects against MPTP, suggesting that superoxide formation is important in MPTP-induced cell death (Przedborski et al., 1992). Increased levels of 3-nitrotyrosine, as evidence for RNS damage, were also measured in mice after MPTP treatment, and NO synthase-inhibited or deficient mice were protected against MPTP toxicity, suggesting an additional role for nitrative damage in the model (Schulz et al., 1995; Hantraye et al., 1996; Przedborski et al., 1996; Liberatore et al., 1999; Pennathur et al., 1999). Since the reaction of superoxide with NO results in peroxynitrite formation, which can also inhibit mitochondrial complexes I, II, and III (Radi et al., 1991), both ROS and RNS production by MPTP could lead to further mitochondrial dysfunction, increasing levels of ROS. Therefore, ROS and RNS production likely plays a role in the cause and effect of MPTP-induced toxicity.

DA plays a vital role in MPTP-induced neurotoxicity. The selectivity for MPTP-induced toxicity *in vivo* is due to DAT expression, since DAT deficient mice were shown to be resistant to toxicity (Gainetdinov et al., 1997; Bezard et al., 1999). Once in dopaminergic cells, MPP+ can be taken up into vesicles by VMAT, which displaces DA from the vesicles to the cytoplasm where it can be oxidized, leading to increased formation of the hydroxyl radical and to cell death (Chiueh et al., 1992; Lotharius and O'Malley, 2000). Sequestering of MPTP into vesicles aids in reducing toxicity, since VMAT expression has been shown to suppress MPTP toxicity *in vitro*; conversely, mice in which VMAT-2 was inhibited or expressed at lower levels were more susceptible to MPTP toxicity (Liu et al., 1992; Takahashi et al., 1997; Gainetdinov et al., 1998; German et al., 2000; Staal and Sonsalla, 2000; Chen et al., 2005a). Increased levels of DAQ protein modification have also been observed in MPTP mouse models (Teismann et al., 2003), showing that endogenous DA released by MPTP can form DAQ and modify protein. MPTP has also been shown to lead to extracellular DA efflux, increased DA turnover and reduced intracellular levels of DA and DA metabolites, suggesting that DA homeostasis is also adversely affected by MPTP (Mihatsch et al., 1988; Santiago et al., 1991; Schmidt and Ferger, 2001; Teismann and Ferger, 2001).

## 1.4 DA OXIDATION AND PD

### 1.4.1 DA Oxidation

DA can lead to oxidative stress through two pathways of ROS production. First, the normal metabolism of DA by MAO produces dihydroxyphenylacetic acid (DOPAC) and leads to

the production of $H_2O_2$ (Maker et al., 1981) (see Figure 1a). Secondly, spontaneous or enzymatic oxidation of DA into DAQ (see Figure 1b) leads to the formation of $H_2O_2$, increasing levels of oxidants that can further damage protein, DNA, and lipids (Graham, 1978). The electron-deficient DAQ can also lead to further oxidative damage, since it readily reacts with cellular nucleophiles; the most reactive nucleophiles in cells are reduced sulfhydryl groups, which can be found on free cysteine residues, GSH, and on proteins (Tse et al., 1976; Graham, 1978). DAQ modification of reduced sulfhydryls on cysteines forms covalently bound cysteinyl-DA conjugates (See Figure 1c) (Tse et al., 1976; Fornstedt et al., 1990a; Hastings and Zigmond, 1994). DAQ modification of free thiols and GSH will decrease the availability of antioxidants, adding to the oxidative stress. In addition to reducing the antioxidant capacity, DA oxidation can affect vital proteins containing cysteine in their active sites, altering the function of these proteins, thus leading to inactivation and cell death.

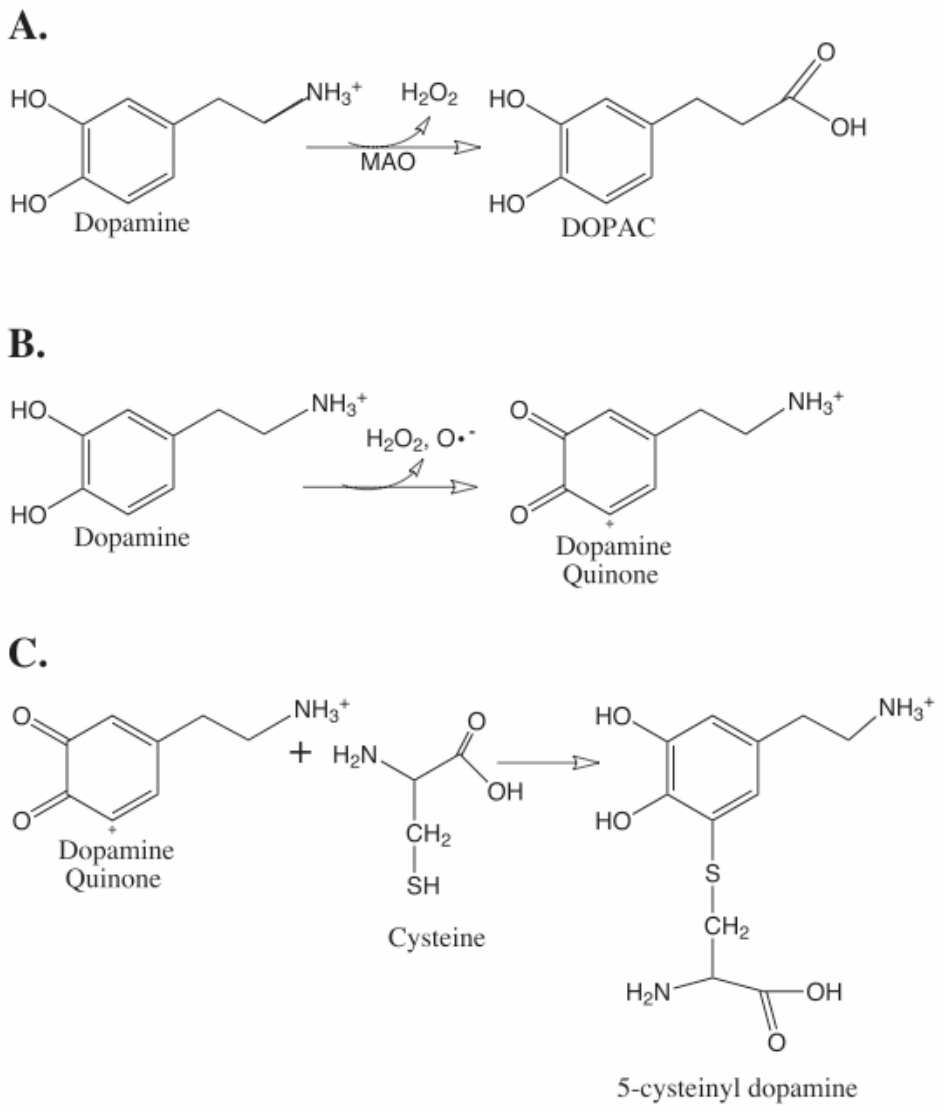


Figure 1: Formation of ROS and other reactive species via DA metabolism and oxidation.

**A.** DA metabolism by MAO leads to DOPAC formation and ROS production. **B.** Spontaneous or enzymatic DA oxidation leads to the formation of DAQ and ROS. **C.** DAQ can react with reduced sulfhydryl groups of cysteine residues, leading to the formation of 5-cysteinyl-DA conjugates.

### 1.4.2 Evidence for DA Oxidation in PD

The presence of high levels of DA combined with a reduced ability to cope with oxidative stress place dopaminergic neurons of the SN in a position of vulnerability. Therefore, it is not surprising that increased cysteinyl-catecholamine conjugates have been found in PD brain lysates (Spencer et al., 1998; Sidell et al., 2001), showing that DAQ modification occurs and is accelerated in PD. Antibodies to DAQ-modified cysteines and proteins have been observed in PD patients, providing evidence for an immune response to DA modification of proteins (Rowe et al., 1998; Salauze et al., 2005). Neuromelanin (NM) is formed from oxidized catecholamines and is driven by a cytosolic excess of catecholamines; NM levels in the SN increase with age (Sulzer et al., 2000; Zecca et al., 2004). The levels of NM and VMAT2 are inversely correlated in human SN, suggesting that increased VMAT sequestering of DA into vesicles reduces the levels of cytosolic DA and leads to lower DA oxidation and NM synthesis (Liang et al., 2004). NM has also been shown to partially inhibit 26S proteasome activity, linking DA oxidation to dysfunction of the UPS, which can lead to abnormal protein accumulation and aggregation (Shamoto-Nagai et al., 2004). NM levels are significantly reduced in PD SN, and postmortem studies show that the amount of neuromelanin in SN cells may be related to susceptibility, although there is some question as to whether cells containing more neuromelanin are preferentially lost or spared in PD (Mann and Yates, 1983; Gibb and Lees, 1991; Kastner et al., 1992; Zecca et al., 2002a). Since increased NM levels are associated with increased cytosolic DA, increased DA oxidation levels, and increased vulnerability in PD, it is likely that DA levels, DA oxidation, and cell death are linked in PD. However, NM, although made from oxidized DA, is thought to have antioxidant properties, since it has been shown to

bind heavy metals, such as iron III, removing both toxic metals and oxidized DA from the cytosol (Zecca et al., 1994; Zareba et al., 1995). Therefore, the formation of NM may also be a mechanism by which SN cells protect themselves from heavy metal accumulation and DA oxidation.

### 1.4.3 DA Oxidation in Models of PD and other DA toxins

#### *6-OHDA*

The chemical 6-OHDA has a similar chemical structure to DA, and is a substrate for DAT and NET (Luthman et al., 1989; Bove et al., 2005). Thus, 6-OHDA can be taken up by dopaminergic and noradrenergic neurons of both the central and peripheral nervous system (Bove et al., 2005). 6-OHDA does not pass through the blood brain barrier, therefore its administration is typically through injection into the SN, medial forebrain bundle (MFB), striatum, or ventricles (Javoy et al., 1976; Blum et al., 2001; Bove et al., 2005). *In vivo*, 6-OHDA injections into the SN or MFB lead to non-apoptotic cell death within 24 h and a maximal reduction in striatal DA levels after 3-4 d, and if given in the striatum, lead to a retrograde degeneration of the nigrostriatal pathway after 1-3 weeks, in a manner which resembles apoptosis (Faull and Laverty, 1969; Sauer and Oertel, 1994; Jeon et al., 1995; Przedborski et al., 1995; Marti et al., 1997). Thus far, no evidence for Lewy body-like protein inclusions following 6-OHDA administration has been found (Bove et al., 2005). Endogenous levels of 6-OHDA may form from reactions between DA and $H_2O_2$, and may be catalyzed by nitrite and metals like iron and manganese (Slivka and Cohen, 1985; Garner and Nachtman, 1989; Jellinger et al., 1995; Linert et al., 1996; Palumbo et al., 1999). Basal levels of 6-OHDA

have been measured in rat, human brain, and L-DOPA treated PD patient urine (Blum et al., 2001).

The mechanism of 6-OHDA induced toxicity is thought to be due to the formation of ROS, such as $H_2O_2$ and the hydroxyl radical, and is supported by numerous *in vivo* and *in vitro* studies which have found ROS production by 6-OHDA and antioxidant protection against 6-OHDA exposure (Heikkila and Cohen, 1971; Blum et al., 2001). Since 6-OHDA is a substrate for MAO, part of the ROS generated by 6-OHDA is thought to be through its deamination by MAO, leading to $H_2O_2$ production (Breese and Traylor, 1971; Karoum et al., 1993). In addition, like DA, 6-OHDA can auto-oxidize, generating $H_2O_2$, superoxide anion, the hydroxyl radical, and quinones (Saner and Thoenen, 1971; Heikkila and Cohen, 1972; Cohen and Heikkila, 1974; Seitz et al., 2000; Soto-Otero et al., 2000). The auto-oxidation of 6-OHDA is thought to be the major source of toxicity, since MAO inhibition, which would normally help metabolize 6-OHDA, has been shown to exacerbate toxicity (Jonsson, 1980). In addition, 6-OHDA toxicity is not limited to dopaminergic cell lines *in vitro*, and there is also evidence for extracellular oxidation for 6-OHDA (Lotharius et al., 1999; Blum et al., 2001). Lipid oxidation, DNA damage, protein modification, PARP activation, and reduced antioxidant levels are all associated with 6-OHDA exposure, suggesting that ROS formation overpowers the ability of the cells to deal with oxidative stress (Rotman et al., 1976; Jonsson, 1980; Bruchelt et al., 1991; Kumar et al., 1995). In isolated mitochondria, there is evidence that 6-OHDA may inhibit mitochondria directly; although this has not been replicated in cell culture or *in vivo*, there is evidence for a ROS-mediated mitochondrial deficit *in vitro* following 6-OHDA exposure (Glinka and Youdim, 1995; Glinka et al., 1996; Wu et al., 1996; Lotharius et al., 1999; Storch et al., 2000).

### *Methamphetamine*

Methamphetamine (METH) is a drug of abuse, which affects both the central and peripheral nervous system. Both dopaminergic and serotonergic systems are affected by METH, leading to losses in both tyrosine hydroxylase (TH) and tryptophan hydroxylase, the rate limiting enzymes of DA and serotonin syntheses, respectively (Hotchkiss and Gibb, 1980). Depletions in DA, DA metabolites, and DAT levels in striatum, serotonin levels, and serotonin uptake have also been observed *in vivo* following METH administration (Seiden et al., 1976; Wagner et al., 1980; Finnegan et al., 1982; Green et al., 1992; Eisch and Marshall, 1998; LaVoie and Hastings, 1999). Long-term deficits in DA terminals have been observed in both *in vivo* models and in patients who abused METH (Woolverton et al., 1989; McCann et al., 1998). In addition to terminal damage, neurodegeneration has also been observed *in vivo* following METH exposure (Ricaurte et al., 1982; Eisch et al., 1998; O'Dell and Marshall, 2005). Toxicity due to METH is thought to be a combination of many factors including oxidative stress (Kita et al., 2003; Quinton and Yamamoto, 2006).

The production of ROS and RNS has been observed in the METH model, including evidence for the hydroxyl radical and NO formation (Fleckenstein et al., 1997; Imam et al., 1999; Kita et al., 1999; Imam et al., 2001; Fukami et al., 2004; Kawasaki et al., 2006). Chemical antioxidants confer protection against METH (Wagner et al., 1986; De Vito and Wagner, 1989; Yamamoto and Zhu, 1998; Fukami et al., 2004; Kawasaki et al., 2006). Inhibition or genetic knockdown of NO synthase and SOD over-expression has been shown to attenuate and SOD inhibition potentiates METH toxicity, suggesting that ROS and RNS production is a key to METH toxicity (De Vito and Wagner, 1989; Cadet et al., 1994; Hirata et al., 1996; Itzhak and

Ali, 1996; Imam et al., 2001). Downstream effects of oxidative stress such as lipid and protein oxidation products, including 3-nitrotyrosine, protein carbonyls, malondialdehyde, and other TBARs, have all been measured following METH (Acikgoz et al., 1998; Yamamoto and Zhu, 1998; Imam et al., 1999; Gluck et al., 2001; Imam et al., 2001; Kawasaki et al., 2006).

The source of METH-induced oxidative stress seems to be linked to DA. METH reverses DA uptake and reduces VMAT uptake of DA from the cytosol, which leads to DA release, DA accumulation, DA oxidation, and formation of ROS (Raiteri et al., 1979; Ricaurte et al., 1980; Schmidt et al., 1985; Michel and Hefti, 1990; Green et al., 1992; Brown et al., 2001; Quinton and Yamamoto, 2006). In fact, DA-induced increases in oxidative stress and protein modification have been measured after METH administration *in vivo* (LaVoie and Hastings, 1999). Reducing levels of DA using the TH inhibitor α-methyl-*p*-tyrosine, protects against METH, and decreased VMAT2 expression *in vitro* is associated with increased METH toxicity and DA oxidation (Larsen et al., 2002), suggesting that cytosolic DA oxidation plays a major role in METH toxicity.

### *DA-induced toxicity*

The toxicity of DA has been established both *in vitro* and *in vivo* (Barzilai et al., 2001; Asanuma et al., 2004). Intrastriatal DA injections result in the selective loss of TH immunoreactive fibers of the striatum and increased formation of protein cysteinyl-catechols (Filloux and Townsend, 1993; Hastings et al., 1996; Rabinovic and Hastings, 1998; Rabinovic et al., 2000; Gomez-Santos et al., 2006). Similarly, DA has been shown to be toxic to various cell lines (Barzilai et al., 2001). While the exact mechanism of the DA-induced toxicity is not

known, the toxicity of DA has been linked to oxidative stress. Antioxidants have been shown to prevent DA toxicity both *in vivo* and *in vitro* (Passi et al., 1987; Hastings et al., 1996; Hoyt et al., 1997; Si et al., 1998; Luo et al., 1999; Izumi et al., 2005) and reduce protein cysteinyl-catechol formation *in vivo* (Hastings et al., 1996). Administration of L-buthionine sulfoximine to deplete GSH potentiates toxicity both *in vivo* and *in vitro* and increases the formation of cysteinyl-catecholamines *in vivo* (Rabinovic and Hastings, 1998; Si et al., 1998). Therefore, DA-induced toxicity is linked to the formation of ROS and DAQ-modification of proteins.

Several proteins relevant to dopaminergic cell function have been shown to be modified or inhibited by exposure to DA or DAQ, including DAT, the glutamate transporter, TH, and tryptophan hydroxylase (Berman et al., 1996; Berman and Hastings, 1997; Kuhn and Arthur, 1998; Xu et al., 1998; Kuhn et al., 1999; Whitehead et al., 2001). Creatine and adenylate kinases, enzymes involved in maintaining the ATP to ADP ratio, are also both inhibited by DA exposure (Maker et al., 1986; Miura et al., 1999). DNA polymerase was also inhibited by DA, indicating that DNA synthesis is affected, and thus adding a complication to *in vitro* studies using proliferating cells (Wick, 1980). DA has also been shown to affect α-synuclein aggregation, by promoting the formation of a DAQ-α-synuclein adduct, which develops into soluble, SDS-resistant oligomers (Conway et al., 2001; Cappai et al., 2005). Recently, covalent modification and inactivation of parkin by DA was reported, linking DA oxidation to inheritable PD (LaVoie et al., 2005). Therefore, DA may exert some of its toxicity by modifying and inactivating critical cytosolic proteins.

In addition to these proteins, DA has also been shown to lead to mitochondrial dysfunction, which could lead to more oxidative stress. Respiration from isolated rat brain mitochondria becomes uncoupled and formation of the permeability transition pore (PTP) was

triggered following exposure to DAQ, which can be protected by antioxidants (Berman and Hastings, 1999; Kim et al., 1999; Dolder et al., 2001; Lee et al., 2002; Youn et al., 2002). Both uncoupled respiration and PTP formation lead to decreases in the mitochondrial membrane potential and decreased ATP formation, and PTP formation can lead to cell death through cytochrome c release (Brookes, 2005; Tsujimoto et al., 2006). DA and cysteinyl-DA metabolites have also been shown to inhibit complex I respiration, complex IV respiration, alpha-ketoglutarate dehydrogenase activity, and pyruvate dehydrogenase activity in isolated mitochondria and in cell culture (Ben-Shachar et al., 1995; Li and Dryhurst, 2001; Khan et al., 2005). As previously mentioned, mitochondrial dysfunction is known to lead to ROS production, and DA-inhibited isolated mitochondria also have been shown to produce $H_2O_2$ and superoxide (Kim et al., 1999). Thus, oxidative stress, DA oxidation, and mitochondrial dysfunction all contribute to further oxidative damage in the mitochondria and the cell, ultimately resulting in cell death.

# 2.0 THESIS GOALS

The goals of the work presented in this thesis were to explore the role of dopamine (DA) in mediating vulnerability to toxicity in dopaminergic cells. Previously, DA-induced neurodegeneration has been established *in vivo*, and DA toxicity *in vitro*, with evidence for DA oxidation in human PD patient brain and PD animal models. I focused on establishing the DA-induced toxicity model in differentiated PC12 cells, a cell line that stops proliferating and extends processes following NGF differentiation, and can synthesize and store DA (Greene and Tischler, 1976; Greene and Rein, 1977). Using the PC12 cell line instead of primary cultures or *in vivo* studies was advantageous for measuring the changes that occurred in only DA-containing cells following DA- and rotenone-exposure. I then used this cell model to further explore the role of DA in differentiated PC12 cell toxicity, in changes in levels of proteins of the mitochondrial-enriched fraction, and in rotenone-induced toxicity, to better understand potential vulnerabilities affected by the presence of DA in cells.

First, I hypothesized that DA exposure would lead to increased DA oxidation and toxicity in differentiated PC12 cells. Thus, I established the DA-induced toxicity model by measuring the viability, catechol levels, cysteinyl-catechol levels, protein cysteinyl catechol levels, and ATP levels following DA exposure in differentiated PC12 cells. Due to the high levels of protein modification and ATP loss detected, I further hypothesized that intracellular DA oxidation was associated with toxicity. Therefore, the role of DA metabolism, DA uptake, and

the oxidative stress resulting from these pathways were also examined. These results are discussed in Chapter 3.

DA quinone and oxidative modification of proteins could lead to the inactivation, misfolding, and increased degradation of proteins. Since I observed increases on DA-modified protein following DA exposure in PC12 cells (Chapter 3), I hypothesized that DA-exposure leads to the modification of critical proteins, resulting in the loss of normal cellular function and increased vulnerability to cell death. I focused on mitochondrial proteins, since mitochondria play a vital role in cell death signaling, and since mitochondrial function is essential to cell survival. Using a new proteomics technique, 2D-difference in-gel electrophoresis (2D-DIGE), I measured protein changes in a mitochondrial-enriched fraction of PC12 cells following DA exposure. These data are described in Chapter 4.

Lastly, I wanted to determine the role of DA in rotenone-induced toxicity. Rotenone, a mitochondrial complex I inhibitor, is a potent dopaminergic toxin and has been established as a PD model. Rotenone also has been shown to increase reactive oxygen species formation and decrease ATP, which could lead to increased DA oxidation and decreased DA vesicular uptake. Therefore, I hypothesized that rotenone-induced toxicity may be associated with the presence DA, and thus rotenone may particularly affect dopaminergic cells. These observations are discussed in Chapter 5.

# 3.0 DOPAMINE-INDUCED TOXICITY IN DIFFERENTIATED PC12 CELLS INVOLVES INTRACELLULAR DOPAMINE OXIDATION AND REQUIRES DOPAMINE UPTAKE BUT NOT MONOAMINE OXIDASE METABOLISM

## 3.1 SUMMARY

Degeneration of dopaminergic cells in Parkinson's disease has been linked to oxidative stress and mitochondrial dysfunction. Dopamine (DA) can cause oxidative stress through production of $H_2O_2$, a byproduct of DA metabolism by monoamine oxidase (MAO), and through auto-oxidation into DA quinone (DAQ). Reduced free cysteine and cysteines incorporated into glutathione or protein can react with DAQ, forming cysteinyl-catechols. We have previously observed dysfunction in isolated mitochondria following DAQ and increased protein cysteinyl-catechol levels and selective dopaminergic degeneration after intrastriatal DA injections *in vivo*. DA toxicity is established in cell culture, but the mechanism by which DA induces cell death remains unknown. In this study, we measured the effects of DA exposure and the roles of MAO and the DA transporter (DAT) on DA-induced toxicity in differentiated PC12 cells. We found that DA exposure leads to 35% loss in cell viability, 8.6-fold increase in protein cysteinyl-catechols (24h, 150 μM), and 54% decrease in ATP levels (18h, 150 μM). We also observed that DA-induced toxicity was completely attenuated by blocking DA uptake. However, inhibiting MAO did not affect DA-induced toxicity. We concluded that DA uptake is required

for toxicity in differentiated PC12 cells. Thus, intracellular protein oxidation may play a major role in DA-induced toxicity.

## 3.2 INTRODUCTION

Parkinson's disease (PD) pathology has been characterized by the degeneration of dopamine (DA) containing neurons of the nigrostriatal pathway. The cause of this degeneration remains unknown, although it has been linked to oxidative stress (Koutsilieri et al., 2002; Jenner, 2003; Sayre et al., 2005). The relationship between DA oxidation and oxidative stress in degeneration has provided a possible link between the selective vulnerability of DA neurons and PD. Since DA is unstable, easily oxidized into reactive oxygen species (ROS) and quinones, it can add to the oxidative stress of a cell. DA can generate ROS and quinones through the normal metabolism of DA by monoamine oxidase (MAO), which leads to the production of $H_2O_2$ and dihydroxyphenylacetic acid (DOPAC) (Maker et al., 1981). In addition, spontaneous or enzymatic oxidation of DA into DA quinone (DAQ) forms ROS, such as $H_2O_2$. Both pathways of DA-related ROS formation can lead to cellular damage. Transition metals can react with $H_2O_2$ that is not reduced by glutathione (GSH) and the GSH-dependent detoxifying enzyme GSH-peroxidase, leading to the formation of the hydroxy radical, which can react with protein, DNA, and lipids (Bolton et al., 2000; Halliwell, 2001). Furthermore, the electron-deficient DAQ readily reacts with cellular nucleophiles, including reduced sulfhydryl groups, which can be found on free cysteine residues, GSH, and proteins (Tse et al., 1976; Graham, 1978; Fornstedt et al., 1990a; Hastings and Zigmond, 1994). Modification of free thiols and GSH can lead to the reduction in the amount of antioxidants available to protect the cells from oxidative stress. Many

vital proteins contain cysteine in their active sites, therefore modification of cysteine residues in proteins may alter the function of these proteins, leading to protein inactivity and cell death (Bailey et al., 2005).

*In vivo* DA oxidation is supported by the presence of neuromelanin, an oxidized polymer of catecholamines and by the presence of cysteinyl-catechol conjugates in PD brain lysates (Spencer et al., 1998; Zucca et al., 2004). Endogenous DA oxidation and protein modification has also been observed following exposure to DA toxins, such as methamphetamine (METH) and 1-methyl-4-phenylpyridinium (MPP+), in the form of increased protein cysteinyl-catechols (LaVoie and Hastings, 1999; Teismann et al., 2003). Exogenous DA application increases the formation of protein cysteinyl-catechols *in vitro* and *in vivo*, and leads to selective damage to DA terminals *in vivo* (Graham, 1978; Hastings et al., 1996; Rabinovic et al., 2000). DA has also been shown to be toxic in both dopaminergic and non-dopaminergic cell culture systems, including mesencephalic cell cultures, cortical neurons, cultured rat forebrain neurons, SH-SY5Y cells, SK-N-MC cells, mouse thymocytes, and undifferentiated PC12 cells (Pardo et al., 1995; Hoyt et al., 1997; Stokes et al., 2000; Barzilai et al., 2001; Blum et al., 2001; Asanuma et al., 2004; Chen et al., 2004b; Moussa et al., 2006). However, only a few studies have shown DA toxicity in differentiated PC12 cells (Jones et al., 2000; Koshimura et al., 2000; Ishisaki et al., 2001). DA-induced toxicity *in vivo* and *in vitro* has been established. The oxidation of DA to ROS and reactive quinones has previously been implied in several cell culture studies, but it has never been measured directly.

In this study, we sought to determine the effect of DA exposure in differentiated PC12 cells focusing on whether DA-induced oxidation, either through $H_2O_2$ production from the metabolism of DA by MAO, or by oxidation of the catechol ring into reactive quinones with

subsequent protein modification was responsible for inducing cell death. We found that DA exposure is toxic to PC12 cells, and leads to the formation of free cysteinyl-catechols and protein-cysteinyl catechols. In addition, DA exposure leads to ATP deficits, indicating that DA exposure may affect mitochondrial respiration. DOPAC, a metabolite of DA that can also form quinones and modify reduced sulfhydryls, is not a substrate of the DA transporter (DAT) and was not toxic to PC12 cells. We also observed that DA-induced toxicity could be completely attenuated by blocking DA uptake into PC12 cells, but that inhibiting MAO did not affect DA-induced toxicity. Therefore, we conclude that DA must be taken up by differentiated PC12 cells to be toxic, and that quinone modification of intracellular protein targets is likely a major contributor to DA-induced toxicity.

## 3.3 EXPERIMENTAL PROCEDURES

*Chemicals and Reagents:*

Cell culture media, Dulbecco's Modified Eagle Medium (DMEM, Gibco brand), fetal bovine serum (HyClone brand), horse serum (HyClone brand), and trypsin (Gibco brand) were purchased from Invitrogen (Carlsbad, CA). Type I rat tail collagen and nerve growth factor (NGF) were purchased from BD Bioscience (San Diego, CA). $^{14}$C-labeled DA was purchased from ICN Biomedicals (Costa Mesa, CA). All other non-specified reagents were purchased from Sigma. All solutions were made in distilled water purified with a Milli-Q system (Millipore Corp., Bedford, MA) unless otherwise noted.

*PC12 Cell Culture:*

PC12 cells, DA-containing rat adrenal pheochromocytoma derived cell-line, were plated at a density of 19,000 cells/$cm^2$ and differentiated in DMEM supplemented with 1% fetal bovine serum, 1% horse serum, and 100 ng/ml NGF over 6 d, with a change of differentiation media after 72 h. After differentiation, cells were treated with DA (150 μM) in the presence and absence of NET inhibitors, DAT inhibitors, or MAO inhibitors (MAOIs) in differentiation media over 2-48 h. Differentiated PC12 cells were also treated with 150 μM DOPAC for 24 h. Control cultures underwent a media change at the same time as treated cultures. The concentration of DA (150 μM, up to 48 h) was selected, since that treatment regimen resulted in significant, but not excessive (>50%) amounts of cell death, so that biochemical measurements and potentially protective treatments could be utilized.

*DA Uptake Inhibition Treatment*:

Cells were treated with 150 μM DA; 10 μM GBR12909 and 1μM desipramine (dopamine transporter [DAT] inhibitor and norepinephrine transporter [NET] inhibitor, respectively); 10 μM GBR12909 and 1μM desipramine for 30 minutes prior to 150 μM DA in the presence of DAT and NET inhibitors; or control media, on the last day of differentiation. Cell viability was determined by cell counting using the trypan blue exclusion method.

*DA Uptake Radioassay:*

Differentiated PC12 cells were treated for 30 min or 24 h with 150 μM DA + 18.18 μM $^{14}$C-DA (10 μCi) alone, or with a 30 min pretreatment and co-treatment of 10 μM GBR12909, a DAT inhibitor, and 1μM desipramine, a NET inhibitor. PC12 cells were then collected, rinsed

several times in PBS, and lysed in 6.5% trichloroacetic acid (TCA) with high-speed centrifugation. Radioactivity was measured in an aliquot of the media, the cell supernatant, and the cellular pellet (dissolved in 0.1 N NaOH) in a Beckman LS 6500 scintillation counter.

*MAOI Treatment*:

Cells were treated with 150 μM DA alone; 100 μM clorgyline alone (monoamine oxidase A inhibitor); 100 μM pargyline alone (monoamine oxidase A/B inhibitor); 100 μM clorgyline 30 min prior to 150 μM DA plus clorgyline; 100 μM pargyline 30 min prior to 150 μM DA plus pargyline; or control media, on the last day of differentiation. Cell viability was determined by cell counting using the trypan blue exclusion method.

*Biochemical analysis:*

DA and DOPAC measurements were obtained from collected PC12 cells following treatment. PC12 cells were force-pipetted off the plates, rinsed in PBS, and gently pelleted by centrifugation with the treatment medium. PC12 cellular protein was acid precipitated in 0.1 N perchloric acid (PCA) and centrifuged at 14,000 x g for 25 min. An aliquot of the supernatant was extracted with alumina, and injected into an HPLC system containing an ESA (Chelmsford, MA) Coulochem II coulometric detector (+280 V). Free cysteinyl catechol (free cys-DA, free cys-DOPAC, and GSH-DA) measurements were obtained from an alumina extracted aliquot of the supernatant injected into an HPLC system containing a Waters Associates (Milford, MA) 460 amperometric detector set at an oxidizing potential of 0.6 V. Protein cysteinyl-catechols (protein cys-DA and cys-DOPAC) were measured from the protein pellet, as described previously (Hastings and Zigmond, 1994). In brief, protein from the acid precipitated pellet was hydrolyzed

in 6 N HCl containing 1 mg/ml BSA at 110$^{o}$C for 22 h. The hydrolyzed protein samples were extracted with alumina prior to analysis on HPLC with a Waters 464 amperometric detector set at an oxidizing potential of 0.6 V. Peaks for catechols and cysteinyl-catechols were identified and quantified by comparison to standards.

*ATP measurement:*

Following 2-48 h DA treatment, cells were force-pipetted off the plates, rinsed in PBS, gently pelleted by centrifugation with the treatment medium. PC12 cell protein was precipitated in 2% TCA and centrifuged at 14,000 x g for 25 min. A luciferase-based assay was used to measure ATP levels in an aliquot of the resulting supernatant (Ronner et al., 1999). A Monolight 3010 luminometer (Pharmingen, San Diego, CA) was used to measure the light output resulting after an aliquot of diluted cell sample, 30mM HEPES, pH 7.75, and Enlighten rLuciferase/Luciferin reagent (Promega, Madison, WI) were mixed in a cuvette. Protein amounts were determined by the Bradford assay (Bradford, 1976).

*Western Blot Analysis:*

Control PC12 cells were cultured as described above. Following 6 d of differentiation, media was removed and PC12 cells were collected in PBS using force-pipetting. Cells were combined with the media before pelleting, to collect any floating cells. Cells were re-suspended once in PBS and re-pelleted prior to lysis in a buffer containing: 9M urea, 2% w/v CHAPS, 30 mM Tris-base, and protease inhibitor cocktail [PIC] (2.5 μL/mg protein), pH 8.5. Protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes using a Trans-blot SD Semi-Dry Electrophoretic Transfer Cell (Biorad). Following transfer, the blots were washed

in PBS, blocked overnight at 4°C with 1:1 Odyssey blocking buffer (Li-Cor, Lincoln, NE): PBS-T (PBS + 0.1% Tween 20), then incubated with primary antibody at RT. The blots were then washed in PBS-T and incubated at RT with IR-Dye 680 or 800 secondary antibody (Li-Cor, Lincoln, NE). Blots were then washed again in PBS-T, with a final wash in PBS prior to the scanning on the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE). Antibodies used were DAT (1: 1000), NET (1:750), actin (1:100,000; Sigma). Actin was used as a loading control. There was no significant difference in the actin band densities between lanes for each blot (data not shown).

Statistical Analysis:

Differences among group means were determined by ANOVA followed by post-hoc student's t-test with significance determined at $p<0.05$.

## 3.4 RESULTS

### 3.4.1 DA Induces Toxicity in PC12 Cells

To determine whether DA was toxic to differentiated PC12 cells, the viability of DA exposed PC12 cells was measured using trypan blue exclusion. PC12 cell viability was significantly different from matched control following 4 to 48 h of 150 μM DA treatment (Figure 2). The largest difference in cell viability (-35% compared to control) occurs after 24 h of DA exposure. Control PC12 cell viability ranged from 83.6% - 94.4 % viable cells, with the earlier

time-points having the highest control viability and the later time-points having the lower viability.

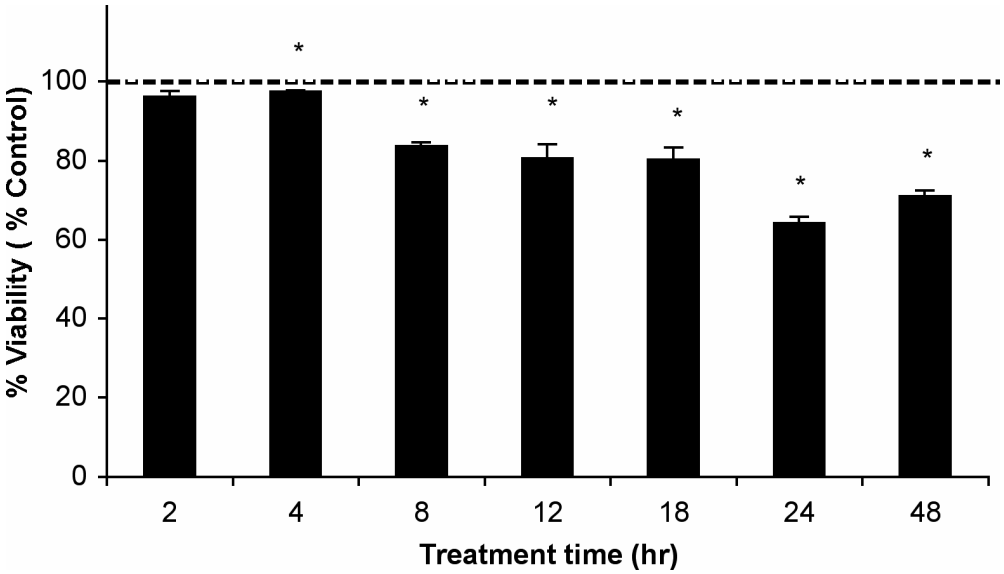


Figure 2: PC12 cell viability following DA treatment.

Viability of PC12 cells treated with 150 μM DA for 2-48 h was measured using trypan blue exclusion. Values are listed as mean % time-matched control ± SEM, n= 3-4. *, significantly different from control, $p<0.05$.

### 3.4.2 DA Treatment Increases Catechol Levels

To determine the effect of DA treatment on catechol levels and to show that DA can be taken up and metabolized by PC12 cells, DA and DOPAC levels were measured by HPLC in PC12 cells following 2-48 h, 150 μM DA treatment. Average control levels of DA in PC12 cells

were 6.07 nmol/mg protein. DA levels were significantly elevated 6-fold above time-matched control, following a 2 h exposure to DA; these levels remained elevated approximately 7-fold above control after 4 and 8 h DA exposure (Figure 3A). At later time-points (24 h and 48 h), the DA levels in treated cells remained above control (2.5 and 1.8-fold, respectively). However, the magnitude (fold change) of the DA level increase was reduced from the shorter DA exposure times (Figure 3A). Intracellular DOPAC levels were also increased following DA treatment. The average control level of DOPAC was 0.39 nmol/mg protein (6.4% of the intracellular DA levels). DOPAC levels were elevated 20-fold above control levels following a 2 h exposure to DA (Figure 3B). DOPAC levels following 4 h and 8 h DA exposure remained elevated above control 13-fold and 18-fold, respectively (Figure 3B). Cellular DOPAC levels remained elevated above control following 24 h DA exposure (4-fold), but were significantly reduced from the earlier time-points (Figure 3B). After 48 h DA treatment, DOPAC returned to control levels (Figure 3B). These data show that DA and DOPAC levels are increased in PC12 cells following DA treatment, indicating that DA is being taken up by the cells and metabolized into DOPAC. The levels of DA and DOPAC in the remaining cells approach control levels after 48 h of DA exposure, suggesting that over time cells can break down the excess DA and DOPAC into other metabolites, such as homovanillic acid.

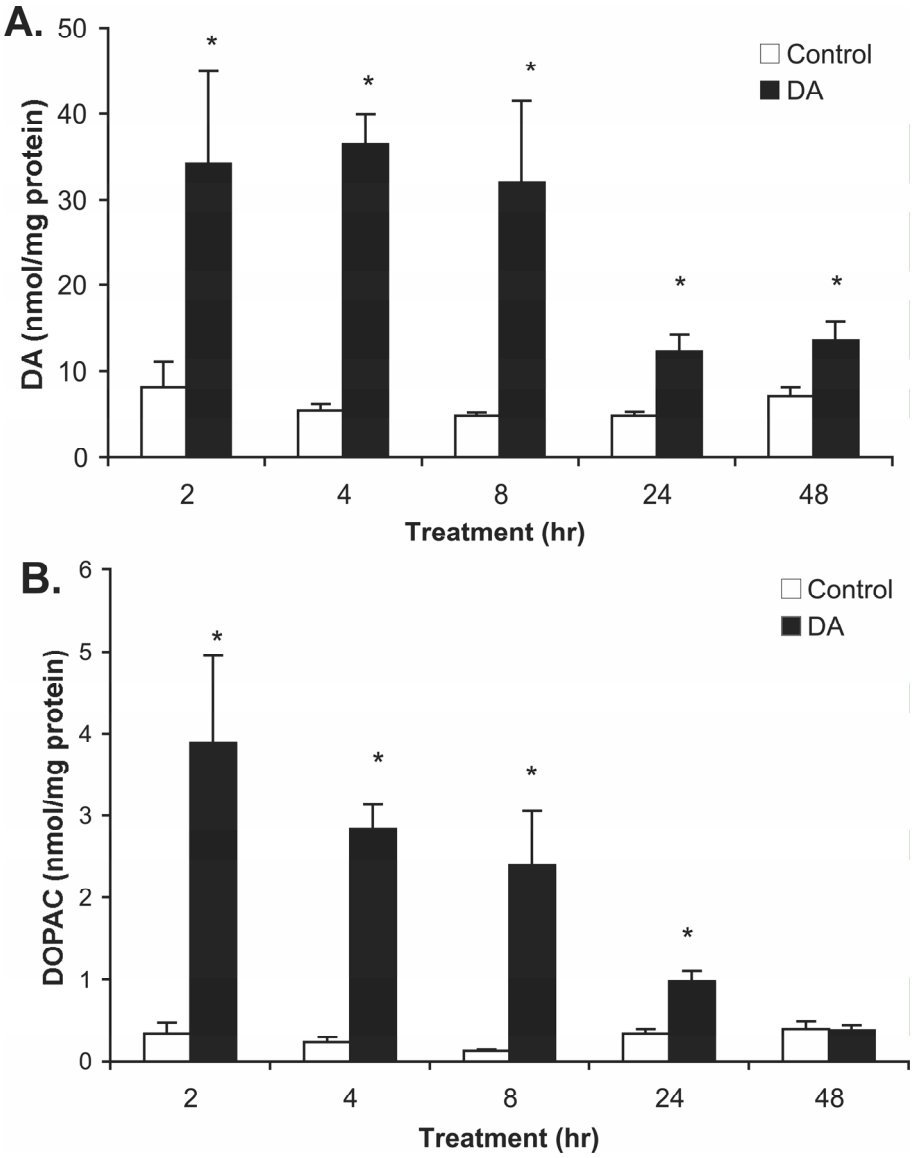


Figure 3: Catechol levels following DA treatment.

PC12 cells were treated with 150 μM DA for 2-48 h, were collected, and assayed for (A) DA or (B) DOPAC levels by HPLC with electrochemical detection. Values are listed as mean ± SEM, n = 3-6. *, Significantly different from control, $p<0.05$.

### 3.4.3 Exogenous DA Treatment Increases DA Oxidation Products in PC12 Cells

We have previously shown that *in vivo* intrastriatal DA injections lead to increased DAQ modified cytosolic free cysteine, GSH, and cysteine on protein (Hastings et al., 1996; Rabinovic et al., 2000). To determine if DA treatment in PC12 cells increased levels of DA oxidation products, free and protein cysteinyl-catechol levels were measured using HPLC with electrochemical detection. Free cysteinyl-catechols are formed when oxidized catechol quinones react with free cysteine residues in the cytosol, or with cysteines found on GSH. All free cysteinyl-catechols were elevated above control following 4 to 8 h application of DA (Figure 4). The average control free cysteinyl-DA (cys-DA) level was 5.7 pmol/mg protein in PC12 cells. Free cys-DA levels were not significantly different from control in PC12 cells treated for 2 h, but after 4 h levels were elevated 3.7-fold above control and were increased to 6.8-fold above control after 8 h of 150 μM DA exposure (Figure 4A). At longer time-points (24 and 48 h), free cys-DA levels remained approximately 2-fold above control (Figure 4A). The average control free cysteinyl-DOPAC (cys-DOPAC) level was 3.6 pmol/mg protein in PC12 cells. Free cys-DOPAC was significantly higher than control in the early DA treatment time-points; after 2h of 150 μM DA exposure, cys-DOPAC levels were elevated 16.5-fold above control, and the levels remained elevated above control after 4 h and 8 h of DA treatment (Figure 4B). Free cys-DOPAC levels were not significantly different from control after 24 h and 48 h DA treatments (Figure 4B). Free GSH-DA levels were significantly higher in DA-treated PC12 cells at all time-points examined (2-48 h). The average control free GSH-DA level was 52.7 pmol/mg protein in PC12 cells. GSH-DA levels rose 2.4-fold above control following 2 h and were elevated to 3.5-fold and 5.8-fold above control after 4 h and 8 h DA exposure (Figure 4C). GSH-DA levels

remained increased after longer exposure times (24 h and 48 h) at 2.9- and 2.7-fold above control, respectively (Figure 4C).

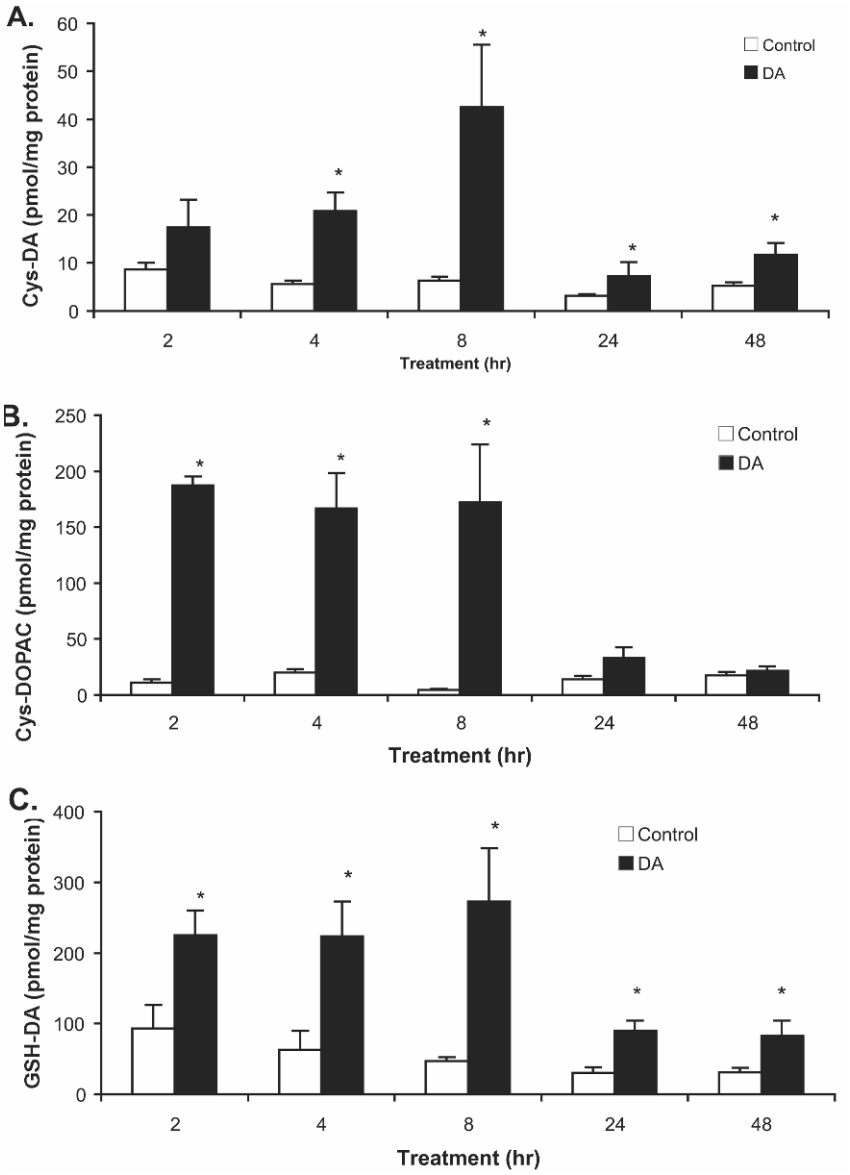


**Figure 4:** Free cysteinyl-catechol levels in PC12 cells following DA treatment.

PC12 cells were treated with 150 μM DA for 2-48 h, were collected, and assayed for (A) free cys-DA, (B) free cys-DOPAC, and (C) GSH-DA levels by HPLC with electrochemical detection. Values are listed as mean ± SEM, n= 3-6. *, Significantly different from control, $p<0.05$.

Protein cysteinyl catechols are formed from oxidized catechols reacting with cysteine residues on proteins. Both protein cys-DA and protein cys-DOPAC levels were elevated significantly above control levels after 4-24 h, 150 μM DA exposure. The average control cys-DA level was 34.7 pmol/mg protein in PC12 cells. Protein cys-DA was not significantly increased in PC12 cells exposed to DA until 4 h of DA exposure. At 4 h, cys-DA levels were elevated 5.6-fold above control, and remained above control at all later time-points, ranging from 4.4- to 7.8-fold above control (Figure 5A). The average control protein cysteinyl DOPAC level was 34.0 pmol/mg protein in PC12 cells. Protein cys-DOPAC levels were significantly higher than control at all DA treatment time-points (Figure 5B). After 2 h, 150 μM DA exposure, cysteinyl-DOPAC levels were elevated 3.2-fold above control, and the levels ranged from 4.8-to 12.5-fold above control at the later time-points (Figure 5B). Protein cysteinyl-catechol levels rose early and remained elevated after longer DA exposure times, indicating that DA oxidation occurs quickly and persists while exogenous DA is present.

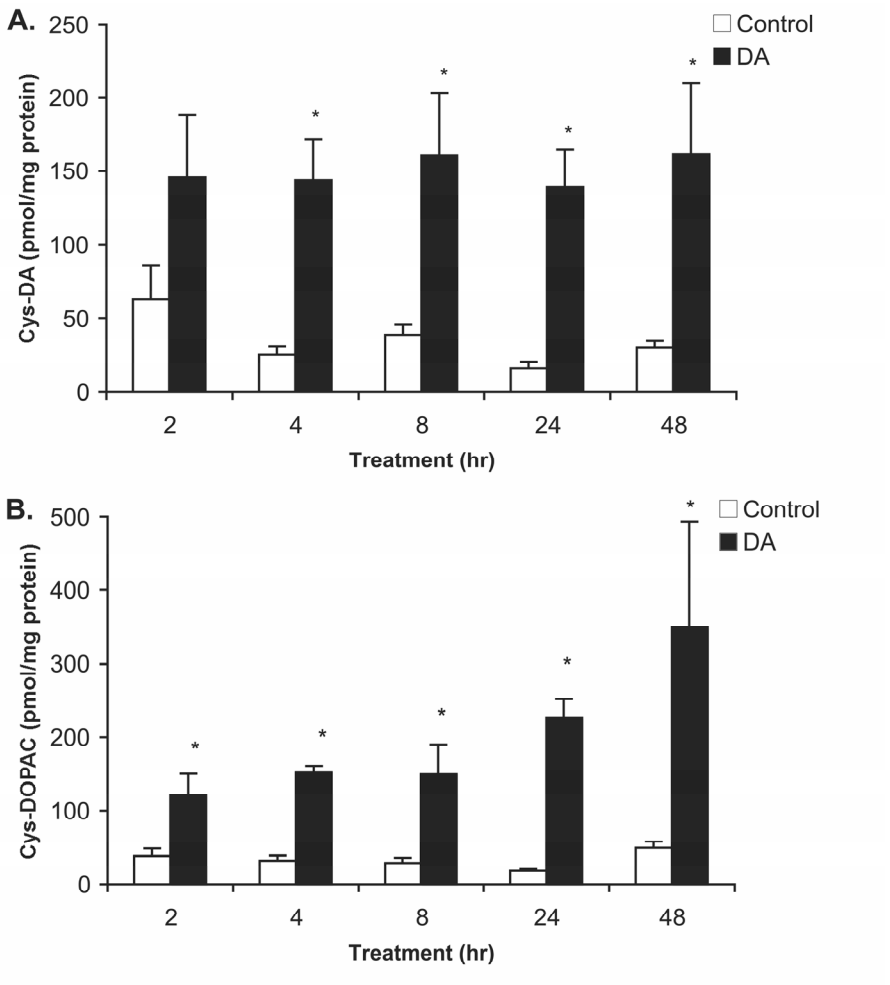


Figure 5: Protein cysteinyl-catechol levels in PC12 cells following DA treatment.

PC12 cells were treated with 150 μM DA for 2-48 h, were collected, and acid precipitated protein pellets were hydrolyzed and assayed for (A) protein cys-DA and (B) protein cys-DOPAC levels by HPLC with electrochemical detection. Values are listed as mean ± SEM, n= 3-6. *, Significantly different from control, $p<0.05$.

### 3.4.4 ATP Depletion in PC12 Cells Following DA Exposure

To determine whether DA treatment leads to energy deficits in PC12 cells, ATP was measured in the cells following 0-48 h, 150 μM DA exposure (Figure 6). The average control ATP level was 37.9 nmol ATP/mg protein. ATP levels were not significantly different from control following 2 to 8 h of DA exposure. However, following an 18 h DA exposure, PC12 cell ATP levels were significantly decreased (-54%) from time-matched controls (Figure 6). Interestingly, the ATP levels following 24 h of DA exposure rose and were not significantly different from control levels. At 48 h, ATP levels dropped again and were decreased (-36%) from control. The transient increases in ATP levels following 24 h of DA exposure may be the result of increased ATP production via glycolysis, stimulated by DA-induced oxidative damage of mitochondria. PC12 cells have previously been shown to resort to increased glycolysis when challenged with mitochondrial inhibitors (Kang et al., 1997).

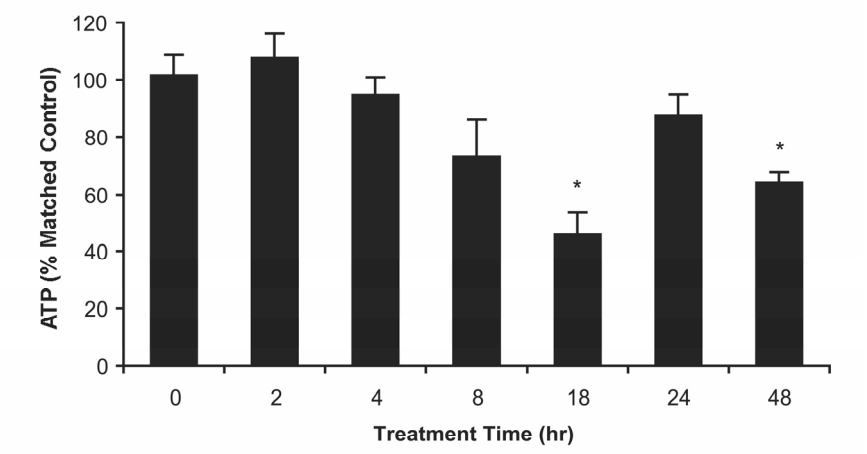


Figure 6: ATP levels in PC12 cells following DA treatment.

PC12 cells were treated with 150 μM DA for 0-48 h, were collected, and assayed for ATP using a luciferase-based assay. Values are listed as mean % matched control ATP ± SEM, n= 3-4. *, significantly different from control, $p<0.05$.

### 3.4.5 MAO Inhibition Does Not Effect DA-Induced Toxicity

There are two pathways for DA to increase oxidation and result in cell death. DA can directly oxidize inside the cell into DAQ, modifying GSH and proteins, or DA metabolism by MAO results in the formation of $H_2O_2$ inside the cell, leading to increased oxidative stress. Therefore, we wanted to determine whether DA metabolism by MAO (or DA uptake) plays a role in DA-induced toxicity in PC12 cells. To determine how blocking DA metabolism by MAO affected the formation of protein-cysteinyl catechols, differentiated PC12 cells were exposed to 150 μM DA in the presence or absence of 100 μM clorgyline, a MAO-A inhibitor. PC12 cells contain MAO-A and are insensitive to some MAO-B inhibitors (Youdim et al., 1986). Treatment with clorgyline reduced DOPAC to non-detectable levels, as measured by HPLC with electrochemical analysis (data not shown). Analysis of DA oxidation was measured in PC12 cellular protein assayed for cysteinyl-DA and cysteinyl-DOPAC adducts using HPLC. Following 24 h exposure to 150 μM DA, cys-DA levels were increased 5.2-fold above control and cys-DOPAC levels were increased 4.0-fold above control (Figure 7A). Exposure to 100 μM clorgyline alone for 24 h did not affect cys-DA levels, and decreased cys-DOPAC levels -38% compared to control (Figure 7A). However, exposure to 150 μM DA in the presence of 100 μM clorgyline for 24 h led to a 15.2-fold increase, above control, in cys-DA levels and no change in cys-DOPAC levels (Figure 7A). The large increase in cys-DA levels after DA and MAOI treatment reflect the inability of the PC12 cells to metabolize DA into DOPAC, resulting in high levels of DA oxidation and cys-DA protein modification.

To determine the effect of MAO inhibition of DA-induced toxicity, differentiated PC12 cells were treated for 24 h with 150 μM DA in the presence or absence of 100 μM clorgyline or

100 μM pargyline, which are both MAO inhibitors, then assayed for viability using the trypan blue exclusion assay. The viability of PC12 cells treated with 150 μM DA for 24 h was significantly decreased -31% compared to control (Figure 7B). Neither pargyline nor clorgyline treatment alone had any effect on PC12 cell viability (data not shown). Viability was decreased when the cells were pre- and co-treated with 100 μM pargyline or 100 μM clorgyline and 150 μM DA (-28% and –34%, respectively; Figure 7B). This data suggests that DA-induced toxicity is not due to the production of $H_2O_2$ via DA metabolism by MAO.

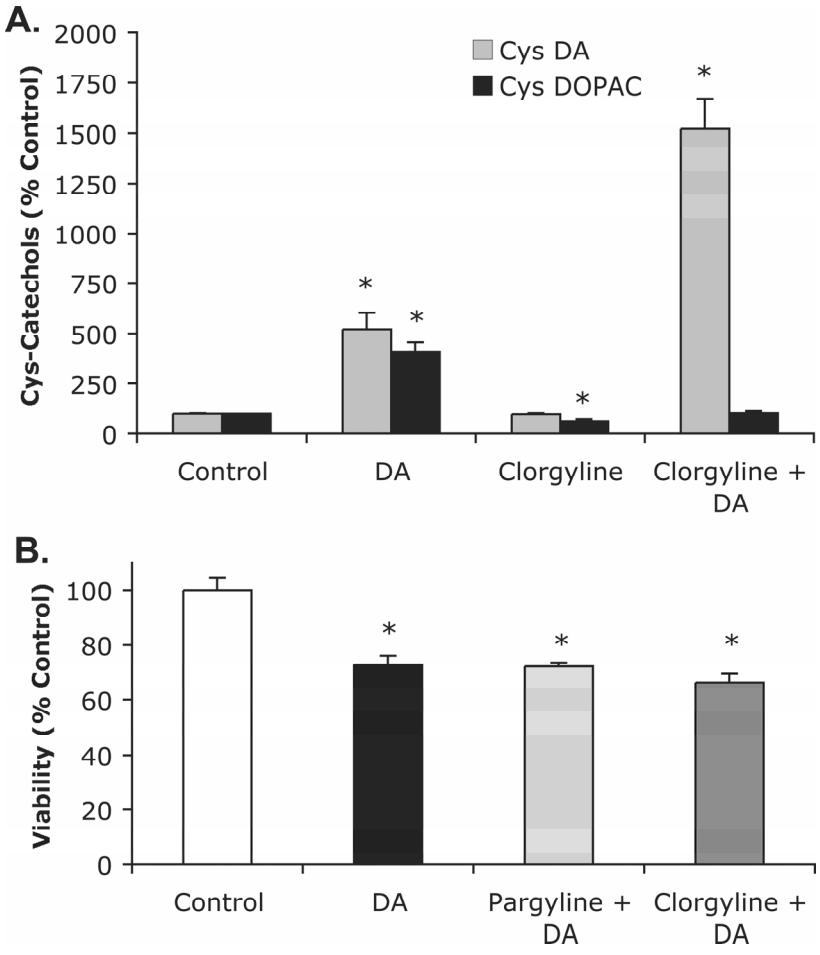


Figure 7: The effect of MAO in inhibition on DA oxidation and toxicity.

PC12 cells were treated with 150 μM DA in the presence or absence of 100 μM clorgyline or 100 μM pargyline for 24 h. **A.** PC12 cells were collected and assayed for protein cys-DA and

cys-DOPAC levels by HPLC with electrochemical detection. Values are listed as mean % control ± SEM, n= 4-5. *, Significantly different from control, $p<0.05$. **B.** Viability of PC12 cells was measured using trypan blue exclusion. Values are listed as mean % matched control ± SEM, n= 3-5. *, significantly different from control, $p<0.05$.

### 3.4.6 DOPAC Is Not Toxic to PC12 Cells

To determine whether extracellular oxidation also leads to toxicity, PC12 cells were exposed to DOPAC and assayed for protein cysteinyl-catechols and viability. DOPAC is a metabolite of DA, has a similar catechol ring that can oxidize and form DOPAC quinones, and thus can react with reduced sulfhydryls in the cell. However, DOPAC is not a substrate for DAT, and therefore is not transported into the cells. PC12 cells were treated with 150 μM DA or DOPAC for 24 h and protein cysteinyl-catechols were measured by HPLC. Protein cys-DA was increased 14-fold above control following DA, but was not affected by DOPAC treatment (Figure 8A). Protein cys-DOPAC levels were increased 11-fold above control following DA, and 6.5-fold above control following DOPAC exposure (Figure 8A). The level of protein cys-DOPAC formed following DA treatment versus DOPAC treatment was not significantly different, but the total amount of protein modification (cys-DA + cys-DOPAC) was much higher in the DA treated group. The formation of protein cys-DOPAC following DOPAC treatment indicates that DOPAC was oxidized into DOPAC quinone and reacted with reduced sulfhydryls on PC12 cell proteins, likely on the outside plasma membrane surface. PC12 cell viability was

not affected by 24 h exposure to DOPAC (Figure 8B), indicating that the extracellular oxidation of DOPAC and modification of proteins was not sufficient for PC12 cell death.

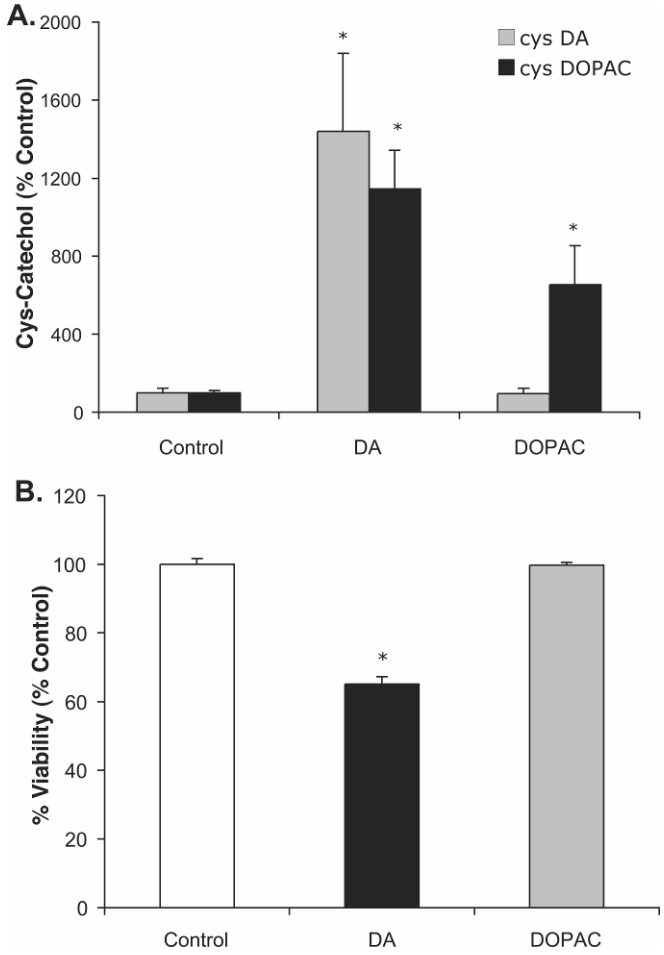


Figure 8: The effect of DOPAC treatment on catechol oxidation and PC12 cell viability.

PC12 cells were treated with 150 μM DA or 150 μM DOPAC for 24 h. **A.** PC12 cells were collected and assayed for protein cys-DA and cys-DOPAC levels by HPLC with electrochemical detection. Values are listed as mean % time-matched control ± SEM, n= 3-9. *, Significantly different from control, $p<0.05$. **B.** Viability of PC12 cells was measured using trypan blue exclusion. Values are listed as mean % time-matched control ± SEM, n= 3-6. *, significantly different from control, $p<0.05$.

### 3.4.7 DA Uptake Inhibition Completely Attenuates DA-Induced Toxicity

To determine whether intracellular or extracellular DA oxidation was toxic to PC12 cells, DA uptake was blocked by GBR12909, a DAT inhibitor, and desipramine, a NET inhibitor. The presence of DAT and NET in our differentiated PC12 cells was determined by Western blot (Figure 9A). Since NET can transport DA into cells, we needed to inhibit NET in addition to DAT to make sure that DA uptake was being blocked efficiently. First, we wanted to determine whether 10 μM GBR12909 and 1 μM desipramine would sufficiently block DA uptake into PC12 cells. Therefore, differentiated PC12 cells were treated for 30 min or 24 h with 150 μM DA + 18 μM $^{14}$C-DA in the presence or absence of the DAT and NET inhibitors, and then the amount of intracellular radioactivity was determined by scintillation counting. Levels of intracellular exogenously applied $^{14}$C-DA were decreased 57±3%, n=3 following 30 min and 80±1%, n=3 following 24 h in PC12 cells pre-treated with DAT and NET inhibitors compared to PC12 cells treated with DA alone (data not shown). Therefore, 10 μM GBR12909 and 1 μM desipramine were used in all other experiments to block DA uptake.

To determine how blocking DA uptake affected the formation of protein-cysteinyl catechols, differentiated PC12 cells were exposed to 150 μM DA in the presence or absence of 10 μM GBR12909 and 1μM desipramine. The cellular protein was collected and assayed for cysteinyl-DA and cysteinyl-DOPAC adducts using HPLC. Following 24 h exposure to 150 μM DA, cys-DA levels were increased 5.2-fold above control (Figure 9B). Exposure to DA in the presence of 10 μM GBR12909 and 1μM desipramine for 24 h led to a 3.5-fold increase, above control, in cys-DA levels (Figure 9B). Exposure to 10 μM GBR12909 and 1μM desipramine alone for 24 h led to a significant decrease (-47% compared to control) in cys-DA (Figure 9B).

Following 24 h exposure to 150 μM DA, cys-DOPAC levels were increased 4.0-fold above control (Figure 9B). However, exposure to 150 μM DA in the presence of 10 μM GBR12909 and 1μM desipramine for 24 h led to a 4.9-fold increase, above control, in cys-DOPAC levels (Figure 9B). Exposure to 10 μM GBR12909 and 1μM desipramine alone for 24 h did not affect cys-DOPAC levels (Figure 9B). Both protein cys-DA and cys-DOPAC conjugates were formed even when DA uptake was blocked. Although we expect some DA was taken up into the cells despite using uptake inhibitors, most protein modification likely occurred on external proteins of PC12 cells.

To determine the role of DA uptake on DA-induced toxicity, differentiated PC12 cells were treated with 150 μM DA for 24 h in the presence or absence of DAT and NET inhibitors, and the viability of the cells was assayed using the trypan blue exclusion assay. The viability of PC12 cells treated with 150 μM DA for 24 h was significantly decreased -32% compared to control (Figure 9C). This toxicity was completely attenuated when the cells were pre- and co-treated with 10 μM GBR12909 and 1μM desipramine, DAT and NET inhibitors (Figure 9C). GBR12909 and desipramine treatment alone had no effect on PC12 cell viability (Figure 9C). This data suggests that DA must be taken up into PC12 cells to be toxic, and thus uptake of DA plays a major role in DA-induced toxicity. Since we still observe DA oxidation, which is probably mostly extracellular, following DA exposure with DA uptake inhibition, the complete attenuation in toxicity we observe due to DA uptake inhibition can not be due to only blocking catechol oxidation and protein cys-catechol formation. These results lend evidence that the intracellular oxidation of catechols, and perhaps the resulting protein modification of critical proteins by catechol-quinones, may play an important role in cell death in the DA-induced toxicity model. However, additional experiments will be required to determine differences in

proteins targeted by DAQ modification in DA exposed PC12 cells in the presence or absence of DAT/NET inhibition.

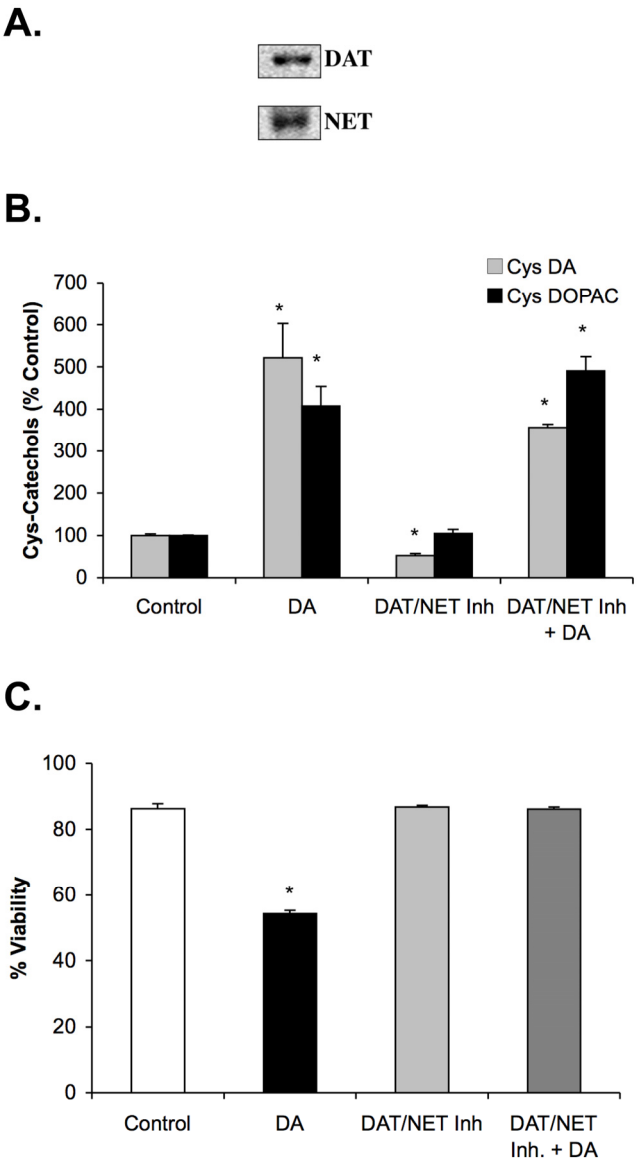


Figure 9: The effect of DA uptake inhibition on DA oxidation and toxicity.

PC12 cells were differentiated for 6 d in NGF **A.** PC12 cells were collected and lysed. Samples containing 30 µg protein were separated by 10% SDS-PAGE and transferred to nitrocellulose. Blots were probed for DAT and NET immunoreactivity. Each lane represents total cellular protein from a different plate of differentiated PC12 cells. **B.** PC12 cells were treated with 150

μM DA in the presence or absence of 10 μM GBR12909 and 1 μM desipramine for 24 h. PC12 cells were collected and assayed for protein cys-DA and cys-DOPAC levels by HPLC with electrochemical detection. Values are listed as mean % time-matched control ± SEM, n= 3-9. *, Significantly different from control, $p<0.05$. **C.** Viability of PC12 cells was measured using trypan blue exclusion. Values are listed as mean % time-matched control ± SEM, n= 3-6. *, significantly different from control, $p<0.05$.

## 3.5 DISCUSSION

### 3.5.1 DA-Induced Toxicity Model

We have found that differentiated PC12 cells are susceptible to DA-induced toxicity. Viability of PC12 cells was affected as early as 8 h (-16% control) following DA exposure. Toxicity reached a maximum after 24 h, with a 35% decrease in viability from time-matched control (Figure 2). Following DA, treatment levels of DA and DOPAC increased in PC12 cells, indicating that DA was being taken up into the cells and metabolized into DOPAC (Figure 3).

Although DA toxicity in PC12 cells is not a novel finding, most DA-toxicity studies are performed with undifferentiated PC12 cells undergoing division. Therefore viability assays in these studies may also reflect differences in the number of cells initially plated and changes in the ability of PC12 cells to proliferate following DA exposure, in addition to changes in cell loss. This may explain the wide range of toxicity reported in undifferentiated PC12 cells, which can range from approximately 10% (Perez et al., 2003; Wang et al., 2005) to 70% (Song et al., 2004;

Xiao-Qing et al., 2005) cell loss for 24 h, 100 μM DA exposure. Very few studies have looked at DA-induced toxicity in differentiated PC12 cells. One study by Ishisaki et al., 2001 found a 25% cell loss in differentiated PC12 cells following 24 h of 250 μM DA exposure, which was attenuated by over-expression of GSH S-transferase class Pi, a detoxifying protein that conjugates GSH to various hydrophobic and electrophilic compounds, including catechol quinones (Baez et al., 1997). Another quinone-reducing protein, NAD(P)H quinone oxidoreductase 1 (NQO1), has also been shown to protect SK-N-MC cells against DA exposure (Zafar et al., 2006b). Decreased levels of catechol-O-methyl transferase (COMT), an enzyme that catalyzes the methylation of the 3-position hydroxyl group on catechols, is also associated with increased DA toxicity in SK-N-SH cells (Ogburn et al., 2006). COMT assists the cell in preventing the formation of quinones, since the methylation of one hydroxyl group prevents the ability of catechols, like DA, to oxidize into quinones. Increased oxidative stress is also associated with potentiating DA-induced toxicity. The exacerbation of DA-induced toxicity by potassium cyanide (KCN), which has been shown to increase ROS and NO and inhibit antioxidant systems and mitochondrial function, has also been reported in PC12 cells (Jones et al., 2000). Another study examining the effect of DA on cell death induced by serum and NGF withdrawal, found that a low amount of DA (10-30 μM) was protective, but that 100 μM DA alone induced cell death and combined with NGF and serum withdrawal, exacerbated toxicity in differentiated PC12 cells (Koshimura et al., 2000). Although we found higher levels of cell death, these other studies support our finding that DA is toxic to differentiated PC12 cells, and that oxidative stress induced by DA is likely involved in cell death in this model.

### 3.5.2 DA Oxidation and the Formation of Cysteinyl-Catechol Conjugates

We are the first to measure direct DA and DOPAC quinone modification of reduced cysteines both free and protein-bound in cell culture. Free cysteinyl catechols, including cys-DA, cys-DOPAC, and GSH-DA were all increased following DA exposure (Figure 4), indicating that DA and DOPAC quinones were formed and reacted with free reduced cysteine and GSH in PC12 cells. Levels of free cysteinyl catechols rose early, but after 24 and 48 h of DA treatment, all free cysteinyl catechol levels dropped from the initial increased amounts. At these later time-points, the intracellular DA and DOPAC levels are also lower (See Figure 2), which is likely due to the metabolism of DA into DOPAC and the subsequent diffusion of DOPAC into the media. Lower DA and DOPAC levels likely influence the free cys-DA, cys-DOPAC, and GSH-DA levels, since these conjugates may be cleared quickly by the cells. We have previously observed the transient nature of free cysteinyl-catechols following striatal injections of DA, in which free cysteinyl-catechol levels also dropped 24 h after DA intrastriatal injections (Hastings et al., 1996; Rabinovic et al., 2000). Therefore, free cysteinyl-catechol levels are not a stable measure for long-term oxidative changes in the DA-induced toxicity model.

Increased protein oxidation in the form of carbonyls have previously been measured following DA exposure in undifferentiated PC12 cells; these levels were observed after 6 h of DA exposure, however since there was only one time point, it is possible that carbonyl formation occurred even earlier (Keller et al., 2000). Other markers of oxidative stress, such as increased ROS, carbonyl formation, decreased free thiol groups, decreased GSH, and decreased NADPH have been observed following DA exposure in various cell lines (Gabby et al., 1996; Offen et al., 1997; Jones et al., 2000; Keller et al., 2000; Pedrosa and Soares-da-Silva, 2002; Grima et al., 2003). However, we are the first to measure direct catecholamine quinone oxidation of proteins

on their cysteinyl residues. We found that both protein cys-DA and cys-DOPAC levels were increased as soon as 4 h following DA exposure and remained elevated after longer (24-48 h) DA treatments in PC12 cells (Figure 5). In previous *in vivo* studies (Rabinovic et al., 2000), protein cysteinyl-catechol levels also remained high after 24 h following a DA intrastriatal injection. Therefore, this cell culture study supports the *in vivo* finding that protein-cysteinyl catechols are a better measure of long-term damage due to DA-induced oxidative stress. One would expect that protein cysteinyl-catechol levels would decline over time, since modified proteins are more likely to be quickly targeted for degradation. However, oxidative stress in general and dopaminergic toxins (including DA) have been shown to decrease proteasome activity (Keller et al., 2000; Asanuma et al., 2004; Bader and Grune, 2006). One protein in the ubiquitin proteasome pathway linked to inheritable PD, parkin, has been shown to be modified by DA, leading to decreased E3 ligase activity (LaVoie et al., 2005). Therefore, DA oxidation may induce toxicity both through modification of target proteins and by inhibiting protein degradation, leading to protein aggregation and cell death.

### 3.5.3 DA-induced Toxicity and Energy Deficits

The critical protein targets that are responsible for DA toxicity remain unknown, although many likely candidates may be found in mitochondria, since mitochondrial function is critical to cell survival. Many neurodegenerative disorders are related to deficiencies of mitochondrial complex activities (Orth and Schapira, 2001; Beal, 2005; Kwong et al., 2006). Mitochondrial respiration, and therefore ATP production, can be disrupted by ROS and quinones, and ROS or sulfhydryl modifying agents have been shown to inhibit electron transport chain enzymes (Kenney, 1975; Yagi and Hatefi, 1987; Zhang et al., 1990; Benard and

Balasubramanian, 1995). We have previously shown that DAQs uncouple mitochondria respiration, lead to a decrease in state 3 respiration, and result in the opening of the PTP (Berman and Hastings, 1999; Gluck and Zeevalk, 2004). Therefore, we wanted to determine if ATP levels were altered following DA exposure. We found that ATP levels in PC12 cells were decreased -54% and -36% from time-matched controls following 18 h and 48 h, but were not significantly different after 24 h of 150 μM DA exposure (Figure 6). We believe that the increases in ATP levels after DA exposure for 24 h may be due to the stimulation of glycolysis induced by the oxidative damage of mitochondria. Mitochondrial inhibitors have been shown to increase glycolysis in PC12 cells (Kang et al., 1997). We have also observed this boost of ATP levels in PC12 cells after 24 h exposure to rotenone, a mitochondrial complex I inhibitor, and therefore we believe it is a compensatory mechanism to cope with mitochondrial dysfunction (Dukes et al., 2005). However following longer exposures to toxins, this compensatory mechanism seems to fail, resulting in decreased levels of ATP after 48 h of exposure to DA (Figure 6) or rotenone (Dukes et al., 2005). DA toxicity may be partially due to mitochondrial dysfunction from protein modification and inactivation by DA quinone and ROS production.

### 3.5.4 MAO Contribution to DA-Toxicity

DA metabolism into DOPAC by MAO, which leads to the production of $H_2O_2$, has been proposed to play a role in dopaminergic neurodegeneration (Graumann et al., 2002). Hydrogen peroxide, one of the less reactive forms of ROS, is itself toxic to all cells and can be oxidized to more reactive ROS, such as superoxide anion and hydroxyl radical, leading to oxidative damage (Szeto, 2006). Hydrogen peroxide has been shown to be toxic to PC12 cells, with 0.5 mM $H_2O_2$ leading to a 50% cell loss in undifferentiated PC12 cells (Halleck et al., 1992; Clement et al.,

2002). Therefore, we wanted to determine what part, if any, of DA-induced toxicity was due to MAO metabolism of DA. Differentiated PC12 cells exposed to 150 μM DA for 24 h in the presence of 100 μM clorgyline, a MAO-A inhibitor, led to 15.2-fold increased protein cys-DA conjugates compared to 5.2-fold above control for DA alone treated cells. Protein cys-DOPAC levels were not different from control when cells were treated with DA and clorgyline, but were increased 4.0-fold after DA alone (Figure 7). Previous studies in PC12 cells have shown a mixed response to MAOI on DA-induced toxicity, including significant protection (Cantuti-Castelvetri and Joseph, 1999; Keller et al., 2000), very little protection (Perez et al., 2003), and in one study MAOIs potentiated DA toxicity (Weingarten and Zhou, 2001). In this study, MAOI inhibition with either pargyline or clorgyline co-treated with DA did not affect DA-induced toxicity, indicating that MAO metabolism of DA does not play a role in cell death induced by DA.

### 3.5.5 DA Uptake Contribution to Toxicity

Since MAO inhibition did not affect DA-induced toxicity, we wanted to examine the contribution of extracellular versus intracellular catecholamine oxidation in toxicity. Previous experiments in our lab have shown that intrastriatal DOPAC injections led to cys-DOPAC formation, but did not damage the dopaminergic fibers of the striatum (Hastings, unpublished observations). In addition, intrastriatal injections of DA into DAT knockout mice, also did not cause degeneration (Hastings, unpublished observations). Therefore, we hypothesized that PC12 cell exposure to DOPAC would not be toxic. We observed increased protein cys-DOPAC levels, but no change in viability after DOPAC exposure in PC12 cells (Figure 8), indicating that cell death did not rely only on extracellular oxidation of catechols and modification of proteins.

We utilized GBR12909, a DAT inhibitor, and desipramine, a NET inhibitor, to block DA uptake into PC12 cells, to determine whether DA uptake was necessary for DA-induced toxicity. Increases in protein-cysteinyl catechol levels after DA exposure remained similar even when DA uptake was blocked (Figure 9). Although we expected most of the protein cysteinyl-catechols formed were extracellular, the increased levels of cysteinyl-DOPAC indicated that DOPAC was also being oxidized. Whether this cysteinyl-DOPAC originated from increased DA metabolism inside the cell, or was due to some of the DA being taken up into the cell, we found that blocking most of DA uptake completely attenuated DA-induced toxicity. The role of DA uptake in DA-induced toxicity has been previously explored using DAT inhibitors with varying results, including no protection (Clement et al., 2002), partial protection (Jones et al., 2000; Keller et al., 2000; Perez et al., 2003), and complete protection (Cantuti-Castelvetri and Joseph, 1999). However, NET uptake of DA was not taken into account. NET has been shown to transport DA *in vivo* (Moron et al., 2002), NET and DAT mRNA expression has been measured in PC12 cells (Lorang et al., 1994; Kadota et al., 1996), and we found both DAT and NET immunoreactive bands in differentiated PC12 cells (Figure 9). Therefore, differences in the ability of DAT inhibition to protect against DA-induced toxicity in various studies may be due to incomplete inhibition of DA uptake. In addition, the question as to whether DA toxicity may be a product of cell culturing conditions was raised by a study that reported production of $H_2O_2$ due to DA oxidization in cell culture medium (Clement et al., 2002). However, according to this study, 100 μM DA only produced 10 μM $H_2O_2$ in DMEM after 2 h of incubation (the longest reaction time reported) (Clement et al., 2002), which is a relatively low amount of $H_2O_2$ that has previously been shown not to be toxic to PC12 cells (Chen et al., 2005b; Chai et al., 2006).

Therefore, we believe that DA-induced toxicity in differentiated PC12 cells was due to DA uptake, and not through a non-specific effect.

Other than DA-induced oxidative stress leading to toxicity, DA could also affect cells via D1 receptor activation, which has been found in other cell culture systems (Chen et al., 2004b; Moussa et al., 2006). Our DA uptake inhibition data suggest that extracellular DA has no effect on DA-induced toxicity, which would include both extracellular DA oxidation and DA signaling through extracellular receptors. This concurs with another study which reported that the D1 antagonist SCH-23390 did not affect DA-induced toxicity (Clement et al., 2002). Although D1 and D2 receptors have been shown to be involved in striatal cell degeneration following 6-OHDA, methamphetamine, dopamine, L-DOPA, and MPTP treatment *in vivo* and *in vitro* (Araki et al., 2000; Muralikrishnan and Ebadi, 2001; Angulo et al., 2004; Ishida et al., 2004; Mark et al., 2004; Wersinger et al., 2004; Taylor et al., 2005; Xu et al., 2005b; Fiorentini et al., 2006), our results suggest that intracellular DA oxidation may play a role in dopaminergic cell death following exogenous application of DA.

### 3.5.6 Relevance of DA Oxidation to PD Neurodegeneration

The DA-induced toxicity model employs the addition of exogenous DA, which results in oxidative stress and dopaminergic cell death. In disease, increased levels of endogenous cytoplasmic DA could occur though various mechanisms, including vesicular dysregulation. Impaired DA storage has been reported in α-synuclein knockout mice (Cabin et al., 2002), and recently, increased cytosolic catecholamine levels were measured in A30P α-synuclein over-expressing but not wild type α-synuclein over-expressing chromaffin cells (Mosharov et al., 2006), possibly linking genetic PD to DA vesicular dysfunction. Decreased vesicular

monoamine transporter (VMAT2) gene expression levels have also been observed in remaining neurons of PD patient substantia nigra (Harrington et al., 1996). Vesicular DA concentration has been estimated around 1 mM in Planorbis corneus (snail) neurons (Anderson et al., 1999a). Therefore, if vesicular storage of DA fails, large amounts of DA could be potentially released inside a neuron, where it can oxidize into quinones and modify proteins. These data suggest that changes in DA storage, which would lead to DA release from the vesicles, could contribute to the dopaminergic selectivity of neurodegeneration in PD, by increasing the availability of DA for oxidation. The presence of excess DA in the cytoplasm, either through exogenous application or due to release of intracellular vesicular stores, may add to the oxidative stress of a cell through ROS and DAQ production and through the subsequent oxidation of important biomolecules, making dopaminergic neurons in the substantia nigra more susceptible to cell death.

# 4.0 INCREASED LEVELS OF ENDOPLASMIC RETICULUM STRESS MARKERS AND DECREASED LEVELS OF ALDOLASE A IN MITOCHONDRIAL-ENRICHED FRACTIONS OF PC12 CELLS FOLLOWING EXPOSURE TO DOPAMINE

## 4.1 SUMMARY

Oxidative stress is implicated in protein misfolding and aggregation, which may activate the unfolded protein response (UPR) by the endoplasmic reticulum (ER). Dopamine (DA) can initiate oxidative stress via $H_2O_2$ formation by DA metabolism and oxidation into DA quinone (DAQ). We have previously shown that oxidative protein modification, mitochondrial dysfunction *in vitro,* and dopaminergic cell toxicity *in vivo* and *in vitro* are induced by DAQ. In this study, we used cysteine- and lysine-reactive fluorescent dyes with 2-D difference in-gel electrophoresis (2D-DIGE), mass spectrometry, and peptide mass fingerprint analysis to identify altered PC12 cell mitochondrial-enriched proteins following DA exposure (150μM, 16h). Quantitative changes in proteins labeled with cysteine- or lysine-reactive dyes indicated increases in a subset of proteins: calreticulin, ERp29, ERp99, Grp58, Grp78, Grp94, and Orp150 (149-260%), and decreased levels of aldolase A (-58% to -61%) in 2D-DIGE experiments. Changes after DA exposure in levels of ER chaperones Grp78 and Grp58, and the glycolytic enzyme aldolase A were also measured using Western blot analysis on PC12 cell mitochondrial-enriched fraction and whole-cell lysate. Using an unbiased proteomics approach, our findings

suggest that in PC12 cells, DA exposure leads to a cellular response indicative of ER stress prior to the onset of cell death.

## 4.2 INTRODUCTION

Oxidative protein modification has been implicated in several neurological disorders, including Alzheimer's disease, Parkinson's disease (PD), and ischemia (Butterfield, 2004; Greenamyre and Hastings, 2004; Schapira, 2004; Christophe and Nicolas, 2006). Dopaminergic neurons may be especially susceptible to oxidative damage due to the reactive nature of dopamine (DA) and its metabolite, dihydroxyphenylacetic acid (DOPAC), which are easily oxidized into DA quinone (DAQ) or DOPAC quinone, respectively. Sulfhydryl groups on cysteinyl residues in free cysteine, glutathione, and on protein are especially vulnerable to quinone modification, leading to the formation of cysteinyl-DA and cysteinyl-DOPAC conjugates (Tse et al., 1976; Graham, 1978; Fornstedt et al., 1990a; Hastings and Zigmond, 1994). DA exposure in PC12 cells and *in vivo* has been shown to increase protein cysteinyl-catechol levels, indicating direct DAQ modification of cysteines in cellular proteins (Hastings et al., 1996; Dukes and Hastings, 2002). In addition, DA exposure has also been shown to be toxic to PC12 cells (Walkinshaw and Waters, 1995; Cantuti-Castelvetri and Joseph, 1999; Jones et al., 2000; Koshimura et al., 2000; Dukes and Hastings, 2002; Xiao-Qing et al., 2005) and to selectively damage DA neurons *in vivo* (Rabinovic et al., 2000). The presence of neuromelanin in normal brain and cysteinyl-catechol conjugates in PD brain suggests that DA oxidation occurs *in vivo* during normal aging and PD disease progression (Spencer et al., 1998; Zecca et al.,

2002b). Therefore, DA-induced toxicity may be an effective model to observe the effects of an endogenous toxin on the delicately balanced redox system in dopaminergic cells.

DAQ modification of protein cysteinyl residues can be especially detrimental, since many vital proteins, including mitochondrial and endoplasmic reticulum (ER) proteins (Ellgaard, 2004; Bailey et al., 2005), contain cysteine residues whose reduced state is essential for activity; any modifications to these cysteines may lead to altered function or inactivation of critical protein pathways. Some of these pathways, including pro- and anti-apoptotic signaling pathways, are shared by the mitochondria and the ER, and can influence one another because of their proximity (Breckenridge et al., 2003; Rao et al., 2004). PD and other neurodegenerative disease models are connected by ER stress, the accumulation of misfolded and oxidized proteins, along with activation of the unfolded protein response (UPR) by the ER (Lindholm et al., 2006). Mitochondrial dysfunction, oxidative stress, protein modification, and UPR activation are all interrelated cell death pathways involved in disease pathogenesis.

In this study, we utilized two dimensional-difference in-gel electrophoresis (2D-DIGE) to observe changes in mitochondrial-enriched fractions isolated from PC12 cells following DA exposure. We used cysteine reactive maleimide-CyDyes and lysine reactive NHS-ester-CyDyes to label proteins, followed by mass spectrometry (MS) and peptide mass fingerprint analysis. Using this unbiased approached, we identified a subset of proteins in the mitochondrial-enriched fraction that were increased following DA exposure, most of which are ER chaperone proteins. Aldolase A was the only identified protein that was decreased in the mitochondrial-enriched fraction following DA. Therefore, the relative increase in the levels of several ER stress proteins in PC12 cells following DA exposure suggests that ER stress is correlated with DA-induced toxicity.

## 4.3 EXPERIMENTAL PROCEDURES

*Chemicals and Reagents:*

Dulbecco's Modified Eagle Medium (DMEM, Gibco brand), fetal bovine serum (HyClone brand), and horse serum (HyClone brand) were purchased from Invitrogen (Carlsbad, CA). Nerve growth factor (NGF) was purchased from BD Bioscience (San Diego, CA). Immobiline Drystrips pH 3-10, maleimide Cy3/Cy5 dyes, N-hydroxysuccinimidyl (NHS)-ester Cy3/Cy5 dyes, and IPG buffer pH 3-10 were purchased from GE Healthcare (Piscataway, NJ). Trypsin for digesting proteins in gel plugs was from Promega (Madison, WI). Protease inhibitor cocktail (PIC) included in mitochondrial isolation was obtained from Sigma (P2714; St. Louis, MO), and PIC added to PC12 cell whole cell lysate was obtained from Roche. All other non-specified reagents were purchased from Sigma. All solutions were made in distilled water purified with a Milli-Q system (Millipore Corp., Bedford, MA) unless otherwise noted.

*Cell culture:*

Proliferating plates of PC12 cells, a dopaminergic cell line, were grown in media containing 7% horse serum (HS) and 7% fetal bovine serum (FBS). For differentiation, PC12 cells were plated at a density of 19,000 cells/cm2 in differentiation media (DMEM + 1% HS, 1% FBS, and 0.1 μg/ml NGF) for 6 days. The differentiated PC12 cells were then exposed to control media or 150 μM DA in media for 2-24 h for Western blot analysis and 16 h for isolated mitochondrial experiments. Control cultures underwent a media change at the same time as DA treated cultures.

*Mitochondrial-enriched Fraction Preparation:*

PC12 cell mitochondrial-enriched fractions were prepared by a modified version of a mitochondrial isolation protocol (Berman and Hastings, 1999). Following exposure to control or DA-containing media, 10-12 100 mm diameter plates (1,500,000 cells/plate) were pooled for each group, collected in PBS, and combined with the treatment media prior to gentle centrifugation into a pellet (800 x g, 3min). The supernatant was discarded, and the cell pellet was re-suspended in isolation buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mg/mL BSA, PIC (2 μL/mL), pH 7.4 and homogenized. The cell homogenate was then centrifuged (12,000 x g) multiple times at 3°C to isolate the mitochondrial-enriched fraction. Both control and DA exposed mitochondrial-enriched pellets were lysed in buffer containing 9M urea, 2% w/v CHAPS, 30 mM Tris-base, and PIC (2.5 μL/mg protein), pH 8.5. The amount of protein in the lysed control and DA exposed mitochondrial-enriched fraction was measured using the Bradford assay (Bradford, 1976). The isolation procedure utilized with the DA and control treated PC12 cells was identical, but the mitochondrial-enriched fractions from the two treatment groups were not pooled until after the completion of the reaction with the fluorescent dyes.

*2D Gel Electrophoresis:*

For first dimension separation of proteins, Immobiline DryStrips (linear pH 3-10; GE Healthcare) were rehydrated overnight at RT, according to the manufacturer's protocol for cup loading of samples. Mitochondrial-enriched lysate from control and DA-exposed PC12 cells were reacted separately with either fluorescent Cy5 or Cy3 maleimide or NHS ester dyes (GE

Healthcare) prior to reduction by DTT. Delaying the addition of DTT to the sample until after exposure to the maleimide dye allows us to look at reduced sulfhydryl labeling differences between control and DA-exposed samples by leaving any oxidized cysteines in their maleimide-unreactive, oxidized form. To confirm that the dyes exhibited no preferential chemistries, control and DA-exposed samples were reciprocally labeled with either Cy5 and Cy3 dyes, and direct comparison showed no evidence of any differential labeling affinities. Maleimides react with reduced sulfhydryl groups, and thus label reduced cysteines. In addition to being sensitive probes for protein levels, any cysteines modified by ROS or DAQ will be unavailable to react with the maleimide dye, and thus result in reduced fluorescence. Maleimide-reactive dyes thus have the potential to detect altered protein levels as well as probe the redox state of constituent cysteine residues. The optimal dye:protein ratio was chosen so that protein spots visualized using the Typhoon 9400 scanner with PMT=600 were clearly visible and not overexposed, and no dye-shifting of spots was visible. Preliminary trials using variable dye to protein ratios indicated that a maleimide reaction ratio of 1 pmol dye to 2 μg protein for 45 min was optimal. After incubation, each maleimide reaction was quenched by the addition of an equal volume of sample loading buffer containing 130 mM DTT, 9 M urea, 2% w/v CHAPS, 2% v/v IPG buffer (pH 3-10; GE Healthcare), and trace amounts of bromophenol blue.

The NHS-ester dye reaction ratio of 2 pmol dye to 1 μg protein was determined to be sufficient for lysine labeling. After the addition of the NHS-ester dye to the mitochondrial-enriched protein (prior to DTT reduction), the reaction was incubated on ice for 30 min and quenched by the addition of 10 nmol lysine (in 1 μL), at 4$^{o}$C. Sample loading buffer was then added in a 1:1 ratio. NHS-ester dyes react with lysine residues, and are used as an indicator of protein amounts. Therefore, if the relative concentration of a protein is altered following DA

treatment, a change in the fluorescent labeling of DA-treated mitochondria as compared to control will be observed.

Following the addition of sample loading buffer for either maleimide or NHS-ester reacted samples, 125 μg of protein from the control mitochondrial-enriched fraction were combined with 125 μg protein from DA exposed PC12 cell mitochondrial-enriched fraction, and the total 250 μg protein were loaded onto the first dimension strip using sample cup loading. To separate proteins by isoelectric point, the first dimension was run on a Multiphor II system (GE Healthcare) with a 3501XL electrophoresis power supply (GE Healthcare) using 4 different phases for a total of 75 kVhr. The strips were prepared for the second dimension by washing with equilibration buffer (75 mM Tris-HCl, pH 6.8, 6M urea, 30% glycerol, 1% w/v SDS) containing 30 mM DTT followed by 240 mM iodoacetamide. The strip was trimmed to 13.5 cm, the molecular weight standards were loaded, and the second dimension was run on a 12% SDS polyacrylamide gel on a Hoeffer SE600 Ruby Electrophoresis Unit (GE Healthcare).

*Visualization of Difference Gels and Spot Picking:*

Gels were initially scanned on a Typhoon 9400 laser scanner using ImageQuant software (GE Healthcare), measuring both Cy5 and Cy3 fluorescence. After the gels were scanned, they were washed twice in Milli-Q H2O, then fixed in 40% methanol, and 1% acetic acid. The gel was then scanned into the automated spot picker in the Genetics and Proteomics Core Laboratories at the University of Pittsburgh (built by Dr. Jonathan Minden of Carnegie Mellon University). Target protein spots that were differentially labeled as visualized by changes in relative fluorescence, plus several spots that appeared not to change, were picked, and gel plugs were placed in a 96-well plate.

*Trypsin Digest and Mass Spectrometry of Target Proteins:*

Gel plugs were washed twice in 50% methanol, 50% ammonium bicarbonate solution (50mM), dehydrated in acetonitrile, and dried by speed-vacuum prior to in-gel trypsin digest at 42oC for 4 h, using Promega Gold trypsin reconstituted in 50% acetonitrile, 0.3% trifluoroacetic acid (TFA), and 1 mM ammonium citrate (200 ng/sample). The gel plug was then rinsed twice in extraction buffer (1% TFA, in 50% acetonitrile and 50 % H2O). The trypsinized protein solution and extraction washes were dried by speed-vacuum.

The dried trypsinized protein was reconstituted in extraction buffer containing 100 μM ammonium citrate and mixed with a saturated solution of CHCA (α-cyano-4-hydroxycinnamic acid) and spotted on a metal MALDI plate along with a trypsin blank and standards (Applied Biosystems). The identification of the protein was determined using a 4700 MALDI-TOF-TOF (Applied Biosystems) mass spectrometer. The resulting ion peak spectra were used for peptide mass fingerprinting, searched against the NCBI database by GPS Data Explorer™ MS analysis software (Applied Biosystems), with the peptide mass tolerance set to 50 ppm and allowing a maximum of 1 missed trypsin cleavage. A positive protein identification was accepted when a confidence interval of >90% of the probability-based MOWSE score was determined by the GPS Data Explorer™ MS analysis software and the identification was replicated in two or more experiments. All listed identified proteins corresponded to only one protein and related isoforms. No limitations other than species designation (All species and *Rattus*) were used in the database search, with no bias towards cell type or organelle.

*Decyder Analysis of Fold Change:*

Identified proteins were analyzed using the DeCyder DIA software (GE Healthcare). Gel images from the Typhoon 9400 laser scanner (GE Healthcare) using equal PMT levels (typically 600 V) between the Cy5 and Cy3 scans, were loaded on the software. Each individual spot was identified and mapped using the DeCyder DIA software. Previously MS-identified spots were located on each gel, and the normalized volume ratio calculated by the DeCyder DIA software was recorded for each spot. The normalized volume ratios were determined by the DeCyder DIA software by first calculating the direct volume ratio of the DA-exposed /control volume densities for every spot on the gel. All of the ratios were then normalized so that the modal peak of all the ratios equals 1, because most proteins should not be up- or down-regulated. Percent change of DA-exposed PC12 cell mitochondrial protein from control mitochondrial protein were determined from the normalized volume ratios, and the percent change was averaged over all gels in which the identified proteins could be determined. Limitations in the DeCyder software led to inaccurate spot mapping, and prevented analysis of one protein. This protein is designated as UN in Table 1. In addition, not all proteins were identified in both cysteine and lysine gels, and therefore some proteins do not have data from both labeling experiments, and is listed as ND (no data) in Table 1. Eight (8) maleimide (cysteine) dye gels from 6 separate experiments and 5 NHS-ester (lysine) dye gels from 4 separate experiments were used in the DeCyder analysis.

*Collection of PC12 Whole-Cell Lysate for Western Blotting:*

PC12 cells were cultured and treated with 150 μM DA as described above. Following 2, 4, 8, 16, and 24 h of DA exposure or treatment with control media, the media was removed and PC12 cells were collected in PBS using force-pipetting. Cells were combined with the media

before pelleting, to collect any floating cells. Unlike the mitochondrial-enriched protein procedure, each 10 cm plate was collected separately. Cells were re-suspended once in PBS and re-pelleted prior to lysis in a buffer containing: 20mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM DTT, 1mM sodium orthovanadate, and 1X PIC (Roche).

*Western Blot Analysis of MS-identified spots:*

Protein from control and DA-exposed PC12 whole cell and mitochondria-enriched lysates, were separated by 10, 12, or 15% SDS-PAGE and transferred to nitrocellulose membranes using a Trans-blot SD Semi-Dry Electrophoretic Transfer Cell (Biorad). Following transfer, the blots were washed in Tris buffered saline (50 mM Tris, pH 8.0, 150 mM NaCl; TBS), blocked with 0.2% w/v dry milk in TBS-T (TBS + 0.1% Tween 20), then incubated overnight at 4$^{o}$C with primary antibody in TBS-T with 0.2% w/v dry milk. The blots were then washed in TBS-T and incubated at RT with the appropriate alkaline phosphatase conjugated secondary antibody (Biorad). Blots were then washed again in TBS-T prior to the application of chemiluminescent substrate (Biorad), and exposure to Biomax MR Film (Kodak) for visualization of bands. Antibodies used were Grp78 (1: 2000; Stressgen), Grp58 (1:2000; Stressgen), aldolase-A (1:1000; Abnova), actin (1:50,000; Sigma), and tubulin (1:12,500; Sigma). The densities of immunoreactive bands were quantified using UN-SCAN-IT software (Silk Scientific; Orem, UT). Actin and tubulin were used as loading controls. Data from both the DeCyder analysis and Western blots indicated that actin levels were not different in control and DA-exposed samples.

*Statistical Analysis:*

Statistical analyses of DA-exposed cysteine and lysine Cy-dye volume densities from isolated mitochondrial MS-identified proteins were performed using a 1-sample two-tailed Z-test on the DA-treated mitochondrial protein spot volume intensities expressed as percent of control. The Z-test was chosen because the DeCyder DIA software calculates changes between control and treated samples as a normalized volume ratio of the two groups, generating one value comparing both groups and normalizing the entire constellation of labeled spots. Significance for each DA-exposed protein from control (valued at 100% control) was determined when $p<0.05$ and the change was greater than ± 1.2-fold from control (<83.3% or >120%). The percent control values were directly calculated from the normalized DeCyder volume ratios.

Differences among group means for Western blot data analysis were determined by ANOVA followed by post-hoc student's t-test, with significance determined at $p<0.05$.

## 4.4 RESULTS

### 4.4.1 Comparison of 2-D DIGE using Cysteine-Reactive to Lysine-Reactive CyDyes

To determine which proteins were changed following a 16 h exposure to 150 μM DA, control and DA-exposed PC12 cell mitochondrial-enriched protein fractions were analyzed together by 2D-DIGE. The advantages of using 2D-DIGE methods are that two different samples (control and treated) can be analyzed and compared within one gel. The 16 h time-point was chosen because we observed significantly increased levels of protein cysteinyl-catechols and low levels of cell death (-20% cell loss from control) following 16 h of 150 μM DA exposure

(Hastings, unpublished observations). The increased DAQ protein modification combined with low toxicity allows us to measure protein modifications in the mitochondrial-enriched fraction obtained from living cells, prior to excessive cell death.

Control and DA exposed mitochondrial-enriched protein samples were incubated separately with the cysteine-reactive maleimide Cy3- and Cy5-conjugated dyes (GE Healthcare) prior to sulfhydryl reduction by DTT. The control and DA-treated fluorescent-labeled samples were pooled and proteins separated in 2-dimensions, first by isoelectric point, and then by molecular weight. In Figure 10A, the fluorescent scan of a representative DIGE gel is displayed with inverted colors (control=Cy3=cyan and DA treated=Cy5=pink). Selected spots were excised, trypsinized and subjected to MS studies for identification. All protein identifications were confirmed in multiple cysteine-labeled gels, and labeled in Figure 10A.

Ideally, if a reduced cysteine residue on protein was either directly modified by DAQ or oxidized by $H_2O_2$, the modified protein would have a reduced number of sulfhydryls available to react with the maleimide dye, resulting in a less labeling in the DA-treated pool. At the onset of this study, we intended to focus on identifying proteins that had reduced cysteine dye labeling in the DA-treated fraction, and we identified aldolase A (Figure 10A, D) as significantly decreased in PC12 cell mitochondria following DA. However, we observed a subset of proteins, mainly ER stress proteins, that displayed strikingly increased cysteine dye binding in the DA-treated mitochondrial fraction, including calreticulin, ER protein 29 (ERp29), ER protein 99 (ERp99), Grp58, Grp 78, Grp94, and oxygen regulated protein (Orp150) (Figure 10A, 10B, 10C, and Table 1). Since increases in cysteine dye-labeling following DA treatment may most likely be attributed to increased protein levels (either due to up-regulation of the protein or a reduced rate

of degradation), we utilized a second dye labeling lysine residues to evaluate total protein amounts in each group.

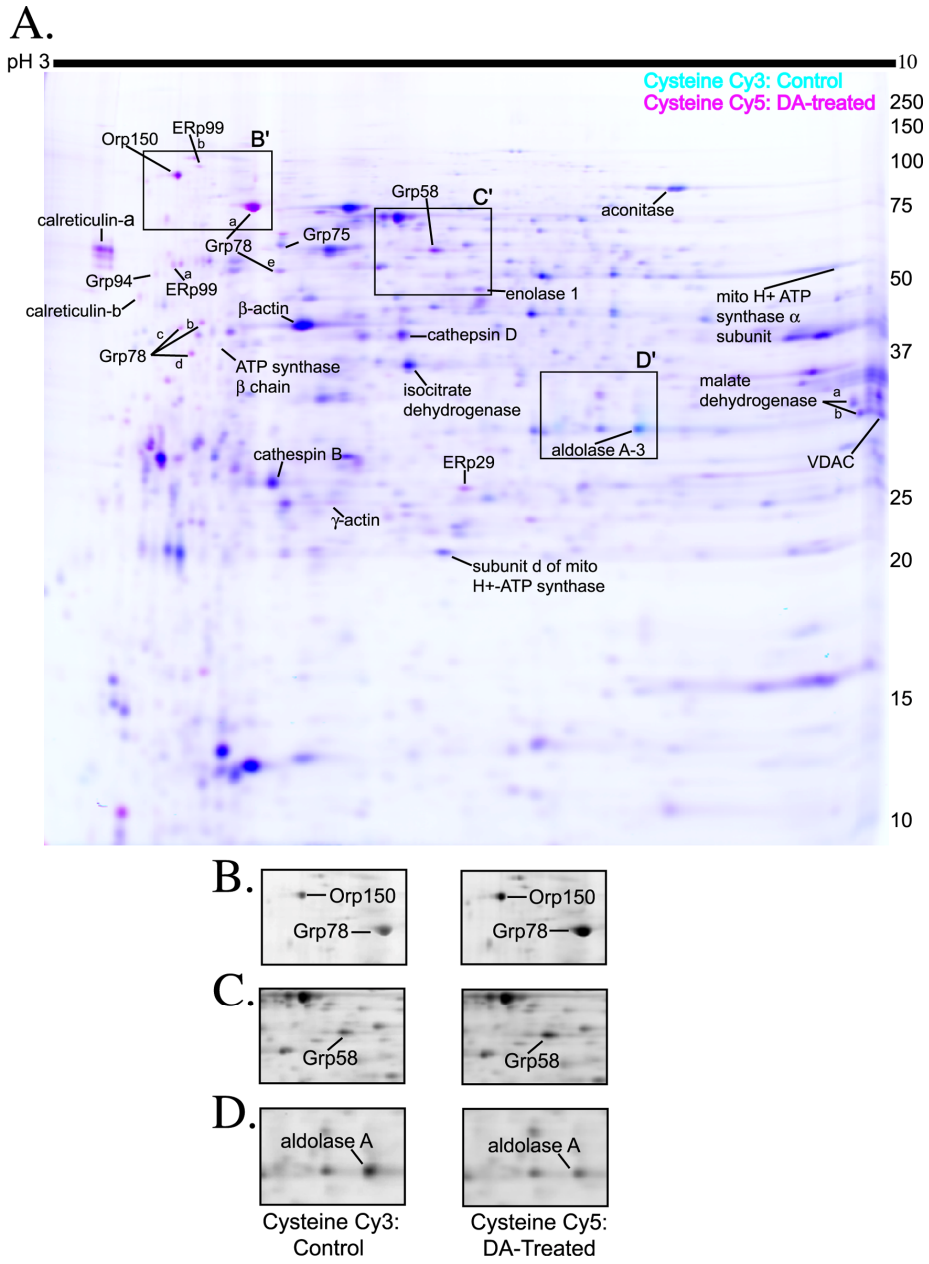


Figure 10: 2D-DIGE of PC12 cell mitochondrial-enriched fractions using cysteine-reactive dyes with insets of sample proteins.

**A**. Mitochondrial-enriched fractions from control and 16 h, 150 µM DA-exposed PC12 cells were isolated by differential centrifugation, and equal protein amounts were reacted with either

Cy3 (cyan scan; control) and Cy5 (pink scan; DA-exposed) maleimide dyes. Pink spots designate proteins in which more cysteine labeling occurred in the DA-exposed sample, indicating increased protein levels following DA exposure. Cyan spots designate proteins in which more cysteine labeling occurred in control, indicating cysteine modification or decreased protein levels following DA exposure. Dark blue spots indicate proteins in which equal cysteine labeling occurred in the control and DA-exposed mitochondrial-enriched fraction. MS-identified proteins are indicated on the gel and listed in Table 1. Gel is representative of n=8 cysteine 2D-DIGE gels. Boxes outline inset pictures in figures B-D. **B**. Black and white representation of the control protein, Cy3 fluorescent scan and the DA-treated protein, Cy5 fluorescent scan with Orp150 and Grp78 spots indicated. **C**. Black and white representation of the control protein, Cy3 fluorescent scan and the DA-treated protein, Cy5 fluorescent scan with the Grp58 spot indicated. **D**. Black and white representation of the control protein, Cy3 fluorescent scan and the DA-treated protein, Cy5 fluorescent scan with the aldolase A-3 spot indicated.

To determine which mitochondrial proteins were up-or down-regulated (the latter via reduced expression, increased degradation, and/or precipitation) following DA-exposure, control and DA-exposed PC12 cell mitochondrial-enriched fractions were incubated separately with the lysine-reactive NHS-ester Cy3 and Cy5-conjugated dyes (GE Healthcare), and analyzed by 2D-DIGE. In Figure 11A, the fluorescent scan of a representative lysine gel is displayed with inverted colors (control=Cy5=pink and DA treated=Cy3=cyan) with all confirmed protein identifications. In both cysteine and lysine labeled 2D-DIGE gels, the vast majority of proteins

exhibit constant levels in the DA treated group as compared to control, indicating that there was no change in most protein levels following DA exposure. Most visibly unchanged spots were not specifically selected for protein identification.

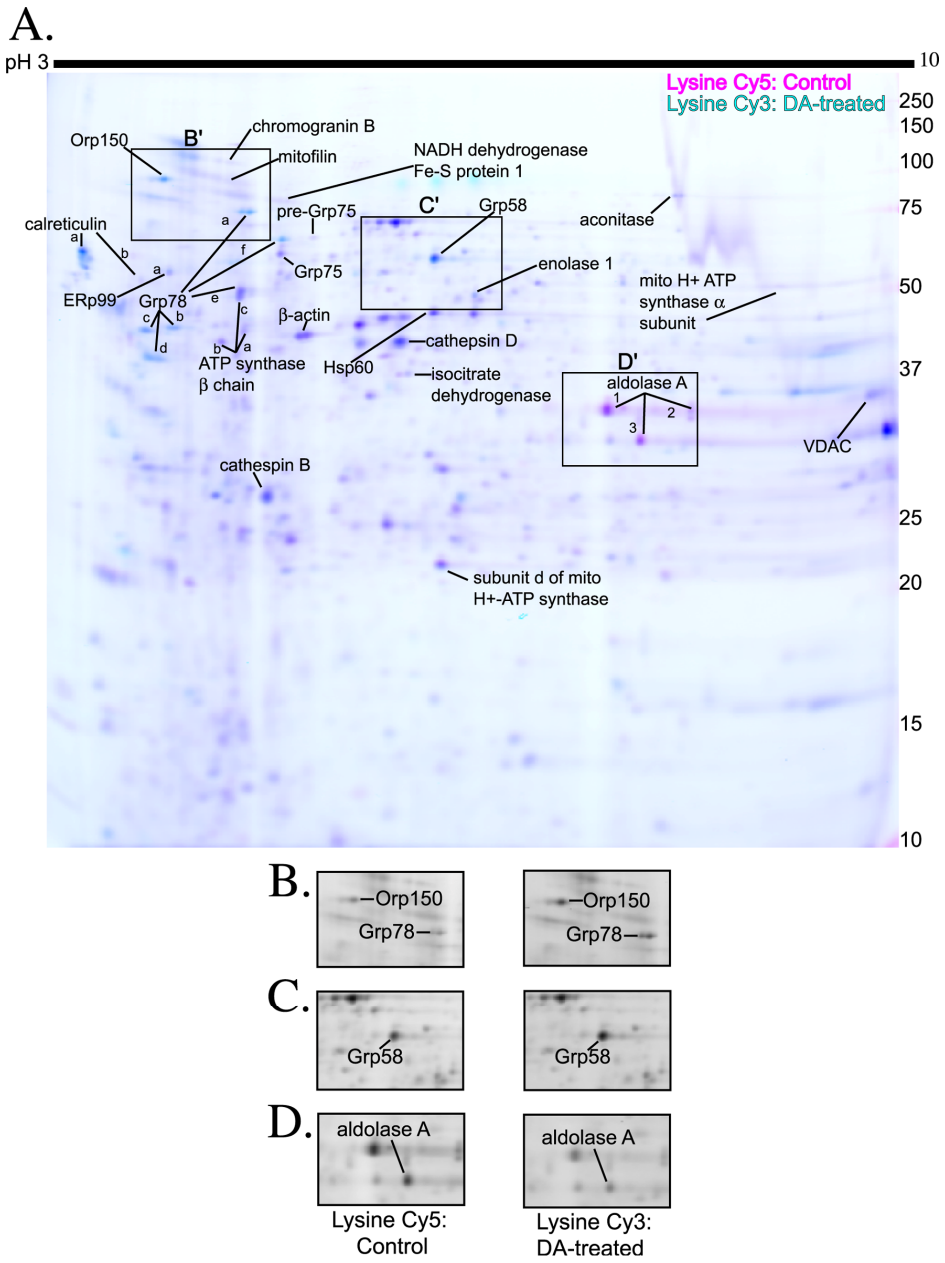


Figure 11: 2D-DIGE of PC12 cell mitochondrial-enriched fraction using lysine-reactive dyes with insets of sample proteins.

**A**. Mitochondrial-enriched fraction from control and 16 h, 150 μM DA-exposed differentiated PC12 cells were isolated by differential centrifugation. Mitochondrial protein was reacted with either Cy5 (pink scan; control) and Cy3 (cyan scan; DA-exposed) NHS-ester dyes to label lysine residues. Cyan (blue) spots designate proteins in which more lysine labeling occurred in the DA-exposed sample, indicating increased protein levels. Pink spots designate proteins in which more lysine labeling occurred in the control sample, indicating decreased protein levels induced by DA exposure. Dark blue spots indicate proteins in which equal lysine labeling occurred in the control and DA-exposed samples. MS-identified proteins are indicated on the gel and listed in Table 1. Gel is representative of n=5 lysine 2D-DIGE gels. Boxes outline inset pictures in figures B-D. **B**. Black and white representation of the control protein (Cy5 fluorescent scan) and the DA-treated protein (Cy3 fluorescent scan) with Orp150 and Grp78 spots indicated. **C**. Black and white representation of the control protein (Cy5 fluorescent scan) and the DA-treated protein (Cy3 fluorescent scan) with the Grp58 spot indicated. **D**. Black and white representation of the control protein (Cy5 fluorescent scan) and the DA-treated protein (Cy3 fluorescent scan) with the aldolase A-3 spot indicated.

Our results showed that most of the proteins that changed in the cysteine-label binding also changed similarly in the lysine labeling studies (Figure 12). In fact, most proteins that displayed increased cysteine dye binding in the DA-treated mitochondrial fraction, like calreticulin A, Orp150, Grp78, and Grp58 (Figure 10A, 10B, and 10C) also displayed increased lysine dye binding in the DA-treated mitochondrial fraction (Figure 11A, 11B, 11C). Decreases

in cysteine and lysine labeling of aldolase A following DA exposure were also observed (Figure 10A, 10D, 11A, 11D). Equivalent increases in both cysteine and lysine dye-labeling following DA treatment indicate increases in protein levels. Similarly, corresponding decreases in both cysteine and lysine dye-labeling following DA treatment, strongly indicate the down-regulation or degradation of proteins. Utilizing both cysteine and lysine dyes allowed us to control for modifications on proteins either on cysteine residues or on lysine residues, both of which could lead to changes in dye labeling and not changes in protein levels (though altered protein levels may be a consequence of the chemical modification).

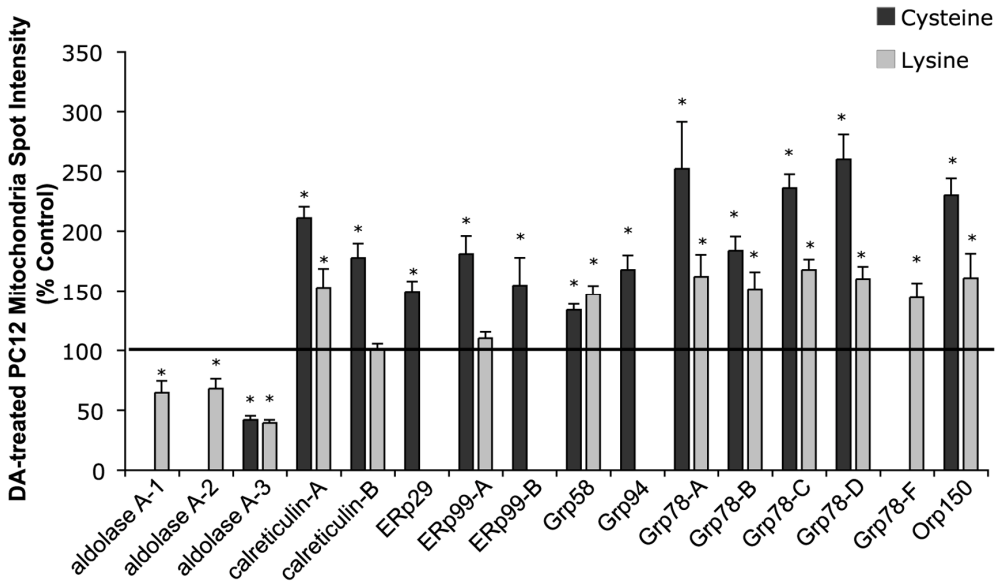


Figure 12: Changes in protein spot intensity of PC12 cell in the mitochondrial fraction following DA-exposure.

Mitochondrial-enriched fractions from control and DA-exposed (150 μM, 16 h) differentiated PC12 cells were isolated by differential centrifugation, and the enriched mitochondrial protein was reacted with Cy3 or Cy5 maleimide or NHS-ester dyes. Proteins listed above were identified by MALDI peptide mass fingerprint. The density of protein spots was analyzed using Decyder DIA software, and changes in DA exposed compared to control protein were

determined in both maleimide (cysteine) and NHS-ester (lysine) reacted protein for most spots shown. DA treated PC12 cell protein intensity is measured as average % control ± SEM, n=5-8. *, significance $p<0.05$.

Levels of cysteine and lysine labeling did not correlate for two identified proteins following DA exposure. Calreticulin-B and ERp99-A had increased cysteine dye labeling, while lysine dye labeling remained unchanged following DA exposure (Figure 12 and Table 1). Both protein spots have lower dye labeling intensities compared to other protein spots in both cysteine and lysine dye experiments, and thus may be present at low levels, close to the limit of detection. Since the cysteine dye has previously been shown to have an order of magnitude greater range of detection (Shaw et al., 2003), it is possible that the lysine dyes are outside their quantitative range.

| Protein Identifications | Subcellular Localization | Accession # | MW/pI | Protein Score†/ % Confidence | % Control Cysteine Gels | % Control Lysine Gels |
|---|---|---|---|---|---|---|
| **Chaperones** | | | | | | |
| calreticulin | ER | gi116 93172 | 47.97 / 4.33 | 218 / 100% | A. 211.3% ± 9.7%*<br>B. 177.75% ±12.2%* | A. 152.7% ± 15.9%*<br>B. 101.0% ± 4.5% |
| ERp29 | ER | gi167 58848 | 28.6 / 6.23 | 315 / 100% | 149.25% ± 8.8%* | ND |
| ERp99 | ER | gi348 62435 | 92.7 / 4.72 | 102 / 100% | A. 181.1% ± 15.2%*<br>B. 154.8% ± 23.3%* | A. 110.2% ± 5.4% |
| Grp58 | ER | gi383 82858 | 56.6 / 5.88 | 212 / 100% | 134.0% ± 4.8%* | 147.0% ± 7.4%* |
| Grp94 | ER | gi178 65698 | 92.4 / 4.75 | 88 / 99.675% | 167.9% ± 12.1%* | ND |
| Grp78 | ER | gi257 42763 | 72.3 / 5.07 | 232 / 100% | A. 211.3% ± 9.7%*<br>B. 184.0% ± 12.0%*<br>C. 236.25% ± 11.6%*<br>D. 260.3% ± 20.7%*<br>E. 114.3% ± 7.4% | A. 162.2% ± 18.5%*<br>B. 151.6% ± 14.1%*<br>C. 167.8% ± 8.8%*<br>D. 160.2% ± 10.1%*<br>F. 144.2% ± 12.2%* |
| Orp150 | ER | gi203 02024 | 111.22 / 5.11 | 58 / 99.882% | 230.3% ± 14.2%* | 161.1% ± 20.2%* |
| **ATP Synthesis** | | | | | | |
| aldolase A | cytoplasm, associated with mitochondria | gi697 8487 | 39.3 / 8.31 | 85 / 99.352% | 1. ND<br>2. ND<br>3. 42.0% + 3.4%* | 1. 64.6% ±10.0%*<br>2. 68.1% ± 8.3%*<br>3. 39.4% ± 2.6%* |

Table 1: Identified proteins labeled with maleimide or NHS-ester conjugated Cydyes using 2-D DIGE and MS.

Proteins identified that were significantly changed from control as quantified by DeCyder analysis and that were changed at least ± 1.2-fold from control in cysteine (maleimide) and/or lysine (NHS-ester) DIGE experiments are grouped by function. Actual molecular weight (MW in kDa) and isoelectric point (pI), best protein score and % confidence for each identified protein are also listed. Data for which no positive identification of proteins and thus no DeCyder data was obtained is listed as ND. † Protein MOWSE score was obtained from the search engine MASCOT. Decyder analysis of DA-treated sample spot intensity for both cysteine and lysine labeled experiments are listed as average % control ± SEM. *, significance $p<0.05$.

### 4.4.2 Quantitative Analysis of Differential Labeling of Proteins Following DA Treatment

Differences between Cy3 and Cy5 spot intensities were determined visually and were later quantified using DeCyder DIA software (GE Healthcare). Protein spots that were visually more strongly labeled in the control or DA-treated, in addition to a few unchanged test spots were picked for identification. Using the fluorescent scanned image, DeCyder software was used to map and label MS-identified spots, and then to calculate the volume density ratio of those spots between treatment groups. The percent change in density of DA-exposed protein spots compared to control protein spots were calculated from the DeCyder volume ratios, and the percent change for each spot was averaged over all gels in which the identified proteins could be determined. The identified proteins that were significantly changed in the DA-treated fraction compared to control are graphed in Figure 12 and listed in Table 1. Some proteins whose levels changed as little as ± 10% from control were statistically different according to the Z test, but were deemed non-significant because they fell within the 83.3% - 120% range. These proteins and the proteins found unchanged are listed in Table 2. All of the significantly changed spots except one (aldolase A) were significantly increased in the DA-treated samples as compared to control, while aldolase A was the only spot identified to be significantly decreased in the DA-treated sample (Figure 12). Most of these proteins showed similar patterns of change on both the cysteine-labeled and lysine-labeled gels. We were unable to positively identify every spot selected for MS analysis, including some that seemed to correspond to previously identified proteins in either a cysteine or lysine gel. Therefore, some DeCyder data for identified spots have only a cysteine or lysine dye intensity value, with the other value listed as "ND", indicating that no data for that protein spot was collected (see Table 1 and Table 2). In one case, the outline

of an identified protein spot was incorrectly defined by the DeCyder program, and thus we were unable to collect the data, and the value was listed as "UN" (Table 2).

| Protein Identifications | Subcellular Localization | Accession # | MW/pI | Protein Score†/ % Confidence | % Control Cysteine Gels | % Control Lysine Gels |
|---|---|---|---|---|---|---|
| **Mitochondrial Chaperones** | | | | | | |
| GRP75 precursor | mitochondrial matrix | gi896232 | 73.8 / 5.97 | 124 / 100% | ND | 90.5% ± 8.9% |
| GRP75 | mitochondrial matrix | gi2119726 | 73.7 / 5.87 | 154 / 100% | 104.0% ± 4.5% | 93.1% ± 11.1% |
| Hsp60 | mitochondrial matrix, cytoplasm | gi11560024 | 60.9 / 5.91 | 187 / 100% | ND | 86.4% ± 9.8% |
| **ATP Synthesis** | | | | | | |
| aconitase | mitochondrial matrix | gi38541404 | 85.4 / 7.87 | 211 / 100% | 98.45% ± 12.1% | 85.7% ± 14.5 % |
| isocitrate dehydrogenase | mitochondrial matrix | gi16758446 | 39.6 / 6.47 | 60 / 96.707% | 98.4% ± 5.5% | 83.0% ± 7.3% * |
| malate dehydrogenase | mitochondrial matrix | gi42476181 | 35.7 / 8.83 | 124 / 100% | 1. 99.5% ± 4. 7%<br>2. 97.2% ± 4. 3% | ND<br>ND |
| ATP synthase subunit d | mitochondria inner membrane | gi9506411 | 18.75 / 6.17 | 140 / 100% | 92.8% ± 4.2% | 80.4% ± 11.2% |
| ATP synthase β chain | mitochondria inner membrane | gi92350 | 50.7 / 4.9 | 247 / 100% | A. ND<br>B. UN | A. 96.5% ± 6.1%<br>B. 93.1% ± 4.2% |
| Mito H+ ATP synthase α subunit | mitochondria inner membrane | gi40538742 | 59.7 / 9.22 | 135 / 100% | 131.3% ± 23.8% | 104.2% ± 29.7% |
| enolase 1 | cytoplasm, plasma membrane, nucleus | gi50926833 | 47.1 / 6.16 | 98 / 100% | 110.6% = 4.2% * | 103.1% ± 7.2% |
| **Structural Protein** | | | | | | |
| β actin | cytoplasm | gi71670 | 41.7 / 5.29 | 159 / 100% | 102.0% ± 3.9% | 84.3% ± 4.9% * |
| γ actin | cytoplasm | gi34875636 | 43.08 / 5.11 | 107/1 00% | 105.8% ± 3.8% | ND |
| **Proteases** | | | | | | |
| cathepsin B | lysosome | gi1127276 | 27.7 / 5.14 | 97 / 99.956% | 92.7% ± 4.8% | 94.0% ± 6.1% |
| cathepsin D | lysosome | gi115720 | 44.7 / 6.66 | 83 / 99.973% | 96.1% ± 6.0% | 91.7% ± 6.9% |
| **Secretory Protein** | | | | | | |
| Chromogranin B | Large dense core vesicles | gi6978547 | 77.3 / 4.98 | 48 / 97.268% | ND | 104.8% ± 2.3% |
| **Mitochondrial Morphology** | | | | | | |
| Mitofilin | mitochondria inner membrane | gi34855983 | 82.3 / 5.37 | 117 / 100% | ND | 101.2% ± 6.2% |
| **Molecule Transport** | | | | | | |
| VDAC | outer mitochondrial membrane | gi48734887 | 32.2 / 8.31 | 123 / 100% | 100. 3% ± 6.2% | 87.0% ± 14.6% |

Table 2: Unchanged identified proteins labeled with maleimide or NHS-ester conjugated Cy dyes using 2-D DIGE and MS.

Proteins identified that were not significantly changed from control in cysteine (maleimide) and/or lysine (NHS-ester) DIGE experiments are grouped by function. Actual molecular weight (MW in kDa) and isoelectric point (pI), best protein score and % confidence for each identified protein are also listed. Data for which no positive identifications of proteins and thus no DeCyder data was obtained is listed as ND. Data that could not be determined due to inaccurate spot definition in the DeCyder software is listed as UN. † Protein MOWSE score was obtained from the search engine MASCOT. Decyder analysis of DA-treated sample spot intensity for both cysteine and lysine labeled experiments are listed as average % control ± SEM, significance $p<0.05$.

### 4.4.3 Most of the Identified Proteins Function in ATP Synthesis or as Chaperones

We successfully identified many proteins in the mitochondrial fraction that are changed following PC12 cell DA exposure (Figures 10 and 11, Table 1). Most of the proteins identified are involved in ATP synthesis or are chaperone proteins (Table 1). Many ER localized chaperone proteins were identified in the mitochondrial-enriched fraction (Table 1). Previously, ER proteins have been found associated with isolated mitochondria (Fountoulakis et al., 2002). Other proteins including some proteases, cytosolic structural proteins, and proteins involved in secretion, mitochondrial morphology, and mitochondrial molecule export were also identified (Table 2). Most of these proteins were deemed not significantly changed in the DA-treated mitochondrial fraction as compared to control (Table 2). Seventeen of the twenty-five identified proteins did not meet the two-part criteria of a change greater than ± 1.2-fold from control with

statistical significance on the DeCyder analysis, and three of those seventeen proteins met statistical significance but were changed in dye-labeling less than ± 1.2-fold from control, and thus were deemed not significant.

### 4.4.4 Increased Levels of ER proteins GRP78 and GRP58 Following DA Exposure

Since many of the proteins with the greatest increases in the DA-exposed samples were ER chaperone proteins (Figure 12, Table 1), we were interested in whether the increases were due to an increased ER response to DA-induced toxicity. Because ER may swell following oxidative stress (Atlante et al., 2001; Esrefoglu et al., 2003) and are closely associated with mitochondria (Levine and Rabouille, 2005), it is possible that DA-exposure caused increased ER contamination of a crude mitochondrial preparation, rather than increased total cellular levels of ER stress proteins. To evaluate this, we measured the levels of two ER-associated chaperone proteins, Grp78 and Grp58, using Western blot in PC12 cell whole cell lysate and mitochondrial-enriched fractions (Figure 13). Western blot analysis of Grp78 in the mitochondrial fraction showed a 224% increase compared to control (Figure 13B), validating the results obtained in 2D-DIGE studies. Levels of Grp78 in the DA-exposed mitochondrial fraction measured by 2D-DIGE, DeCyder analysis (cysteine: +184-260%; lysine: +152%-168%) are similar to levels of Grp78 measured by Western blot analysis (+224%; Figure 12 and 13B). We also observed a 224% increase in Grp58on Western blot in the DA exposed mitochondrial fraction as compared to control (Figure 13B). Levels of Grp58 in the DA-exposed mitochondrial fraction measured by 2D-DIGE, DeCyder analysis (cysteine: +134%; lysine: +147%) were significantly elevated, but to a lesser extent than levels of Grp58 measured by Western blot analysis (+224%; Figure 12

and 13B). Lanes from representative Western blots for Grp78 and Grp58 in 16 h control and DA-exposed mitochondrial fractions are shown in Figure 13A.

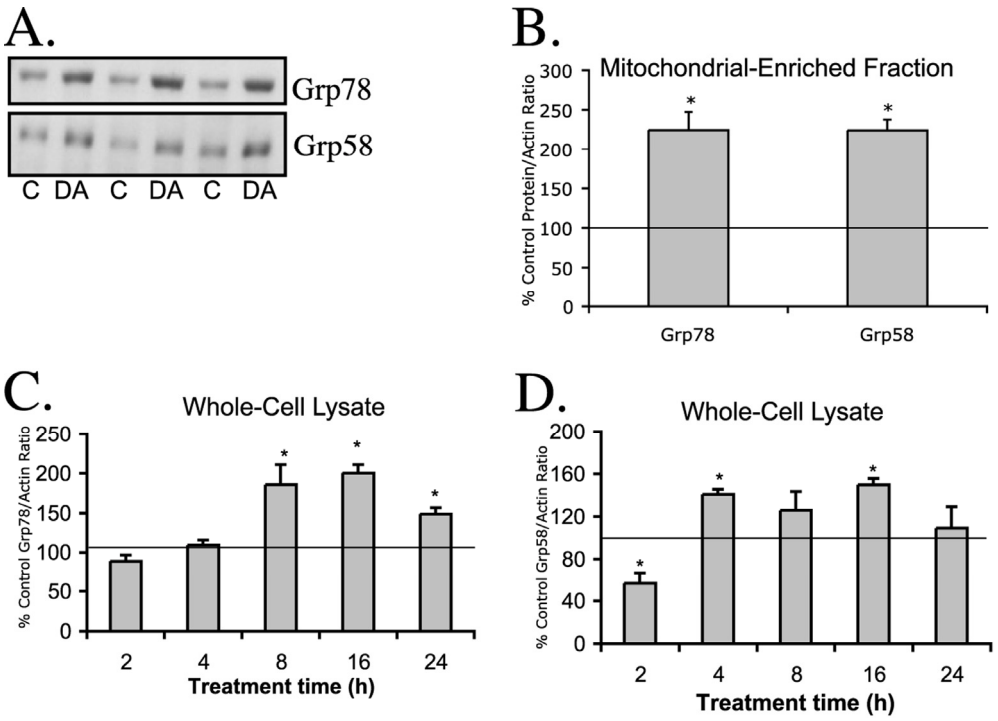


Figure 13: Western blot analysis of Grp78 and Grp58 in PC12 mitochondrial-enriched fraction and whole cell lysate following DA-exposure.

Mitochondria-enriched protein and PC12 whole-cell lysate from control and 150 μM DA-exposed PC12 cells were collected. Samples containing 20 μg of protein were separated by 15% SDS-PAGE. Following transfer to nitrocellulose, blots were probed for actin and either Grp78 or Grp58 immunoreactivity and chemiluminescent band densities were quantitated. **A**. Representative blots for Grp78 and Grp58 are shown for PC12 mitochondrial fractions isolated from 16h control and 150 μM DA-treated cells. **B**. Grp78/actin and Grp58/actin ratios for PC12 mitochondrial fraction DA treated cells were quantified and compared to control. **C**. Grp78/actin and **D**. Grp58/actin ratios in whole cell lysates were quantified at various time-points and

expressed as average DA-treated protein/actin immunoblot intensity in % control ± SEM, n=3 separate experiments, each measured in duplicate. *, significance $p<0.05$.

We utilized Western blot analysis of PC12 cell whole cell lysate to determine whether the DA-induced changes in Grp78 and Grp58 protein were due to increases in the levels of these proteins in the cell or increased association of ER proteins with mitochondria. Differentiated PC12 cells were treated with control media or media containing 150 μM DA for 2, 4, 8, 16, or 24 h prior to collection and lysis for Western blotting. A time-course of Grp78 and Grp58 levels in whole cell lysate allows us to more accurately assess the timing of the ER induced response to DA. Levels of Grp78 were significantly increased from control following 8 h DA exposure (+186%; Figure 14A). The levels of Grp78 remained elevated above control after 16 and 24 h of DA exposure (+200% and +148%, respectively; Figure 14A). Levels of Grp58 in whole cell lysates were significantly decreased from control after 2 h of DA exposure (-44%; Figure 14B). However, levels of Grp58 were increased after 4 h and 16 h of DA exposure (+141% and +150%, respectively; Figure 14B). The levels of Grp78 in the mitochondrial-enriched fraction (224% of control) are comparable to the 16 h DA exposed whole-cell lysate levels (200% of control), and the levels of Grp58 after 16 h DA exposure in whole cell lysate (150% of control) are comparable to the DeCyder analysis (134%-147%) of spot intensity, and similar to Western blot analysis of the mitochondrial enriched fraction (224% of control), indicating that the increases in Grp78 and Grp58 occur throughout the cell and are most likely not due to increased ER contamination of the mitochondrial-enriched fraction in the DA sample.

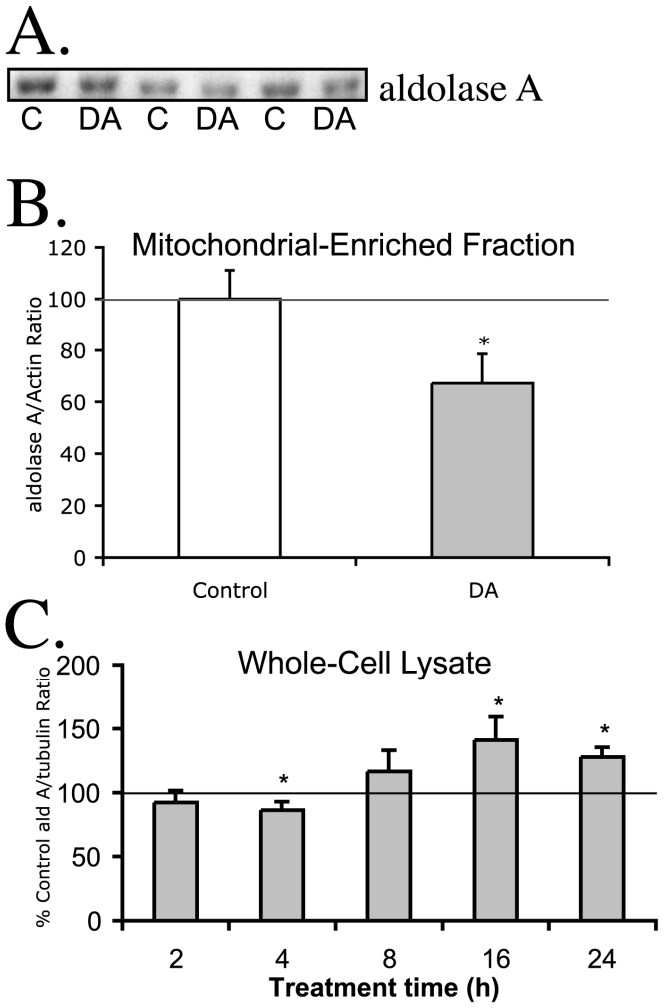


Figure 14: Western blot analysis of aldolase A in PC12 mitochondrial-enriched fraction and whole cell lysate following DA-exposure.

Mitochondria-enriched protein and PC12 whole-cell lysate from PC12 cells were collected after exposure to control media or media containing 150 μM DA. Samples containing 20 μg protein were separated by 10% SDS-PAGE and transferred to nitrocellulose. Blots were probed for aldolase A and actin or tubulin immunoreactivity. Quantification of aldolase A/actin and aldolase A/tubulin ratios from PC12 cell protein was determined from chemiluminescent band density. **A**. Representative blots for aldolase A are shown for PC12 mitochondrial fractions isolated from 16h control and 150 μM DA-treated cells. **B**. The quantification of control and DA

exposed PC12 cell mitochondrial-enriched protein are reported as % control ± SEM of the average immunoblot intensity of aldolase A/actin. **C**. Aldolase A/tubulin ratios from PC12 whole cell lysate were quantified over various time-points. Tubulin levels did not vary under different treatment conditions. Aldolase A/tubulin ratios were reported as % time-matched control ± SEM. n=3 separate experiments, each measured in duplicate. *, significance $p<0.05$.

### 4.4.5 Levels of Aldolase A Differ In Whole Cell Lysate Compared to the Mitochondrial Fraction Following DA Exposure

In addition to the ER chaperone proteins, we were also interested in confirming the levels of aldolase A, since it was the only identified protein significantly decreased following DA-exposure in the PC12-cell mitochondrial fraction, according to DeCyder analysis. Since aldolase A is found both in the cytoplasm (Pfleiderer et al., 1975; Wachsmuth, 1976) and associated with mitochondria (MacDonnell and Greengard, 1974), we were also interested in whether the aldolase A in the cytosolic fraction, the mitochondrial fraction, or both was lost following DA-exposure. We observed that the aldolase A levels in mitochondrial-enriched fraction measured by Western blot also followed a similar trend to levels measured by 2D-DIGE, DeCyder analysis. We observed 67% of control levels of aldolase A following 16 h DA exposure using Western blot (Figure 14A) and 42% of control (cysteine) and 39% control (lysine) using 2D-DIGE, DeCyder analysis (Figure 12). Although Western blotting and 2D-DIGE DeCyder techniques are very different, we were able to confirm the trend of decreased in aldolase A levels

by comparing these two analyses. In whole cell lysates, we observed a slight decrease in aldolase A after 4 h of DA exposure (-14%; Figure 14C). However, after longer DA exposure times, the levels of whole cell aldolase A were increased above control (16 h, +141%; 24 h +128%; Figure 14C). Thus, results using whole cell lysates did not correlate with results observed in the mitochondrial fraction, which are likely due to different DA-induced changes in cytosolic aldolase A separate from the changes observed in mitochondria.

## 4.5 DISCUSSION

### 4.5.1 Proteins with Increased Levels Identified Using DIGE

Using an unbiased proteomics approach in this study, we found that DA exposure increased levels of ER chaperone proteins in differentiated PC12 cells, suggesting activation of an ER stress response. We identified proteins from PC12 cell mitochondrial-enriched fractions that were altered following DA exposure using 2D-DIGE. We utilized cysteine dyes that label reduced cysteine residues from both the control and DA-exposed mitochondrial-enriched fractions to detect changes in cysteine oxidation and protein levels and compared the results with lysine-labeling dyes that specifically detect changes in protein levels. Using both methods, we observed increased levels of various ER chaperone proteins in response to DA exposure in PC12 cells, including: Grp78 (BiP), Grp58, Grp94, Orp150, calreticulin, ERp29 and ERp99. Some of these proteins, including Grp58, Grp78, Grp94, calreticulin, and Orp150 have previously been shown to be up-regulated following oxidative stress and ER stress (Kuwabara et al., 1996;

Kaufman, 1999; Kaneda et al., 2000; Lee, 2001; Nunez et al., 2001; Berridge, 2002). ERp29 has been described as an escort chaperone, with possible involvement in secretion (Mkrtchian and Sandalova, 2006). ERp99 is speculated to be an ER membrane sorting chaperone, since the N-terminus has identical sequence homology to Grp94 (Mazzarella and Green, 1987).

### 4.5.2 ER Chaperone Proteins Grp78 and Grp58

Grp78 up-regulation is a classical marker for induction of the UPR, and serves to protect cells by binding to stress sensor proteins, PERK, IRE1, and ATF6 (Rao et al., 2004). Using 2D-DIGE and Western blot analysis of mitochondrial protein, we found that Grp78 was significantly increased above control, indicating that DA exposure led to Grp78 up-regulation. The time-course of Grp78 levels in whole cell lysate measured by Western blot revealed a significant increase in Grp78 as early as 8 h. Levels of Grp78 remain increased above control at 16 h and 24 h DA exposure, indicating a sustained activation of the UPR. Prolonged UPR activation may contribute to cell death via multiple mechanisms, including ER calcium release, caspase-12 activation, ROS accumulation, the ASK1/JNK stress activated kinase apoptosis pathway, and the p53 apoptosis pathway (Gorlach et al., 2006; Szegezdi et al., 2006; Zhao and Ackerman, 2006).

Grp58 is an ER stress-inducible chaperone protein with thiol oxidoreductase activity that has been shown to be susceptible to oxidation by $H_2O_2$, and works in conjunction with calreticulin and calnexin to promote proper disulfide bond formation of glycoproteins (Mazzarella et al., 1994; Murthy and Pande, 1994; High et al., 2000; van der Vlies et al., 2002; Antoniou and Powis, 2003). In the present study, we found that Grp58 levels were significantly elevated above control in 16 h exposed PC12 cell mitochondria using 2D-DIGE and Western blot analysis. Grp58 protein increases in whole cell lysate were confirmed by Western blot

analysis, which revealed that there was a significant increase in Grp58 at 4 and 16 h of DA exposure. The increases in Grp58 and Grp78 levels shown by 2D-DIGE DeCyder and Western blot analyses and the increased labeling of other ER stress proteins by 2D-DIGE strongly indicate ER stress activation in PC12 cells exposed to DA.

### 4.5.3 Aldolase A

Thus far, only one identified protein, aldolase A, had significantly lower labeling using cysteine dyes and lysine dyes in DA-exposed mitochondria. Aldolase A catalyzes the breakdown of fructose-1,6-bisphosphate during glycolysis to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Lorentzen et al., 2005) and is expressed in all cells throughout the body, including neurons (Buono et al., 2001). It is found both in the cytoplasm and associated with mitochondria (MacDonnell and Greengard, 1974; Pfleiderer et al., 1975). Nitrated aldolase A has been identified *in vivo* in aged skeletal muscle and in light exposed retina, leading to a loss of activity (Miyagi et al., 2002; Koeck et al., 2004; Kanski et al., 2005). Aldolase A has been linked to various diseases, including Alzheimer's disease, where antibodies to aldolase A were found in patient sera (Mor et al., 2005), and schizophrenia, where decreased aldolase A levels were found in patient prefrontal cortex (Prabakaran et al., 2004). Therefore, our finding of decreased levels of aldolase A in mitochondria could be an indication of energy metabolism deficits, correlated with decreased ATP levels, resulting from DA exposure. Recently, aldolase A was found to be associated with PD-linked proteins DJ-1 and α-synuclein in MES cells (Jin et al., 2006). This association with aldolase A was decreased following rotenone exposure in MES cells, suggesting a novel role for aldolase A in a PD model employing selective dopaminergic cell death (Jin et al., 2006).

Result from the Western blot analyses showed that aldolase A is down-regulated or degraded in the mitochondrial fraction, but not throughout the cell (Figure 14) following DA exposure. Aldolase A levels measured by Western blot in whole cell lysate showed an initial decrease following 4 h of DA exposure, but at longer exposure times, aldolase A levels were increased above control. Increased levels of aldolase A have been associated with disease and have been found in brain tissue of patients with psychiatric disorders, including schizophrenia and major depression (Beasley et al., 2006). Since PC12 cells have previously been shown to increase glycolysis when challenged with mitochondrial inhibitors (Kang et al., 1997), our observed differential protein expression in whole cells versus mitochondria could be due to the up-regulation of aldolase A in the cytoplasm as compensation for mitochondrial energy deficits induced by DA. We have previously observed decreases in ATP levels in PC12 cells following 18 h DA exposure that return to control at 24 h DA exposure (Hastings, unpublished observations). Decreased levels of ATP that later return to control indicate that DA exposure leads to an energy deficit that can eventually be compensated by the PC12 cells, presumably by increased glycolysis. Increasing cytoplasmic aldolase A levels after DA exposure may possibly be a component of the compensatory mechanism to aid in returning ATP levels back to normal. Although the role of aldolase A in disease has yet to be determined, changes in levels of an enzyme involved in ATP synthesis is likely to be involved in molecular pathways of protection and/or toxicity.

### 4.5.4 Using Maleimide Dyes to Measure Cysteine Oxidation Changes

In addition to using standard methodologies to examine protein levels using lysine reactive dyes, cysteine dyes were used to measure relative changes in cysteine oxidation states of

mitochondrial proteins following DA exposure in PC12 cells. Since a subset of highly reactive protein thiols may be selectively oxidized in neurons upon oxidative stress, we used minimal dye concentrations (0.125% of GE Healthcare's recommended dye/protein ratio) to compare the oxidation state of reactive cysteine residues in proteins. In addition to proteins whose concentration levels were altered, we were hopeful that we would observe an additional subset of mitochondrial proteins that had reduced cysteine labeling due to specific oxidation of reactive thiols, but had unaltered lysine labeling. What we did observe was overwhelming evidence for up-regulation of ER chaperone proteins following DA exposure, suggesting that the cells were mounting a response to the oxidative stress.

Our inability to observe changes in cysteine modification using 2D-DIGE thus far may be due to saturation of the maleimide CyDye, despite our efforts to use very low dye to protein ratio. Another study using 2D-DIGE to examine redox- and ErbB2-dependent changes in cells found similar results to ours comparing cysteine and lysine dyes, using their own synthesized cysteine dyes, which contained an iodoacetyl cyanine group that binds with better efficiency to reduced thiols than the maleimide group (Chan et al., 2005). In both the Chan et al. (2005) and our studies, cysteine labeling was more indicative of protein levels than changes in cysteine oxidation. In addition, we have collected complementary data from 2D-DIGE experiments in isolated rat brain mitochondria directly exposed to DAQ, which suggest that using cysteine-reactive dyes, even at such low concentrations, also sensitively revealed mostly protein level changes and not changes in the amounts of reduced cysteines (manuscript submitted). Therefore, using cysteine reactive dyes to measure changes in cysteine oxidation states may require additional fine-tuning of the dye to protein ratio or pH, to optimize the reaction towards the most reactive cysteines, which have a lower pKa (Di Simplicio et al., 2003).

### 4.5.5 ER Stress and Mitochondria

The ER plays a role in pro- and anti-apoptotic signaling in conjunction with mitochondria (Berridge, 2002; Breckenridge et al., 2003; Paschen, 2003; Rao et al., 2004). One way in which mitochondria and the ER communicate is via calcium signaling, in which small releases of calcium from the ER is detected and taken up by mitochondria, and this calcium homeostasis is essential for the functions of both organelles (Berridge, 2002; Breckenridge et al., 2003; Paschen, 2003). In addition to calcium, signaling molecules are shared between mitochondria and ER. Following induction of ER stress, Grp78 has been found localized inside the mitochondria, within the intermembrane space, inner membrane, and matrix (Sun et al., 2006). Additionally, a large portion of newly synthesized Grp78 was found localized to the mitochondria in cells under UPR, indicating that Grp78 up-regulation may be another way that ER can relay information to the mitochondria (Sun et al., 2006). Induction of ER stress has also been associated with translocation of c-Abl tyrosine kinase, a stress-inducible apoptosis-regulating signaling molecule, from ER to mitochondria, resulting in cytochrome c release (Ito et al., 2001). Mitochondria can also communicate stress to the ER. Many of the Bcl-2 family of proteins, known to regulate mitochondrial-mediated apoptosis, also seem to influence ER-induced cell death and have been shown to localize to the ER membrane and lead to large calcium effluxes from the ER and concomitant large calcium influxes by mitochondria (Breckenridge et al., 2003; Rao et al., 2004; Gorlach et al., 2006; Szegezdi et al., 2006; Wu and Kaufman, 2006). Mitochondrial energy deficits caused by decreased levels of uridine diphosphoglucose or induced by NO have also been shown to cause the activation of ER stress, including the up-regulation of Grp78 (Flores-Diaz et al., 2004; Xu et al., 2004). Mitochondria and ER communication is essential for the cellular response to stress and ultimately cell survival.

Sustained activation of ER stress can lead to activation of mitochondrial-dependent apoptosis involving cytochrome c release, in addition to a mitochondrial-independent caspase-12 activation pathway, leading to cell death (Breckenridge et al., 2003; Momoi, 2004; Rao et al., 2004; Zhao and Ackerman, 2006) .

### 4.5.6 ER Stress and PD

Using an unbiased approach, this study has provided evidence that DA exposure leads to ER stress protein up-regulation in PC12 cells, which may be involved in the mechanisms of DA-induced toxicity. Previous studies lend evidence that ER stress may be induced by dopaminergic toxins. Exposure to high levels of DA (500 μM), 6-OHDA, or MPP+ in SH-SY5Y cells, and expression of mutant A53T alpha synuclein in PC12 cells have been previously shown to increase levels of Grp78 and Chop/GADD153, an ER stress inducible transcription factor (Chen et al., 2004a; Gomez-Santos et al., 2005; Smith et al., 2005a). Increases in the phosphorylation state of ER stress kinases were observed in MN9D cells following 6-OHDA and in PC12 cells following 6-OHDA, MPP+, and rotenone treatment (Ryu et al., 2002; Holtz and O'Malley, 2003). In MN9D cells, 6-OHDA induced an ER stress response prior to cytochrome c release, indicating that ER stress activation occurred prior to the onset of cytochrome c-mediated apoptotic signaling pathways (Holtz et al., 2006). Others have also suggested ER stress leads to activation of apoptotic cell death pathways (Boyce and Yuan, 2006; Lindholm et al., 2006; Szegezdi et al., 2006; Wu and Kaufman, 2006). A recent finding found that after rotenone exposure in MES cells, increased amounts of calreticulin precursor were associated with both α-synuclein and DJ-1, and increased levels of Grp94 were associated with α-synuclein, linking ER stress proteins to proteins involved in PD pathogenesis (Jin et al., 2006).

The activation of ER stress has also been observed directly in PD and ischemia *in vivo* models (DeGracia and Montie, 2004; Silva et al., 2005), and is thought to be a common pathological pathway in many neurodegenerative disorders (Paschen and Frandsen, 2001; Paschen and Mengesdorf, 2005; Xu et al., 2005a; Lindholm et al., 2006). Many inherited forms of PD involve abnormalities in protein degradation or mitochondrial dysfunction, including the proteins α-synuclein, Parkin, DJ-1, PINK1, ubiquitin carboxyl-terminal hydrolase-1, and LRRK2/dardarin (Lindholm et al., 2006). The relationships between genetic forms of PD and protein degradation and inclusion formation suggest that accumulation of unfolded proteins might contribute to the selective neuronal death observed in PD (Lindholm et al., 2006). The relationship between oxidative stress, mitochondrial dysfunction, and abnormal protein degradation is connected to the activation of ER stress pathways in DA-induced toxicity and likely in the pathology of PD.

# 5.0 THE EFFECT OF ENDOGENOUS DOPAMINE IN ROTENONE-INDUCED TOXICITY IN PC12 CELLS

## 5.1 SUMMARY

Deficiencies in complex I have been observed in Parkinson's disease (PD) patients. Systemic exposure to rotenone, a complex I inhibitor, has been shown to lead to selective dopaminergic cell death *in vivo* and toxicity in many *in vitro* models, including dopaminergic cell cultures. However, it remains unclear why rotenone seems to affect dopaminergic cells more adversely. Therefore, the role of dopamine (DA) in rotenone-induced PC12 cell toxicity was examined. Rotenone (1.0 μM) caused significant toxicity in differentiated PC12 cells, which was accompanied by decreases in ATP levels, changes in catechol levels, and increased DA oxidation. To determine whether endogenous DA makes PC12 cells more susceptible to rotenone, cells were treated with the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine (AMPT) to reduce DA levels prior to rotenone exposure, and then cell viability was measured. No changes in rotenone-induced toxicity were observed with or without AMPT treatment. However, a potentiation of toxicity was observed following co-exposure of PC12 cells to rotenone and methamphetamine. To determine whether this effect was due to DA, PC12 cells were depleted of DA prior to methamphetamine and rotenone co-treatment, resulting in a large attenuation in

toxicity. These findings suggest that DA plays a role in rotenone-induced toxicity and possibly the vulnerability of DA neurons in PD.

## 5.2 INTRODUCTION

The cause of selective dopaminergic degeneration in the substantia nigra of Parkinson's disease (PD) patients remains unknown, however, research has linked cell death in PD to oxidative stress (Dauer and Przedborski, 2003) and mitochondrial dysfunction (Schapira et al., 1990a; Schapira et al., 1990b; Blandini et al., 1998). Direct effects of reactive oxygen species (ROS), including increased lipid peroxidation, protein carbonyls, and DNA damage in PD brain have been observed (Beal, 2003). Increased iron and significant decreases in the major antioxidant, glutathione (GSH), which also promotes oxidative stress, have also been observed in PD (Jenner, 2003).

The relationship between oxidative stress and dopamine (DA) oxidation in degeneration has provided a link between the selective vulnerability of DA neurons and PD. Increased DA metabolism, by both monoamine oxidase (MAO) and DA oxidation into DA quinone (DAQ) will cause increased ROS production, which may lead to oxidation of protein, DNA, and lipids (Graham, 1978; Maker et al., 1981; Halliwell, 1992; Hastings, 1995). In addition to ROS, the electron-deficient DAQ readily reacts with cellular nucleophiles, including reduced sulfhydryl groups, located on free cysteine residues, GSH, and proteins (Tse et al., 1976; Fornstedt et al., 1990b; Fumagalli et al., 1998). Modification of free thiols and GSH can lead to the reduction in the amount of antioxidants available to protect the cells from oxidative stress. In addition, free cysteinyl-DA conjugates can be further oxidized to form 7-(2-aminoethyl)-3,4-dihydro-5-

hydroxy-2H-1,4-benzothiazine-3-carboxylic acid (DHBT-1), a mitochondrial toxin (Li and Dryhurst, 1997). DAQ modification of proteins lead to the formation of covalently bound DA-protein conjugates, often on cysteinyl residues (Hastings et al., 1996). Many vital proteins contain cysteine residues at their active sites, and therefore modification may alter the function of these proteins, leading to inactivation and possibly cell death. Both *in vitro* (Graham, 1978) and *in vivo* (Filloux and Townsend, 1993; Hastings and Zigmond, 1994) studies support the hypothesis that exposure to DA increases protein cysteinyl-catechol levels, and *in vivo* causes selective damage to DA terminals (Rabinovic et al., 2000). Protein modification by DAQ has also been observed following the dopaminergic toxins methamphetamine (METH) (LaVoie and Hastings, 1999) and 1-methyl-4-phenylpyridinium (MPP+) (Teismann et al., 2003), indicative of endogenous DA oxidation. The presence of neuromelanin in the brain and cysteinyl-catechol conjugates in PD brain lysates (Spencer et al., 1998), suggests that DA oxidation occurs *in vivo*. Therefore, the presence of DA in the cytoplasm, especially in a reduced antioxidant environment, will add to the oxidative stress of a cell through ROS and DAQ production and through the subsequent oxidation of important biomolecules, making dopaminergic neurons in the substantia nigra more susceptible to cell death.

Impairment of mitochondrial function is also likely to contribute to oxidative stress and cell death in PD. The link between complex I inhibition and PD was first identified after the active metabolite of the dopaminergic toxin MPTP, MPP+, was discovered to be a complex I inhibitor. Further studies revealed that impaired mitochondrial function, in the form of a complex I deficiency, occurs in PD in the substantia nigra (Mizuno et al., 1989; Schapira et al., 1989) and systemically in platelets and muscle (Shoffner et al., 1991; Blandini et al., 1998). The role of complex I inhibition in PD has been expanded through experimentation with pesticides

and toxins that inhibit complex I, including MPTP, paraquat, and rotenone, all of which cause selective DA degeneration (Dawson and Dawson, 2003; Di Monte, 2003; Greenamyre et al., 2003). The selective toxicity of both MPTP and paraquat is due to their similar structure, which makes them substrates for the DA transporter (DAT) (Pifl et al., 1993; McCormack et al., 2002; Shimizu et al., 2003). Rotenone however is not a substrate for DAT. It is lipophilic and can cross membranes of all cells easily. *In vivo* studies have shown that chronic, systemic administration of rotenone produces dopaminergic degeneration and Lewy body-like cytoplasmic inclusions, which closely mimic the pathology of PD (Betarbet et al., 2000), although less selective effects have also been observed (Hoglinger et al., 2003). The systemic rotenone model does represent both the central and peripheral inhibition of complex I as seen in PD, which leads to nigrostriatal dopaminergic degeneration (Betarbet et al., 2000), α-synuclein aggregation (Sherer et al., 2003a), and glial activation (Sherer et al., 2003b). Rotenone treatment also functions as an effective PD model *in vitro*, resulting in toxicity to dopaminergic cells (Hartley et al., 1994), increasing oxidative stress (Sherer et al., 2002), and decreasing proteasome activity (Shamoto-Nagai et al., 2003).

Partial inhibition of complex I has been shown to increase mitochondrial production of ROS (Pitkanen and Robinson, 1996; Votyakova and Reynolds, 2001), which may be the precipatory event in toxicity models. However, the basis for rotenone-induced selective toxicity to dopaminergic neurons remains ambiguous. The increased oxidative stress within dopaminergic neurons, due to DA metabolism and oxidation, combined with a complex I inhibition-induced ROS production may lead to cell death by overloading the oxidative capacity of dopaminergic cells. Therefore, in this study we sought to investigate whether DA was involved in rotenone-induced toxicity in PC-12 cells. We found that DA depletion prior to toxin

exposure did not protect against rotenone-induced toxicity. However, rotenone toxicity was potentiated by METH-induced increases in cytoplasmic DA in PC12 cells.

## 5.3 EXPERIMENTAL PROCEDURES

*Chemicals and Reagents:*

Cell culture media, Dulbecco's Modified Eagle Medium (DMEM, Gibco brand), fetal bovine serum (HyClone brand), and horse serum (HyClone brand) were purchased from Invitrogen (Carlsbad, CA). Rotenone was obtained from ICN Biomedicals (Costa Mesa, CA), α-methyl-*p*-tyrosine from Fluka (Ronkonkoma, NY), and nerve growth factor (NGF) from BD Bioscience (San Diego, CA) and Accurate Chemical (Westbury, NY). All other reagents were purchased from Sigma (St. Louis, MO).

*PC12 Cell Culture*:

PC12 cells, a rat adrenal pheochromocytoma derived cell-line, were differentiated in DMEM supplemented with 1% fetal bovine serum, 1% horse serum, and 100 ng/ml NGF for 3 d. Cells were then treated with rotenone (dissolved in DMSO) and/or methamphetamine, in differentiation media for 2-48 h. Control cultures underwent a media change at the same time as rotenone treated cultures. Cell viability was determined by cell counting using the trypan blue exclusion method. Vehicle (DMSO) had no effect on cell viability (data not shown).

*Depletion of Cellular DA:*

DA levels were depleted using the TH inhibitor α-methyl-p-tyrosine (AMPT). AMPT was added to the differentiating media in concentrations of 100, 300, or 1000 μM. For subsequent experiments in which DA levels were depleted, 1000 μM AMPT was added 3 d prior to, and then sustained during rotenone treatment.

*Biochemical analysis:*

For DA and DOPAC measurements, PC12 cells were collected following treatment, and the protein was acid precipitated in 0.1 N perchloric acid and centrifuged at 14,000 x g for 25 min. An aliquot of the supernatant was extracted with alumina, and injected into an HPLC system containing an ESA (Chelmsford, MA) Coulochem II coulometric detector (+280 V). Protein cysteinyl catechols (protein cys-DA, cys-DOPAC, and cys-DOPA) were measured following hydrolysis of protein in 6 N HCl containing 1 mg/ml BSA, as described previously (22). Hydrolyzed protein samples were extracted with alumina prior to analysis on HPLC with a Waters 460 amperometric detector set at an oxidizing potential of 0.6 V. Peaks for catechols and cysteinyl-catechols were identified and quantified by comparison to standards.

*ATP measurement:*

Following exposure to DA, protein from PC12 cells was precipitated in 2% trichloroacetic acid and centrifuged at 14,000 x g for 25 min. A luciferase-based assay was used to measure ATP levels in an aliquot of the resulting supernatant (Ronner et al., 1999). A Monolight 3010 luminometer (Pharmingen, San Diego, CA) was used to measure the light output resulting after an aliquot of diluted cell sample, 30mM HEPES, pH 7.75, and Enlighten

rLuciferase/Luciferin reagent (Promega, Madison, WI) were mixed in a cuvette. Protein amounts were determined by the Bradford assay (Bradford, 1976).

*Statistical Analysis:*

Differences among group means were determined by ANOVA followed by post-hoc student's t-test with significance determined at $p<0.05$.

## 5.4 RESULTS

### 5.4.1 Rotenone-Induced PC12 Cell Toxicity

Previous studies have shown the mitochondrial complex I inhibitor rotenone to be toxic to DA-containing cells such as undifferentiated PC12 cells (Hartley et al., 1994), SH-SY5Y cells (Shamoto-Nagai et al., 2003), and primary mesencephalic cultures (Lotharius and O'Malley, 2000). To evaluate the susceptibility of differentiated PC12 cells to rotenone toxicity, cell viability was determined using trypan blue exclusion, following 48 h of rotenone exposure at concentrations ranging from 0.5 μM to 20 μM (Figure 15). Rotenone treatment for 48 h significantly decreased the number of viable cells (from -37% to -70% as compared to time-matched control cells) at all concentrations measured (Figure 15). Future experiments used either the 0.5 μM or 1 μM rotenone concentration, since these were the lowest concentrations that caused significant amounts of cell death.

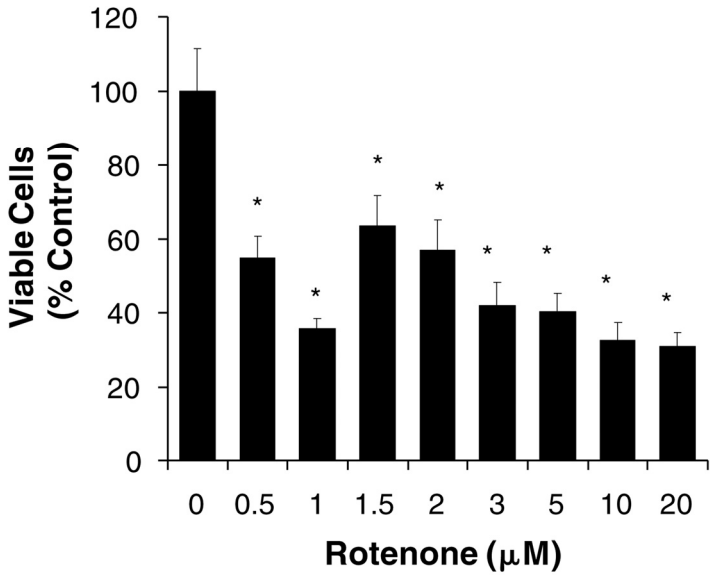


Figure 15: Concentration response curve for rotenone toxicity.

Differentiated PC-12 cells were treated for 48 h with increasing concentrations of rotenone dissolved in DMSO. The number of viable cells was counted using trypan blue exclusion. Data expressed as mean % control viable cells ± SEM, n=3-4. *Significantly different from control, $p<0.05$.

### 5.4.2 Rotenone Reduced ATP Levels in PC12 cells

Rotenone exposure inhibits mitochondrial complex I and in part the electron transport chain, potentially reducing ATP synthesis. Previous studies have shown that rotenone treatment in SK-N-MC human neuroblastoma cells lead to a dose-dependent loss in ATP (Sherer et al., 2002). Therefore, to determine whether the rotenone concentrations that caused decreases in viability also led to reductions in ATP level, we measured ATP levels in differentiated PC12

cells treated with 1 μM rotenone, for 12 to 48 hours (Figure 16). We observed significant decreases in ATP levels following 12, 24, and 48 h of rotenone exposure, ranging from -25% to -65% as compared to time-matched control levels (Figure 16). The greatest depletion occurred after 48 h rotenone exposure.

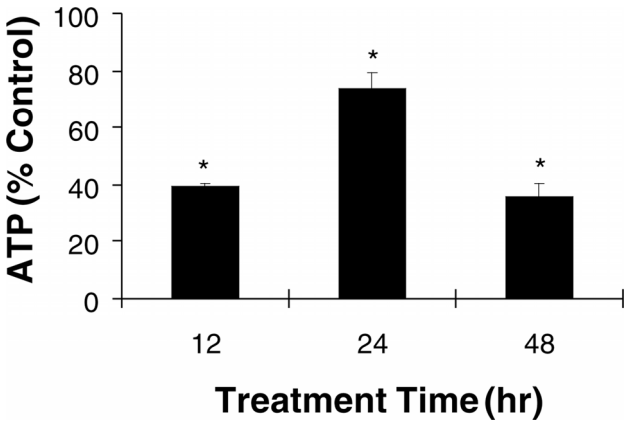


Figure 16: ATP levels in PC12 cells following rotenone exposure.

Differentiated PC-12 cells were treated with 1 μM rotenone for 12, 24, or 48 h. ATP levels were measured from PC-12 cell lysate in a luminometer. Control ATP levels were 13.0 ± 1.5 nmol/mg protein. Data expressed as mean % control ± SEM, n=4. *Statistically significant from control, $p<0.05$.

### 5.4.3 Effects of Rotenone on Catechol Levels

Rotenone has previously been shown to cause catecholamine release in PC12 cells (Taylor et al., 2000), therefore, we wanted to determine whether cellular catecholamine levels

were affected by rotenone. DOPA, DA, and DOPAC amounts were measured in differentiated PC12 cells treated with 1 μM rotenone for 2 to 48 h and compared to time-matched control levels (Figure 17). DOPA levels were significantly increased from control (+130%) following 48 h of 1 μM rotenone treatment. In contrast, DA levels were significantly lower than control following 6 to 24 h of 1 μM rotenone treatment, ranging from -18% to -25% of time-matched control levels. However, the greatest decrease was observed in DOPAC levels, which were significantly lower (-33% to –68%) than control at all time-points observed, following 1 μM rotenone treatment. The sustained low levels of DOPAC suggested that rotenone exposure might alter monoamine oxidase (MAO) activity, the enzyme that metabolizes DA to DOPAC. To evaluate this possibility, we treated isolated rat brain mitochondria with 1 μM rotenone, exposed the mitochondria to 50 μM DA, and measured DA metabolites using HPLC. The rates of DA metabolism were similar in rotenone-treated and untreated mitochondria, suggesting that this was not a direct effect of rotenone on MAO activity (data not shown).

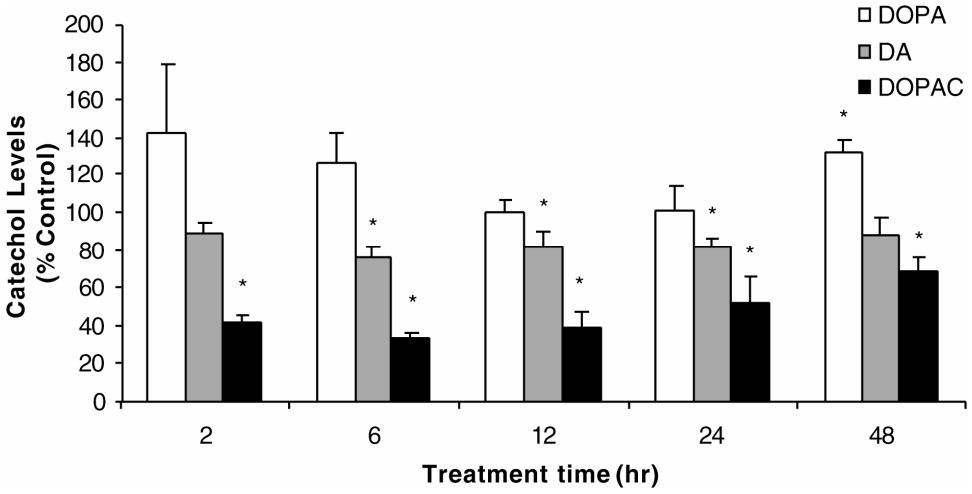


Figure 17: Catechol levels following rotenone exposure.

Differentiated PC-12 cells were treated with 1 μM rotenone for 2 to 48 h. DA, DOPAC, and DOPA levels were determined using HPLC with electrochemical detection. Average control DA

levels were 2.0 ± 0.2 nmol/mg protein. Average control DOPAC levels were 48 ± 4.9 pmol/mg protein. Average control DOPA levels were 511 ± 51 pmol/mg protein. Data expressed as mean % control ± SEM, n=4-5. *Statistically significant from control, $p<0.05$.

### 5.4.4 Effect of Rotenone on DA Oxidation

Rotenone inhibits mitochondrial complex I, leading to ROS production (Pitkanen and Robinson, 1996; Votyakova and Reynolds, 2001). Increased ROS is likely to lead to increased DA oxidation and DAQ formation in DA-containing neurons, which may additionally contribute to the rotenone-induced toxicity. As a measure of DA oxidation and catechol oxidation in general, we evaluated the formation of protein cysteinyl-catechols in PC12 cells treated with 1 μM rotenone (Figure 18). Protein from rotenone-treated cells was acid precipitated and hydrolyzed to break up the protein into its amino acid components. Protein cysteinyl-DA and cysteinyl-DOPAC levels were then measured using HPLC. Protein cys-DA levels were increased above control (+150%) following 48 h rotenone treatment (Figure 18). Protein cys-DOPAC levels were also increased significantly from control (+120%-140% of control) after a 12-48 h rotenone treatment (Figure 18), suggesting that rotenone treatment leads to increased DA and DOPAC oxidation resulting in protein modification in PC12 cells.

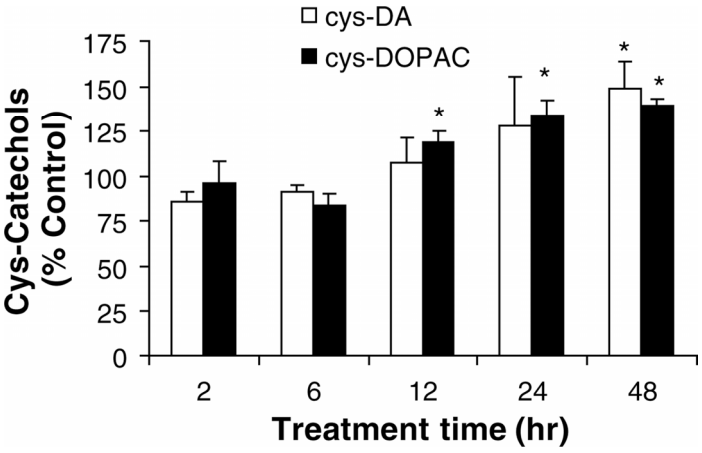


Figure 18: Protein cysteinyl-catechol levels following rotenone exposure.

Differentiated PC-12 cells were treated with 1 μM rotenone for 2 to 48 h. Protein cysteinyl-DA and protein cysteinyl-DOPAC levels were determined using HPLC with electrochemical detection. Data expressed as mean % control ± SEM, n=4-6. *Statistically significant from control, $p<0.05$.

### 5.4.5 DA Depletion Does Not Protect PC12 Cells from Rotenone Induced Toxicity

Because DA may make cells more susceptible to cell death due to the formation of reactive DA metabolites, we examined whether the presence of DA makes PC12 cells more susceptible to rotenone-induced toxicity. To evaluate this initially, DA was depleted in PC12 cells using the tyrosine hydroxylase (TH) inhibitor, α-methyl-*p*-tyrosine (AMPT). Since TH is the rate-limiting step in DA synthesis, blocking TH will stop DOPA, the DA precursor, from being produced, and thus depletes cells of DA and its metabolite, DOPAC. PC12 cells were treated with increasing concentrations of the TH inhibitor, AMPT (0-1000 μM) during the 3 d

differentiation period (Figure 19). Results showed that DA, DOPAC, and DOPA levels were all significantly decreased in a concentration-dependent manner following AMPT treatment (Figure 19). At the highest concentration (1000 μM AMPT), DA levels were only 7.5% of control and DOPAC and DOPA levels were non-detectable. AMPT (1000 μM) treatment alone had no effect on PC12 cell viability (Figure 20), and therefore was chosen for all subsequent DA depletion experiments.

The effect of DA depletion on rotenone toxicity was determined by pretreatment with 1000 μM AMPT for 72 h, followed by 1 μM rotenone or vehicle plus AMPT for an additional 48 h. Rotenone treatment alone lead to a 60% decrease in viable cells. However, AMPT plus rotenone showed a similar decrease in cell viability (-60%), which did not differ from the rotenone-alone treated cells (Figure 20).

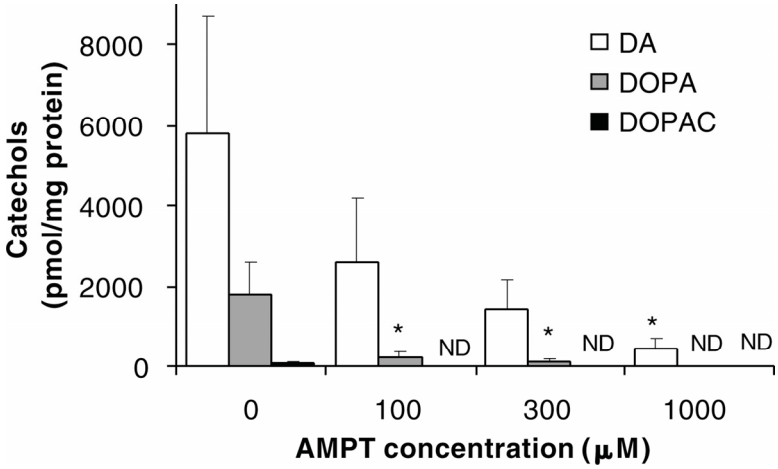


Figure 19: Effect of AMPT on catechol levels.

PC12 cells were treated with 0-1000 μM AMPT, a tyrosine hydroxylase inhibitor, for 72 h during differentiation. DA, DOPAC, and DOPA levels were measured using HPLC with electrochemical detection. Data expressed as mean ± SEM, n=4. *Statistically significant from

control, $p<0.05$.

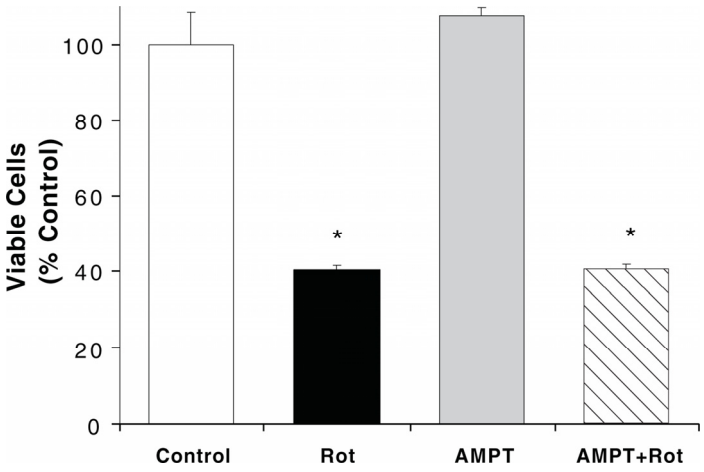


Figure 20: PC12 cell viability following DA depletion and rotenone exposure.

PC-12 cells were treated with 1 mM AMPT or control media for 72 h, followed by 1 μM rotenone or vehicle for 48 h. Cell viability was then determined using trypan blue exclusion. Data expressed as mean % control ± SEM, n=4. *Statistically significant from control, $p<0.05$.

### 5.4.6 Methamphetamine Potentiated Rotenone-Induced Toxicity in PC12 Cells

PC12 cells contain both synaptic-like vesicles and large dense core vesicles (O'Lague et al., 1985), which comprise a large storage capacity for DA, and thus much of the DA would be adequately sequestered away from any rotenone-produced ROS. Because DA depletion did not attenuate rotenone-induced toxicity, we sought to determine whether increasing cytoplasmic DA by treatment with METH would potentiate rotenone-induced toxicity. Previous studies in primary cultures have shown that rotenone potentiated toxicity induced by amphetamine, which

releases DA from vesicles into the cytoplasm (Lotharius and O'Malley, 2001). First, to confirm the mobilization of intracellular DA stores in PC12 cells following METH treatment, we exposed differentiated PC12 cells to control media, 0.5 μM rotenone, 0.5 mM METH, or 0.5 μM rotenone plus 0.5 mM METH in media for 24 h and then measured cellular catechol levels (Figure 21a). METH treatment alone did not affect DOPA levels. However there was a significant increase in DOPA levels to 217% and 310% of control in the rotenone and rotenone plus METH-treated cells, respectively. DOPAC levels were decreased to -55%, -24%, and -72% as compared to control following METH, rotenone, and rotenone plus METH treatment, respectively. DA levels were not affected by rotenone treatment alone, but were significantly decreased (-83%) from control following 24 h METH, and decreased (–73%) after a rotenone plus METH treatment, indicating that exposure to METH but not rotenone is mobilizing intracellular DA stores in PC12 cells, leading to DA depletion.

After confirming that METH was mobilizing DA stores, we examined the effect of increased cytosolic DA on rotenone-induced toxicity. We co-treated PC12 cells with 0.5 mM METH and 0.5 μM rotenone, for 48 h, and measured cell viability (Figure 21b). In these studies, we utilized a lower concentration of rotenone than previous experiments, which led to a 22% loss in viable cells. Methamphetamine treatment alone also led to a small, but significant decrease in cell viability (-12% of control). However, co-treatment of rotenone and METH lead to a 49% loss in cell viability, which was significantly different from control, rotenone alone, and METH alone groups. In addition, rotenone plus METH appeared to potentiate toxicity beyond the sum of the toxicities seen in either treatment group alone.

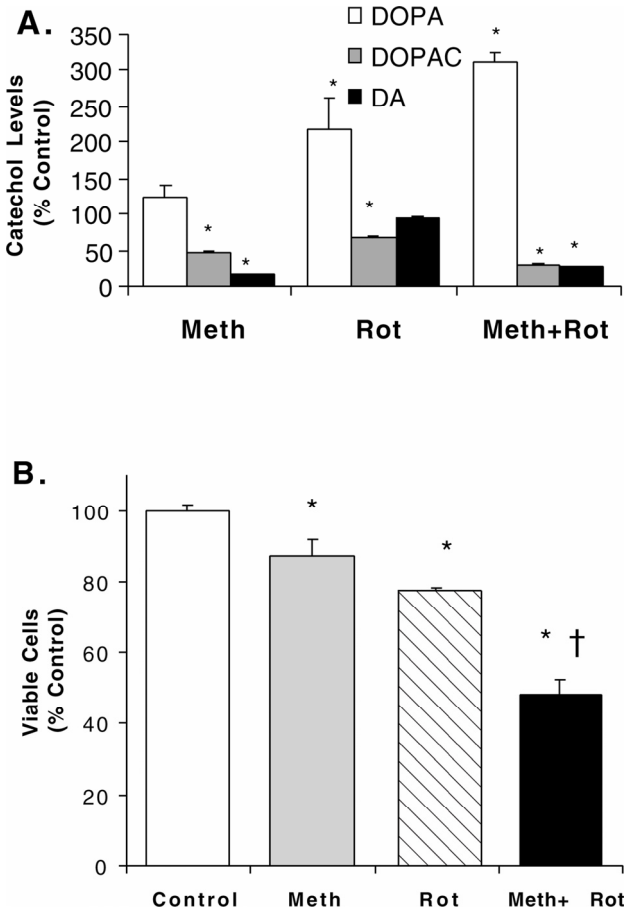


Figure 21: Effect of methamphetamine and rotenone co-treatment on PC12 cell catechol levels and viability.

PC12 cells were treated with control media, 0.5 mM methamphetamine, 0.5 μM rotenone, or co-treated with 0.5 μM rotenone and 0.5 mM methamphetamine for 48 h. **A.** DA, DOPAC, and DOPA levels were determined using HPLC with electrochemical detection. Data expressed as mean % control ± SEM. *Statistically significant from control, $p<0.05$. n=4. **B.** Cell viability was determined using trypan blue exclusion. Data expressed as mean % control ± SEM, n=4. *Statistically significant from control, $p<0.05$. †Statistically significant from methamphetamine- and rotenone-alone treated groups, $p<0.05$.

To determine whether the effect was due to increased cytosolic DA or due to a direct effect of METH, we first depleted DA with AMPT and then treated PC12 cells with METH and rotenone for 48 h. In this experiment, 0.5 mM METH exposure lead to only a 7% loss in viability, which was not significantly different from control (Figure 22). Rotenone exposure (0.5 μM) lead to a 29% loss in cell viability as compared to control, which was again not significantly different from rotenone treatment following DA depletion with AMPT (-34% as compared to control) (Figure 22). Methamphetamine and rotenone co-treatment lead to a 46% loss in cell viability as compared to control, which was again significantly different from control, rotenone alone, and METH alone treated groups (Figure 22). However, 1000 μM AMPT pre-treatment followed by rotenone and METH co-treatment lead to attenuation of toxicity to only a 19% loss in viable cells, which represents the rescue of 60% of the cells lost following rotenone plus METH without pre-treatment (Figure 22). This observation suggests that a large portion of the enhanced toxicity observed in rotenone/METH-induced toxicity could be due to the presence of DA.

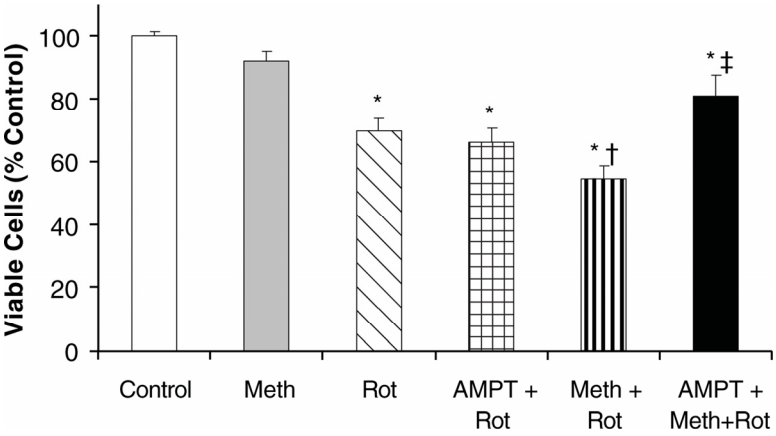


Figure 22: Effect of DA depletion on methamphetamine and rotenone co-treatment on PC12 cell viability.

PC12 cells were treated with control media, 0.5 mM methamphetamine, 0.5 μM rotenone, pre-treated with 1 mM AMPT for 3d followed by 0.5 μM rotenone treatment, co-treated with 0.5 μM rotenone and 0.5 mM methamphetamine, or pre-treated with 1 mM AMPT for 3d followed by 0.5 μM rotenone and 0.5 mM methamphetamine co-treatment for 48 h. Cell viability was then determined using trypan blue exclusion. Data expressed as mean % control ± SEM, n=4. *Statistically significant from control, $p<0.05$. †Statistically significant from methamphetamine- and rotenone-alone treated groups, $p<0.05$. ‡Statistically significant from methamphetamine and rotenone co-treated group, $p<0.05$.

## 5.5 DISCUSSION

### 5.5.1 Mitochondrial Dysfunction, Rotenone, and Dopaminergic Cell Death

In the present study, we wanted to determine the role of DA in rotenone-induced toxicity, to better understand the possible contribution of DA to cell death in PD. Oxidative stress and mitochondrial dysfunction, combined with DA oxidation may make dopaminergic cells a more vulnerable target for toxic stimuli in PD. The rotenone model, with complex I inhibition and selective dopaminergic cell death, possesses many aspects of PD pathology, including evidence for increased oxidative stress (Sherer et al., 2002) and α-synuclein positive protein aggregates (Sherer et al., 2003a). Therefore, we used rotenone, in conjunction with AMPT and METH to examine the contribution of DA to rotenone-induced toxicity in PC12 cells. We found that DA depletion prior to toxin exposure did not protect against rotenone-induced toxicity. However, rotenone toxicity was potentiated in PC12 cells by the intracellular release of DA from the vesicles, induced by METH exposure.

Partial inhibition of complex I has been shown to increase mitochondrial production of ROS (Pitkanen and Robinson, 1996; Votyakova and Reynolds, 2001), which may be the precipatory event in toxicity models. However, the basis for rotenone-induced selective toxicity to dopaminergic neurons remains ambiguous. The increased oxidative stress within dopaminergic neurons, due to DA metabolism and oxidation, combined with enhanced ROS production by complex I inhibition may lead to cell death by overloading the antioxidant capacity of these cells. In addition, DA oxidation may cause mitochondrial dysfunction, since isolated mitochondria exposed to DAQ have increased state 4 (uncoupled) respiration and opening of the permeability transition pore (Berman and Hastings, 1999). DA oxidation,

mitochondrial dysfunction, and mitochondrial ROS production are all processes that can lead to an increasing cascade of oxidative damage to cellular macromolecules, which may lead to total mitochondrial failure and cell death.

We found that the depletion of DA by AMPT did not protect PC12 cells from rotenone-induced toxicity. However co-exposure of PC12 cells to rotenone and METH, which leads to the release of DA stores into the cytoplasm, lead to increased toxicity. Additionally, the potentiation of rotenone toxicity by METH was blocked when PC12 cells were depleted of DA prior to rotenone and METH co-treatment. Although METH may have toxic actions on its own (Brown and Yamamoto, 2003), these data suggest that cytoplasmic DA, and perhaps increased oxidative stress due to DA oxidation and metabolism, may exacerbate rotenone-induced toxicity in differentiated PC12 cells.

Previous studies in primary mesencephalic cultures (Sakka et al., 2003), SH-SY5Y cells (Shamoto-Nagai et al., 2003), and undifferentiated PC12 cells (Hartley et al., 1994) have shown nanomolar concentrations of rotenone to be toxic. However, in this study, we found a 37-70% decrease in cell viability in NGF-differentiated PC12 cells following a 48 h, exposure to 0.5-20 μM rotenone (Figure 15), and very little toxicity prior to 48 h (data not shown). Differentiated PC12 cells seem to be less susceptible to rotenone-induced toxicity than other cellular models, which may be due to the presence of the growth factor NGF throughout exposure.

ATP levels were depleted following 1 μM rotenone exposure (Figure 16). However, the levels of ATP after 24 h rotenone treatment (75% of control) were higher than the ATP levels following 12 h (40% of control) or 48 h (35% of control) of rotenone exposure. The jump in ATP levels may be the result of glycolysis stimulated by rotenone-induced complex I inhibition. PC12 cells have previously been shown to resort to increased glycolysis when challenged with

the mitochondrial complex I inhibitor, MPP+ (Kang et al., 1997). This has also been observed in primary neuronal cultures in response to oxidative stress (Ben-Yoseph et al., 1996). Our observations suggest that the loss of ATP was not responsible for cell death, as also determined by others (Sherer et al., 2003c).

### 5.5.2 Intracellular DA Efflux and Rotenone-Induced Toxicity

Rotenone-induced catecholamine release in PC12 cells has been previously observed (Taylor et al., 2000), and in our study, we observed a slight, but significant, depletion of DA following a 6-24 h, treatment with 1 μM rotenone (Figure 17). However at 24 h, a lower concentration of rotenone (0.5 μM) did not affect DA levels (Figure 21a). Therefore, if DA is being released from PC12 cells following rotenone treatment, it is very small compared to the total DA stored in the cells. We also observed a substantial decrease in DOPAC levels in PC12 cells treated with rotenone (Figure 17 and Figure 21a) but found no direct effect of rotenone on MAO activity in isolated mitochondria. Decreased DOPAC levels have been previously observed in PC12 cells following rotenone treatment (Lamensdorf et al., 2000). Decreased DOPAC following rotenone in that study was accompanied by increased levels of 3,4-dihydroxyphenylacetaldehyde (DOPAL), suggesting that rotenone leads to the inactivation of aldehyde dehydrogenase, the enzyme that converts DOPAL into DOPAC (Lamensdorf et al., 2000). DOPAL exposure has been shown previously to be toxic to dopaminergic cells (Mattammal et al., 1995), and thus may add to the rotenone-induced toxicity. However, we did not observe the presence of DOPAL in PC12 cells following rotenone treatment. We also observed increased DOPA levels following rotenone treatment (Figure 17 and Figure 21a). DOPA-induced toxicity has previously been shown in PC12 cells (Basma et al., 1995). Like

DA, DOPA can oxidize, forming ROS and DOPA quinones, which could add to the oxidative damage in the cell (Graham, 1978).

Rotenone has been shown to increase oxidative stress. In previous cell culture studies, rotenone reduced GSH levels (Seyfried et al., 2000; Sherer et al., 2002), while increasing levels of oxidized glutathione (GSSG) (Seyfried et al., 2000). In addition, acute and chronic rotenone exposure in SK-N-MC cells leads to increased carbonyl formation (Sherer et al., 2002; Sherer et al., 2003c). In this study, we observed evidence for increased DA oxidation following rotenone treatment in PC12 cells, as levels of protein cysteinyl-DA and cysteinyl-DOPAC increased after 12-48 h of rotenone exposure (Figure 18), suggestive of an oxidative environment in the cells.

Previous studies have shown that depletion of DA is protective in MPP+-induced toxicity (Lotharius and O'Malley, 2000), and recent studies in primary mesencephalic cultures have suggested that DA may be involved in rotenone-induced toxicity (Sakka et al., 2003). In addition, rotenone potentiated amphetamine-induced toxicity in primary mesencephalic cultures, which was also thought to be due to DA (Lotharius and O'Malley, 2001). We found that DA depletion did not affect rotenone-induced toxicity in PC12 cells (Figure 20). However, since DA may not have been accessible for oxidation due to PC12 cell's high storage capacity, we utilized a way to mobilize endogenous DA stores in the presence of rotenone, to determine whether DA could play a role in rotenone-induced toxicity. Previous studies have shown that METH is transported into cells by DAT (Fumagalli et al., 1998), where it displaces vesicular DA into the cytoplasm, (Cubells et al., 1994), leading to DA depletion (Fumagalli et al., 1998). In PC12 cells, METH treatment and rotenone/METH co-treatment lead to DA depletion (Figure 21a), suggesting that DA was being released from the vesicles into the cytoplasm, where it could be easily oxidized, metabolized, and/or released from the cell via reversal of DAT. Results showed

that DA potentiates rotenone-induced toxicity following the mobilization of DA by METH (Figure 21b), an effect that was eliminated with prior DA depletion (Figure 22). METH has also been shown to enhance 3-nitropropionic acid and glutamate toxicity (Jakel and Maragos, 2000), an effect thought to be dependent on DA. Although oxidative stress is likely to be involved, the mechanism may be different than the intracellular effects on DA neurons.

Rotenone and other complex I inhibitors are currently being used as PD models both *in vivo* and *in vitro* (Greenamyre et al., 2003). However, the question of why complex I inhibitors seem to target dopaminergic neurons has remained unanswered. The present study demonstrates that unsequestered, intracellular DA could play a significant role in the selective targeting of DA neurons in rotenone-induced toxicity. The ability of a dopaminergic cell to deal with increased oxidative stress, created by complex I inhibition, may be hampered by the presence of DA, which may further increase oxidative stress. The complex I deficiency observed in PD is likely to cause increased ROS production, which in turn will promote DA oxidation, leading to a cycle of increasing oxidative stress and further DA oxidation. This will result in oxidative protein modifications, inactivation of critical protein functions, and/or altered protein degradation, all of which are likely to contribute to the pathological mechanisms involved in PD.

# 6.0 CONCLUDING REMARKS

The underlying cause behind the selective vulnerability of the dopaminergic neurons of the substantia nigra remains a focus of Parkinson's disease research. Both inheritable and idiopathic forms of Parkinson's disease are associated with oxidative stress, mitochondrial dysfunction, and ubiquitin proteasome pathway deficits. A common thread of these three pathways is the oxidation of proteins. Oxidative stress, i.e. the formation of excess ROS, increases protein oxidation. Mitochondrial dysfunction can be the result of proteins inactivated by ROS modifications and can further increase ROS formation, resulting in more protein oxidation. Oxidized and misfolded proteins are degraded by the ubiquitin proteasome pathway. The ubiquitin proteasome pathway can be adversely affected by ROS, leading to dysfunction. Thus, deficits of the ubiquitin proteasome pathway lead to the accumulation and aggregation of oxidized proteins. Therefore, both mitochondrial dysfunction and ubiquitin proteasome pathway deficits can be caused oxidative stress and can lead to further oxidative stress, perpetuating a cycle of cellular dysfunction and injury.

Dopamine (DA) could itself be a factor in neuronal vulnerabilities, since DA adds to the oxidative stress of the cell through ROS and quinone formation, which can modify proteins in addition to damaging DNA and lipids. DA selectively damages dopaminergic terminals *in vivo* (Hastings et al., 1996) and *in vitro* (Chapter 3), and this damage occurs following an increase in DA-protein adduct levels. DA quinone also increases uncoupled respiration and permeability

transition pore formation in isolated mitochondria (Berman and Hastings, 1999) and decreases ATP levels in differentiated PC12 cells (Chapter 3), linking DA oxidation to mitochondrial dysfunction. Although depleting DA did not protect against rotenone, a complex I inhibitor, the release of vesicular DA stores exacerbated rotenone-induced toxicity (Chapter 5). These data suggest that DA may make dopaminergic neurons more sensitive to mitochondrial inactivation, due to increased oxidative stress and also perhaps directly by DAQ-induced protein inactivation. Exposure to DA also led to the up-regulation of endoplasmic reticulum stress proteins, indicating that unfolded proteins were accumulating (Chapter 4). These results indicate that protein modification, mitochondrial dysfunction, and endoplasmic reticulum stress can all result from DA oxidation, and thus DA may play a major role in the susceptibility of dopaminergic neurons in Parkinson's disease. A summary of the events described in this document associated with DA-induced toxicity in differentiated PC12 cells is shown in the illustration below (Figure 23).

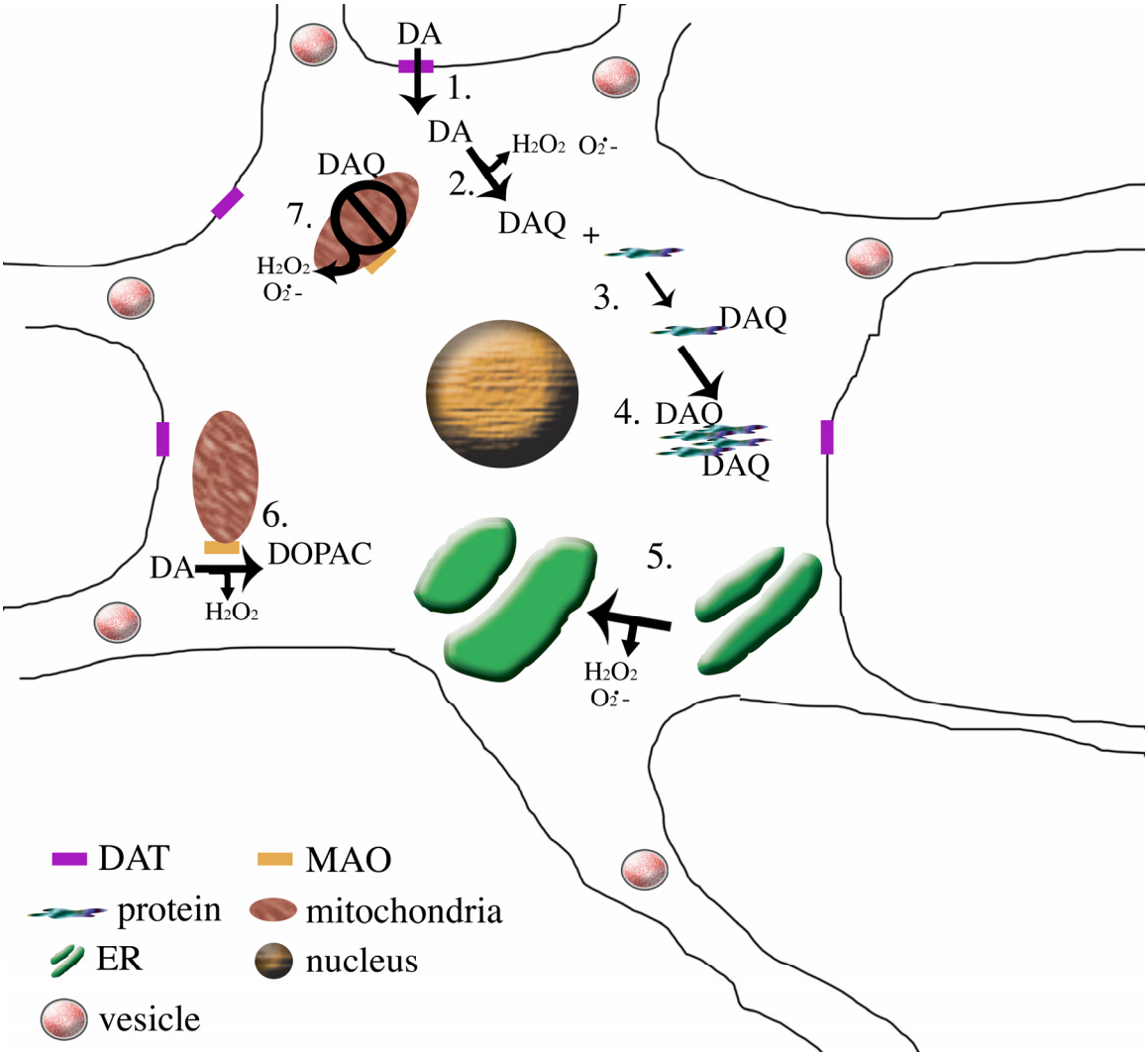


Figure 23: Summary Diagram of DA Toxicity.

**1.** Dopamine (DA) is taken up by the DA transporter (DAT) into the cells. This step is necessary to induce toxicity in differentiated PC12 cells. **2.** Once inside the cells, DA is oxidized into the DA quinone (DAQ), leading to the production of $H_2O_2$ and superoxide anion ($O_2^{\bullet -}$). **3.** DAQ reacts with reduced cysteine residues on proteins, leading to cysteinyl-DA modified proteins on the outside of the cell, in the cytoplasm, or associated with organelles such as mitochondria. **4.** Modified proteins may be inactivated or become misfolded and aggregate. **5.** Misfolded proteins lead to the activation of endoplasmic reticulum (ER) stress causing the ER to swell and up-

regulating levels of chaperone proteins like Grp78 and Grp58. **6.** Intracellular DA can also be metabolized by monoamine oxidase (MAO), producing DOPAC and leading to the production of $H_2O_2$. Inhibition of MAO metabolism of DA does not affect DA-induced toxicity. **7.** DAQ inhibits mitochondrial respiration, leading to reduced production of ATP and increased production of $H_2O_2$ and $O_2\bullet^-$.

## 6.1 THE ROLE OF DOPAMINE IN PC12 CELL DEATH

The data presented in this document provides evidence that DA treatment leads to increased protein modifications, ATP losses, ER stress activation, and toxicity (Chapters 3 and 4). Individually, each of these responses could result in cell death. Therefore, the question remains as to which pathway(s) significantly mediate DA-induced toxicity. To answer this question, the timeline of the measured responses to DA will be discussed below and shown in Figure 24.

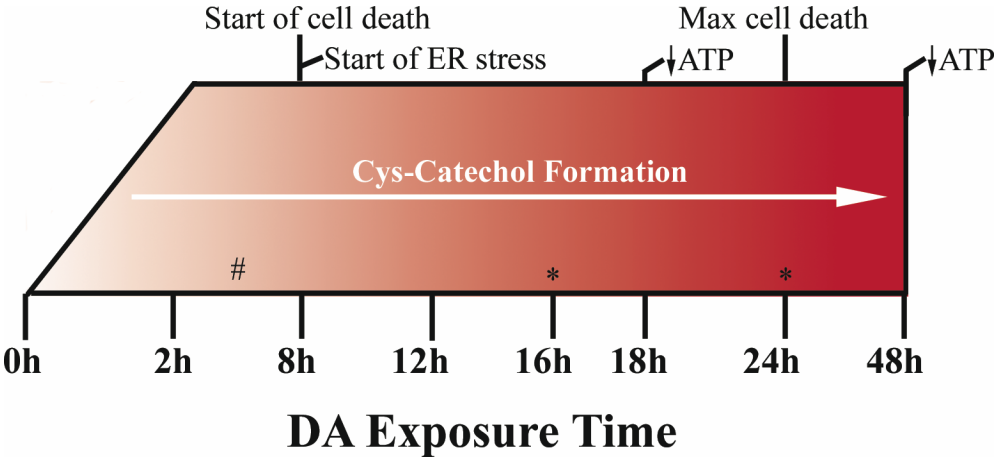


Figure 24: Timeline of PC12 cellular responses to DA treatment.

DA exposure leads to the rapid formation of protein cysteinyl-catechols. These proteins may become unfolded, leading to increased levels of chaperone protein Grp58 (#, 4 h). Unfolded proteins start to accumulate, leading to increases in Grp78 levels, indicating the beginning of ER stress activation after 8 h, correlating to the beginning of trypan blue exclusion-measured cell death. Cellular aldolase A levels increase and mitochondrial aldolase A levels are decreased (*, 16 h) around the same time ATP levels decrease (18h). Cellular aldolase A levels remain elevated after 24h (*) when ATP levels return to normal, and maximum cell death is observed. ATP levels decrease again after 48 h.

DAQ formation and protein modification occurs rapidly, resulting in protein cysteinyl-catechol formation as early as 2 h (Figure 4, Figure 24). The levels of protein cysteinyl-catechols remain elevated even when PC12 cells were treated with DA for 48 h. These DAQ-modified proteins and probably other oxidatively modified proteins may lose their enzymatic

activities or unfold. Increases in Grp58, an ER chaperone protein, after 4 h of DA exposure indicate that the cells are responding to misfolded proteins fairly early (Figure 13, Figure 24). Following 8 h of DA exposure, the differentiated PC12 cells respond by increasing levels of Grp78, a chaperone protein that leads to activation of the UPR when it releases its normal substrates and binds to misfolded proteins (Figure 13, Figure 24). Also after 8 h of DA exposure, cell death becomes apparent in PC12 cells as measured by trypan blue exclusion (Figure 2, Figure 24). Between 8 and 18 h, DA exposure leads to ATP deficits, with ATP levels significantly decreased after 18 h, indicating that mitochondrial respiration may be affected (Figure 6, Figure 24). This drop in ATP occurs around the same time as increased levels of cytosolic aldolase A and decreased mitochondrial aldolase A were observed (16h *, Figure 14, Figure 24). Differential aldolase A levels between the mitochondrial fraction and in whole cell lysate could be due to various mechanisms: 1. Mitochondrial aldolase A degradation could be increased, which may be due to the protein's modification by DAQ or ROS. As compensation for decreased mitochondrial levels, cytosolic levels may then be up-regulated, resulting in increased cellular levels. 2. DA may also affect aldolase A transport to or from the mitochondria, resulting in less aldolase A in the mitochondria. 3. Since aldolase A has been shown to be part of multi-enzyme glycolytic complexes (Beeckmans et al., 1990; Minaschek et al., 1992; Xu et al., 1995; Singh et al., 2004), and thus release of aldolase A from mitochondrial-bound or ER-bound glycolytic complexes could also result in decreased levels in the mitochondrial-enriched fraction. PC12 cells are capable of offsetting ATP deficits through glycolysis, and after 24 h DA exposure, ATP levels return to normal (Figure 6, Figure 24). It is at this time-point that the highest levels of cell death were observed (Figure 2, Figure 24). Over time, PC12 cells are unable to maintain ATP levels, and thus the levels significantly decrease

again after 48 h DA exposure (Figure 6, Figure 24). After 48 h cell viability as measured by trypan blue exclusion is remains fairly similar to levels observed after 24 h, indicating that the population of PC12 cells may be heterogeneous, with a subset of cells susceptible to DA-induced toxicity. Alternatively, the amount of DA remaining in the media after 48 hr may be substantially reduced from the initial insult, and any remaining cells may have initiated compensatory mechanisms for protection against DA, leading to stabilization in the amount of cell death. Even though each differentiated PC12 cell may not have the same vulnerabilities and may be responding to DA differently, the onset of cell death occurred prior to observable ATP loss and during the same timeframe as ER stress initiation by up-regulation of Grp78. Therefore it is possible that neither ATP depletion nor ER stress activation initiate cell death in PC12 cells exposed to DA. Thus, DA exposure may lead to the early inactivation or misfolding of critical protein(s) in combination with oxidative stress-mediated pathways and combined with DA-induced mitochondrial deficits and UPR activation over time, result in cell death.

## 6.2 ROLE OF DOPAMINE IN ROTENONE-INDUCED TOXICITY

Complex I deficits are observed in PD patients, and both complex I inhibitor models of PD, rotenone and MPTP, are associated with selective dopaminergic neuron toxicity. Therefore, to determine whether DA was a factor in rotenone-induced toxicity, both depletion of DA and increased vesicular release of DA were utilized. A diagram of the summary of the events associated with the role of DA in rotenone-induced toxicity in differentiated PC12 cells is displayed below in Figure 25.

PC12 cells were not particularly susceptible to rotenone-induced toxicity; micromolar amounts of rotenone were required to initiate cell death after 48 h (Figure 15), compared to nanomolar amounts used for other cell types. Previously, rotenone has been shown to lead to increased DA extracellular release (Taylor et al., 2000), which could lead to increased DA oxidation. Slightly reduced levels of DA were observed following rotenone (Figure 17), and rotenone exposure did lead to increases in protein cysteinyl-catecholamine levels, to approximately 150% of control levels (Figure 18, Figure 25). Perhaps, the decreased sensitivity of differentiated PC12 cell death to rotenone is related to the inability of rotenone to mobilize DA and thus reducing levels of DA oxidation seen in this model. This hypothesis is supported by the results in which α-methyl-*p*-tyrosine, a TH inhibitor, was used. Inhibition of TH, and almost complete depletion of DA levels, did not affect rotenone-induced toxicity (Figure 20, Figure 25). However, if cytosolic levels of DA were raised by the administration of methamphetamine combined with a lower dose of rotenone, increased cell death was observed (Figure 21, Figure 25). This potentiation of toxicity was due to the presence of DA, and thus likely also due to DA oxidation, since prior depletion of DA attenuated the toxicity to viability levels above rotenone treatment alone (Figure 23, Figure 25). These data suggest that DA is important to rotenone-induced toxicity.

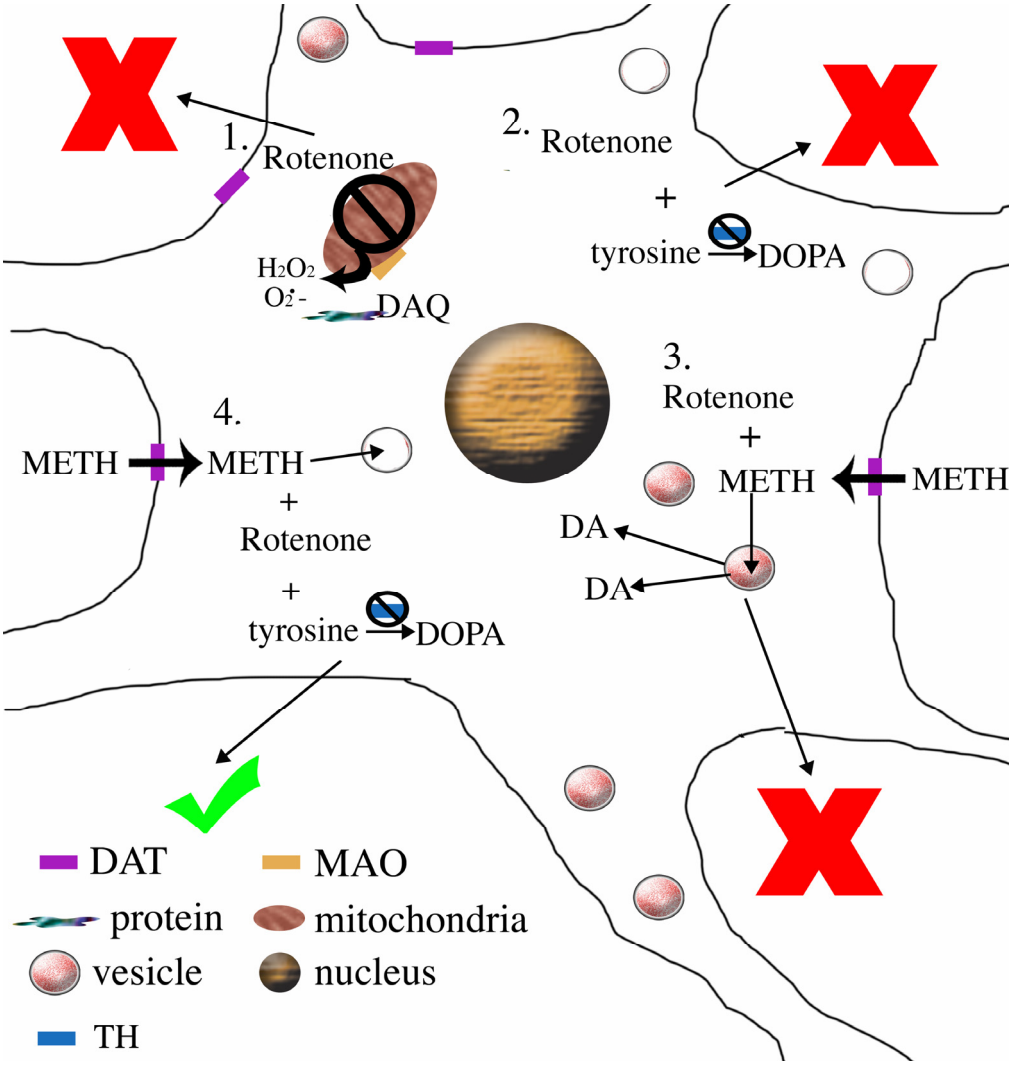


Figure 25: Summary Diagram of the Role of DA in Rotenone-induced Toxicity.

**1.** Rotenone exposure leads to mitochondrial inhibition and slight increases in DAQ-modified proteins and cell death (✖). **2.** Inhibition of TH does not protect against rotenone-induced toxicity. **3.** Displacement of DA by methamphetamine (METH) treatment exacerbates rotenone-induced cell death. **4.** DA depletion prior to METH and rotenone exposure protects cells against toxicity (✓).

## 6.3 DIFFERENTIATED PC12 CELLS AS A CELL MODEL FOR DOPAMINERGIC NEURONS

The differentiation of the chromaffin granule cells by NGF was first described in 1975, leading to the establishment of the PC12 cell line (Tischler and Greene, 1975). The characterization of differentiated PC12 cells revealed that these cells stopped proliferating and extended processes, similar to neurons, following NGF exposure (Greene and Tischler, 1976). Immunohistochemical and biochemical analyses revealed that these differentiated PC12 cells contained all of the proteins necessary for DA synthesis (Greene and Tischler, 1976). Unlike the chromaffin source cells, differentiated PC12 cells contained more DA than norepinephrine, and NGF treatment was shown to increase TH activity (Greene and Tischler, 1976). Differentiated PC12 cells contain both vesicular-like and dense core vesicles, with catecholamine levels estimated from 80 to 550 mM, suggesting that PC12 cells can store a significant amount of catecholamines (Greene and Tischler, 1976; Finnegan et al., 1996; Colliver et al., 2000; Wightman et al., 2002). PC12 cells, like dopaminergic neurons, can take up catecholamines and can release catecholamines in a Ca+ -dependent mechanism (Greene and Rein, 1977).

I used NGF-differentiated PC12 cells to observe the effects of DA on a DAergic cell line, since PC12 cells have high levels of DA and a neuronal phenotype (extends processes). For these studies, we wanted to determine what makes DA neurons more susceptible to toxins in order to better understand why DA neurons of the SN are lost in PD. Although *in vivo* work definitely has advantages over cell culture models, including the preservation of intact neuronal circuits, we wanted to look at changes specifically in DA-containing cells, which is difficult to do when working with the heterogeneous populations of neurons in brain, even when using

primary cultures. Ideally, we can use the knowledge gained from cell culture studies to investigate similar mechanisms *in vivo*, to better understand the pathology of disease.

Comparisons between *in vivo* and *in vitro* studies reveal that not every feature from cell culture studies translate to *in vivo* studies, however comparisons between the two are necessary for critical evaluations of the data and their interpretations. A recent paper that compared various features of PC12 cells to striatum, reported that the number of vesicles and baseline levels of DA, TH, DAT, and VMAT2 are significantly less in PC12 cells (Fornai et al., 2007). This study also reported that methamphetamine exposure led to decreased DA levels in PC12 cells, which is also reported in this document (Figure 21A), but that *in vivo* DA levels are not affected by low-dose METH (1 dose, 5mg/kg). However, extracellular DA levels were found to be elevated in both PC12 cells and in striatum. A comparison of the effect of L-DOPA treatment on DA levels in PC12 cells and striatum, and found that DA levels rose 5-fold above control in PC12 cells, while in striatum, only rose 2-fold (Fornai et al., 2007). Although, such a comparison is important in understanding the relevance of a cell model in comparison to an *in vivo* model, there are some concerns with this study. First, the PC12 cells used in this study were not NGF-differentiated, and thus the levels of DA and DA-related proteins may have been lower than differentiated cells, since these DA markers have previously been shown to respond to NGF. Secondly, the use of the whole striatum complicates a comparison between homogeneous dopaminergic cells, and tissue, which contains dopaminergic terminals, GABAergic neurons, and other non-neuronal cells like microglia. In addition, the differences in intracellular DA levels seen following METH treatment could reflect the differences in extracellular fluid between *in vitro* and *in vivo* studies. METH is known to lead to the release of vesicular DA, leading to extracellular DA release. This could lead to the depletion of DA in PC12 cells, especially since

the cells were treated for 30 min, compared to 7 d *in vivo*. In addition, we and others have shown that high doses of METH lead to DA depletions, and we have also shown that lowering ambient temperature during METH administration can attenuate DA depletions (15 mg/kg, 4 doses) (LaVoie and Hastings, 1999; Kita et al., 2003). Also, the DA levels following DOPA treatment between PC12 cells and striatum differ only in magnitude, and thus may be due to the difference between a treatment in a dish where DOPA can either be taken up into the cells or remain in the medium, and treatment in the animal where DOPA can diffuse to other areas. Therefore, I believe that these discrepancies do not detract from the usage of PC12 cells as a model of dopaminergic cells, but do serve as a needful reminder of the differences that can occur *in vivo* and *in vitro*.

## 6.4 2-D DIFFERENCE IN-GEL PROTEOMICS TECHNIQUE

Proteomics research in neurodegenerative disease is particularly promising, especially in investigations involving altered protein expression and changes in organelles (Hanash, 2003). Reducing the number of proteins by subcellular fractionation of target organelles, such as mitochondria, concentrates a subset of proteins, improving the separation in the 2D gel (Hanash, 2003). Recently, 2D gel electrophoresis has successfully been used in Alzheimer's disease research, leading to the identification of oxidized proteins, detection of changes in levels of energy metabolism enzymes and antioxidant proteins, and identification of altered synaptic and neurotransmitter function in Alzheimer's disease (Butterfield, 2004). Advances in the standardization of 2D protein maps help other researchers locate the position of previously identified proteins, allowing for direct comparisons in protein separation and identification

techniques (Morrison et al., 2002; Vercauteren et al., 2004). The combination of 2D gel electrophoresis with mass spectrometry allows for the identification of new proteins involved in disease processes, especially with the advent of peptide mass spectrometry search engines, which not only help identify proteins, but calculate the probability of accurate identification (Butterfield et al., 2003). Recent improvements in 2D-DIGE allows for two pools of proteins, for example control and treated or diseased samples, to be labeled with different fluorescent dyes, mixed, and separated on the same gel, reducing the inevitable variability that occurs from running samples on separate gels (Patton, 2002; Kim et al., 2004). In fact, our gels from different experiments using 2D-DIGE generate highly reproducible spot patterns, allowing us to be confident in matching identified proteins between gels (data not shown). The applications of proteomic technology for the advancement of research in neurodegenerative disorders are numerous.

In Chapter 4, a comparative investigation using two dimensional-difference in-gel electrophoresis (2D-DIGE) of mitochondrial-enriched proteins from PC12 cells with and without DA exposure was described. Lysine reactive NHS-ester CyDyes were used as a general method to label mitochondrial-associated proteins to assay for altered protein levels following DA exposure. In addition, minimal (sub-stoichiometric) amounts of the cysteine reactive maleimide-CyDyes were used to label mitochondrial proteins, in order to target those which proteins whose highly reactive cysteines are modified following DA treatment. We had expected to find and characterize two different sets of mitochondrial proteins: 1. proteins with similar increased or decreased labeling following DA exposure in both cysteine and lysine labeling, indicating proteins that were increased or decreased without any apparent changes in relative cysteine oxidation, and 2. proteins with differential relative cysteine and lysine labeling, specifically a loss of cysteine labeling compared to lysine labeling following DA treatment, indicating proteins

with cysteinyl residues that were oxidatively modified following DA exposure. Most proteins in the mitochondrial fraction fell into category 1, and appeared to be increased after DA exposure. Following mass spectrometry (MS) and mass peptide peak analysis, the majority of these proteins were identified as chaperone proteins, including ER-associated proteins.

2D-DIGE has been useful in illuminating the up-regulation of various mitochondrial and ER chaperone proteins in response to DA exposure in PC12 cells, suggesting a role for ER stress in DA-induced toxicity. However, identifying proteins with cysteine residues that have been oxidized or bound by DAQ has proved difficult. Several proteins that appear visually control-shifted in the cysteine (maleimide) gels, suggesting cysteine modification by ROS or DAQ, have been picked, but have yet to be identified through MS peptide fingerprinting or upon Decyder analysis are not significantly changed from control dye protein labeling. Despite our efforts to label the most reactive cysteines by decreasing the cysteine dye concentration to 0.125% of the recommended pmol of dye per μg protein, we labeled hundreds of proteins (Figure 10). Since the most reactive cysteines have a neutral pKa (Di Simplicio et al., 2003), at a pH below the maximal maleimide-labeling pH, modification of the pH of the reaction between protein and cysteine dye may optimize the reaction towards labeling only the most reactive cysteines. Two studies looking at $H_2O_2$- and acute DA-induced cysteine oxidation in purified proteins and in PC12 cell lysates indicate that lowering the pH to 6.5 when reacting protein with cysteine-reactive labels, in these studies biotin-conjugated iodoacetamide, can help identify reactive cysteines, albeit not all reactive cysteines (Kim et al., 2000; Kim et al., 2002). As in our methodology, decreased cysteine labeling indicates cysteine oxidation. In the study by Kim et al., 2002, DA exposure (45 min, 3 mM) lead to decreased labeling of cysteine residues by biotin-conjugated iodoacetamide of several proteins, including endoplasmic reticulum protein 72 kDa

(ERp72), phospholipase C-γl (PLC-γl), thioredoxin reductase (Trx-R), endoplasmic reticulum protein 60 kDa (ERp60), creatine kinase (CK), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Since cysteine oxidation was found in two ER chaperone proteins in the Kim et al., 2002 study, it is possible that some of the ER proteins we identified as increased following DA exposure were also modified by DA, but that the large increase in protein levels overshadowed the cysteine modifications. In addition, two energy related proteins, CK and GAPDH, were also identified as having cysteine modifications following DA exposure, suggesting that DA may affect the cell's ability to produce ATP on demand. Thus, future experiments altering the pH of the protein and cysteine dye reactions may identify proteins with oxidatively modified cysteines after DA exposure, helping to determine the critical proteins involved in increased dopaminergic neuron vulnerability to cell death.

## 6.5 ENDOGENOUS DOPAMINE IN PD TOXICITY

The DA-induced toxicity model utilizes exogenously applied DA, resulting in oxidative stress, DAQ formation, protein modification, and cell death. How does this translate to the human disease? Direct evidence for increased DA oxidation and protein modification, namely increased levels of cysteinyl-catecholamine conjugates and the presence of antibodies to DAQ-modified cysteines and proteins, has been measured in PD brain (Rowe et al., 1998; Spencer et al., 1998; Sidell et al., 2001; Salauze et al., 2005). In addition, the SN neurons which are lost in PD contain neuromelanin (NM), which is formed from oxidized catecholamines and increases with age (Sulzer et al., 2000; Zecca et al., 2004). These studies show that increased DA oxidation levels and protein modification are present in the region of the brain (the SN)

associated with the movement dysfunction observed in PD. Levels of cytoplasmic endogenous DA would likely need to be increased for DA to play role in the pathogenesis of PD, therefore, we need to determine which proteins and pathways may play a role in affecting DA homeostasis, leading to increased DA oxidation.

The affinity of the VMATs for monoamines such as DA, which transport monoamines from the cytosol into vesicles, is approximately 1000-fold greater than other transporters (Liu and Edwards, 1997). Since the monoamines, DA in particular, have been shown to be toxic both *in vivo* and *in vitro*, the ability of VMATs to bind and transport monoamines even at low cytoplasmic concentrations is a key step in keeping DA from accumulating and causing oxidative damage. The levels of DA may itself be affected by DA oxidation; proteins relevant to DA synthesis and uptake, such as DAT, the glutamate transporter, TH, and tryptophan hydroxylase, can be modified or inhibited by exposure to DA or DAQ (Berman et al., 1996; Berman and Hastings, 1997; Kuhn and Arthur, 1998; Xu et al., 1998; Kuhn et al., 1999; Whitehead et al., 2001). Enzymes involved in maintaining the ATP to ADP ratio, like creatine kinase and adenylate kinase, and the V-type H+-ATPase, the driving force behind DA uptake into vesicles, are also both inhibited by DA exposure, which could also add to increased cytosolic DA accumulation (Maker et al., 1986; Miura et al., 1999; Terland et al., 2006). In addition, regulatory pathways of DA may be altered through interactions between DA and mutant proteins associated with familial PD, also leading to increased cytosolic levels of DA and adding to the oxidative environment of dopaminergic cells.

Recently, the relationship between α-synuclein, DAT, and microtubules suggests that microtubule stabilization is vital for DAergic cell survival, and that mutations in α-synuclein may lead to alterations in DAT and vesicle accumulation. Over-expression of normal human α-

synuclein, but not the A53T mutant, decreases DA uptake and decreases the amount of DAT associated with the plasma membrane (Wersinger and Sidhu, 2005). The regulation of DAT by α-synuclein appears to be associated with microtubules, since chemicals that disrupt microtubules, also increase DA uptake, increase levels of DAT on the cell surface, and exacerbate DA-induced toxicity (Wersinger and Sidhu, 2005). Formation and/or stabilization of microtubules may also be another role for α-synuclein, since it has been shown to interact with various cytoskeletal proteins, including tubulin, and to promote the formation of microtubules from purified tubulin (Wersinger and Sidhu, 2005). Since microtubules are vital for vesicle transport, it is possible that alterations in α-synuclein may lead to destabilization of microtubules, disrupting the transport of vesicles, leading to alterations in the ability of a cell to store DA. This hypothesis is supported by evidence for decreased levels of VMAT in PD patient brain and impaired DA storage in α-synuclein knockout mice and in A30P α-synuclein over-expressing cells (Harrington et al., 1996; Cabin et al., 2002; Mosharov et al., 2006). Therefore, mutations may disrupt these suggested functions of α-synuclein, leading to increased uptake of DA, increased cytosolic levels of DA, and increased susceptibility of cell death. Rotenone also has been shown to lead to microtubule destabilization (Diaz-Corrales et al., 2005; Ren et al., 2005). Microtubule assembly requires ATP, therefore ATP deficits due to mitochondrial inhibition would adversely affect proper tubulin folding and microtubule formation (Feng, 2006). In addition to rotenone, other dopaminergic neurotoxins like MPTP have also been shown to destabilize microtubules (Brinkley et al., 1974; Marshall and Himes, 1978; Cappelletti et al., 1999; Cappelletti et al., 2001). Both α-synuclein and complex I inhibition appear to affect microtubules, which are important in the transportation various biomolecules and organelles, including DAT, vesicles, and mitochondria.

The systemic mitochondrial complex I inhibition observed in PD patients may also play a role in the dysregulation of DA storage. Since the uptake of DA into vesicles requires ATP, it is possible that mitochondrial dysfunction may affect the ability of cells to store DA. In addition, rotenone and other mitochondrial inhibitors/uncouplers have been shown to lead to the release of DA (Taylor et al., 2000; Milusheva et al., 2005). Rotenone administration *in vivo* also leads to decreased levels of VMAT2 (Zhu et al., 2004). Combined with the fact that oxidized DA has been shown to directly inactivate the V-type H+-ATPase, the driving force behind DA uptake into vesicles, it is likely that mitochondrial dysfunction can lead to increased cytoplasmic DA levels, due to decreased DA uptake and increased DA release (Terland et al., 2006).

## 6.6 DOPAMINE QUINONE VERSUS REACTIVE OXYGEN SPECIES AS A MEDIATOR OF TOXICITY

Numerous *in vitro* and *in vivo* studies support the hypothesis that DA is toxic to dopaminergic cells (Asanuma et al., 2004). Formation of ROS, increased levels of oxidized proteins, lipids, and DNA, and protection by antioxidant molecules and enzymes are all associated with DA-induced toxicity, suggesting that cell death is related to oxidative stress. DA's ability to form quinones in addition to various ROS, both of which can modify and potentially inactivate vital proteins, complicates the role of DA in cell death. This generates an important question for potential therapies: which is the more toxic species, DAQ or ROS?

The role of ROS as the cause or effect in the initiation of cell death in disease is still debated, although evidence for oxidative stress in Parkinson's disease is supported by both *in vitro* and *in vivo* models, in addition to postmortem studies (Andersen, 2004; Krantic et al.,

2005). But, does DA-induced oxidative stress of DAQ formation mediate DA toxicity? Administration of intrastriatal DOPAC injections in rat or intrastriatal DA injections in DAT knockout mice do not result in TH immunoreactivity loss (Hastings, unpublished observations). This study found that both DOPAC treatment and DAT inhibition attenuates DA-induced toxicity completely, although total levels of DAQ-modified protein were not affected by DAT inhibition combined with DA exposure (Figure 8 and Figure 9). Although some of the DAQ-modification is likely on proteins found on the extracellular surface of the plasma membrane, it is also possible that some intracellular protein targets were also modified. Thus, there may be a difference in the protein targets between DA alone and DAT inhibition + DA treated PC12 cells, leading to protection. This difference in protein targets may be the key to understanding the mechanisms of DA-induced toxicity. In addition, since DAT inhibition would also decrease the amount of DA that is taken up by the cell, it is also possible that mediating a lower dose of DA by DAT inhibition could also lead to an increase in compensatory mechanisms by the cell, effectively preconditioning the cells to DA toxicity, resulting in protection. Regardless, both *in vivo* and *in vitro* studies suggest that DA toxicity requires uptake, however, uptake of DA is required for both MAO-mediated ROS formation and DAQ-induced toxicity. What about antioxidant protection? The administration of either ascorbic acid or GSH has been shown to attenuate the loss of TH immunoreactivity in the striatum following DA injections (Hastings et al., 1996; Luo et al., 1999). *In vitro*, the thiol related antioxidants, GSH and N-acetylcysteine, are protective against DA in PC12 cells, SH-SY5Y cells, and primary forebrain cultures (Hoyt et al., 1997; Lai and Yu, 1997; Si et al., 1998; Choi et al., 2000; Jones et al., 2000; Keller et al., 2000; Kim et al., 2002; Emdadul Haque et al., 2003; Izumi et al., 2005). However, ascorbic acid and the antioxidant enzymes catalase and superoxide dismutase, which would most likely affect

mainly ROS, have varied protective effects, from none to almost complete protection in PC12 cells, SK-N-MC cells, SH-SY5Y cells, and primary cultures (Cheng et al., 1996; Alagarsamy et al., 1997; Lai and Yu, 1997; Si et al., 1998; Choi et al., 2000; Jones et al., 2000; Stokes et al., 2000; Clement et al., 2002; Emdadul Haque et al., 2003; Izumi et al., 2005; Colapinto et al., 2006; Zafar et al., 2006a). Antioxidants can protect against both ROS and DAQ-mediated toxicity, and since reducing ROS will aid in preventing DAQ-formation, separating the effects of ROS and DAQ using antioxidants becomes problematic.

MAO metabolism of DA is though to be a major source of ROS. MAO inhibition, which would reduce $H_2O_2$ formed from DA metabolism, has been shown in this study (Figure 7) and by others to have none to very little protection against DA-induced toxicity in PC12 cells and SH-SY5Y cells (Alagarsamy et al., 1997; Jones et al., 2000; Emdadul Haque et al., 2003; Izumi et al., 2005; Gimenez-Xavier et al., 2006; Zafar et al., 2006a). One study found that MAOIs potentiated DA toxicity in PC12 cells (Weingarten and Zhou, 2001). To determine whether MAO inhibition or another non-specific function of MAOIs mediated protection, the effect of either active or inactive pargyl MAOIs on DA toxicity was measured, and both were found to be protective against DA (Cantuti-Castelvetri and Joseph, 1999). These results may explain why there are varied results using MAOIs in cell culture, likely due to non-specific protection mechanisms.

Studies examining proteins that can reduce DAQ and other quinone species have aided in understanding the contributions of DAQ and ROS in DA-induced toxicity. Proteins like GSH-S-transferase and NAD(P)H quinone oxidoreductase 1 (NQO1) can detoxify DAQ, allowing the role of DAQ in DA-induced toxicity to be tested. Increased expression of GSH-S transferase Pi (GSTp), an enzyme that conjugates GSH to many electrophilic substrates including catechol

quinones, was observed following DA exposure in PC12 cells (Ishisaki et al., 2001). When over-expression of GSTp was induced, DA toxicity was attenuated; conversely, knockdown of GSTp exacerbated DA-induced cell death (Ishisaki et al., 2001). These data suggest that detoxification of DAQ by GSH, catalyzed by GSTp, is vital for cell survival following exogenous DA exposure. NQO1, a reducing enzyme which uses NADH or NADPH to catalyze the two electron reduction of various compounds including DAQ, is expressed in human SNpc in astrocytes, endothelium, and in some dopaminergic neurons. In PD, expression of NQO1 in SN was found to be increased, except in the most end-stage cases (van Muiswinkel et al., 2004). Polymorphisms in NQO1 linked to PD have been observed, but appear to be linked to ethnicity, and thus do not affect all populations (Okada et al., 2005). In studies utilizing methamphetamine-induced toxicity, in which DAQ has been shown to play a major role, induction of NQO1 blocked both DAQ formation and METH toxicity *in vivo* (Miyazaki et al., 2006). And, a recent *in vitro* study found that over-expressing NQO1 in SK-N-MC cells was protective against DA-induced toxicity (Zafar et al., 2006b). Therefore, these studies indicate that DAQ seems to play a major role in DA-induced toxicity.

The data presented in this document support the hypothesis that DA may play a role in the susceptibility of dopaminergic neurons to cell death. Dopaminergic cell health may be delicately balanced against the cytosolic levels of the reactive neurotransmitter DA, and combined with some of the already known multiple factors involved in PD dopaminergic cell loss, DA may exacerbate cell death and potentiate the pathogenesis of the disease. Thus, elucidating the pathways in which DA and DAQ mediate toxicity may lead to a better understanding to why DA neurons in the SN are lost in PD, leading to the development of potential therapeutic targets for PD.

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